New Treatment Aspects in Alveolar Cleft Repair

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New Treatment Aspects in Alveolar Cleft Repair

Nieuwe behandelinzichten in het sluiten van de gnathoschisis
(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

General Introduction

Cleft lip and palate

Cleft lip and palate is a congenital malformation that can present itself in a great many ways. It can be unilateral or bilateral, complete or incomplete and almost exclusively affects the lip, alveolus and/or palate. With an incidence of approximately 1:700 live births worldwide, it is the most common congenital craniofacial malformation[1]. Its treatment is performed in a multidisciplinary fashion and individualized per patient taking into account the local treatment protocol. Surgical and non-surgical procedures are spread out over the course of a patient's life taking into account craniofacial growth, speech and psychosocial development. This thesis focuses on closure of the alveolar cleft. The alveolar cleft is the anterior palatomaxillary defect that includes both a persistent oronasal communication and a bony defect of the anterior maxilla. Closure of the alveolar cleft combines two procedures. Firstly, the residual oronasal communication is closed using locally transposed mucoperiosteum. After definitive suturing of the newly derived nasal, palatal and buccal layers, a bone graft is applied. In this way, alveolar cleft closure provides for four clinical parameters: It closes the residual oronasal communication, it provides a stable and continuous maxillary arch, it facilitates eruption of teeth adjacent to the reconstructed cleft, and finally, it grants the alar base on the affected side better skeletal support.

Historical perspective

The first documented cases of alveolar cleft closure in patients with cleft lip and palate originate from German literature in the early 1900's[2-4]. In this pre-antibiotic era in which general anesthesia for children was not yet an option, these surgeons endeavored in free autologous bone transplantation of cleft sites.

Figure 1. Preoperative photograph of one of the patients reported on by Drachter. A periosteum covered tibial bone graft was used to close and bridge the bilateral alveolar cleft defect. The surgeon describes the result as "ein Teilweiser", meaning a partial success.

After the First World War, alveolar bone grafting became less popular. It was until the 1950's that alveolar bone grafting became popular under a broader surgical audience, mostly due to Axhausen's book on cleft surgery[5]. Again, the German cleft surgeons, all maxillofacial surgeons, were the advocates of both a soft tissue and bony reconstruction of the cleft defect. In Millard's epic *Cleft Craft*, Ralph Millard translates the key passage in which Axhausen revived alveolar cleft grafting[6]. Axhausen wrote *'If there were a means of inducing subsequent bony healing between the premaxilla and the lateral fragments, this approach would be preferred; it would then be possible to preserve well-formed incisors. To find such means appears to me to be the final problem in repair of complete clefts at present'*. It needs to be noted that Axhausen is the first to acknowledge the functional and aesthetic importance of a continuous dental arch in patients with cleft lip and palate. Millard describes the revival of alveolar bone grafting in the colorful way only he can do: *"Suddenly almost everyone began bone grafting or apologizing for not doing so. In fact, the world literature on alveolar bone grafting read like a roster of the elite in a German Panzer Division as compared with the sparse but strong guerilla bands from Sweden, U.S.A., Yugoslavia and Britain."* In the Netherlands after the First World War, many cleft patients were operated on by dr. H. de Groot, ENT surgeon, appointed by the Department of dentistry of the Utrecht university. His successor in 1932, professor Tjebbes, head of the Utrecht University Maxillofacial Surgery Department (Kliniek voor Kaakchirurgie) was trained in cleft surgery procedures by Veau in Paris and Pichler in Vienna. Together with the department of orthodontics and maxillofacial prosthetics, patients were treated in a rather holistic manner. As such, however in a primordial phase, the first features of a cleft care unit could be discerned. Back then, alveolar cleft closure was not performed in Utrecht. It was with the arrival of Egyedi in 1972, who received his training in Zürich, that alveolar cleft closure became standard of care.

Timing of the procedure

Whereas surgical techniques in alveolar bone grafting tend to be quite uniform, the greatest two debates in this surgical niche have almost exclusively encompassed the timing of the procedure and the type of bone graft or substitute used. As for timing of the procedure, *grosso modo* two camps can be discerned. The primary alveolar bone grafting adepts and the secondary bone grafting adepts. The latter being far larger than the first[7]. Before getting into the matter of timing of the procedure, the matter of nomenclature of timing needs to be discussed. According to a great deal of authors, primary alveolar bone grafting takes place in infancy at the moment of lip (and sometimes palatal) closure, early secondary alveolar

bone grafting is done before eruption of the permanent canine adjacent to the cleft, late secondary alveolar bone grafting is performed just after eruption of the permanent canine adjacent to the cleft and tertiary alveolar grafting takes place after the mixed dentition phase[8]. In this thesis, this subdivision is upheld. Primary alveolar bone grafting is inspired by the first alveolar bone grafting surgeons mentioned above, but made popular by Tord Skoog in the 60's and 70's[9]. Contradictory to his predecessors, he discovered that when closing the mucoperiosteal layers of the alveolar cleft in infancy, most patients acquired bony bridging of the alveolar process. He nicknamed this procedure 'boneless bone grafting'. The fact that only mucoperiosteal closure of the alveolar cleft can induce alveolar bone formation caused a paradigm shift among practitioners of both primary and secondary alveolar bone grafting in the regard of a bone transplant. Before, the qualities of a bony transplant were regarded as an absolute necessity to facilitate bone formation in the alveolar cleft defect. Since Skoogs publications the role of the transplant as a space maintainer to facilitate the periosteal cambium layers and osteoconduction from the lateral bony walls became widely accepted.

Figure 2. From Millard's Cleft Craft. By early maxillary bone grafting Millard means primary alveolar cleft closure.

The choice between secondary alveolar bone grafting (whether early or late) and primary alveolar bone grafting in by far the greatest amount of centers is made in favor of secondary alveolar bone grafting. Though primary alveolar bone grafting has mostly been abandoned, some centers still advocate this technique or perform a primary gingivoperiostoplasty only[10]. The multicenter Eurocleft and Americleft[11, 12] studies both came with evidence that primary alveolar bone grafting results in higher GOSLON-Yardstick scores, meaning resulting in less favorable dental arch relationships.

As we concluded in the recently published Dutch Guideline for Treatment of Patients with Cleft Lip and Palate[13], there is evidence that secondary alveolar bone grafting gives significantly better results than tertiary alveolar grafting[14-16]. More specifically, within the secondary alveolar bone grafting group, there is evidence that early secondary alveolar bone grafting (timed before eruption of the permanent canine, sometimes before eruption of the lateral incisor, adjacent to cleft) gives superior results in bony bridging, height of the residual bone and provides less occurrence of residual oronasal fistulas compared to late secondary alveolar bone grafting (after eruption of the permanent canine adjacent to the cleft).

In the Wilhelmina Children's Hospital we close the alveolar cleft according to the early secondary alveolar grafting protocol.

The alveolar cleft defect

Focusing on the specific properties of the recipient site seems equally important as focusing on the bone graft or bone graft substitute. The alveolar cleft is a complex three-dimensional structure that has been extensively described in contemporary scientific literature. Parameters for successful restoration of this naso-alveolar-palatal defect consist of re-establishing a continuous and stable maxillary arch, closure of the oronasal fistula and providing stability for the ala nasi on the affected side.

Although literature strongly tends towards bone grafting of the alveolar defect, the fact that not all defects have to be grafted in order to ossify has been proven, as mentioned in the paragraph above. In other words, one can state that not all alveolar cleft defects in which the nasal, buccal and palatal mucoperiosteum have been reconstructed are of critical size. If not all alveolar cleft defects are of critical size, this may imply that over-treatment exists in the number of alveolar bone grafting procedures performed. The more difficult question then remains, which defects do not need to be treated with a bone graft, and: does the alveolar bone graft merely acts as a space maintainer for bony ingrowth?

Furthermore, alveolar clefts vary in all three dimensions. There is significant variation in cleft width, but also in the surrounding bony walls of the cleft defect. The number of bony walls varies within alveolar cleft varieties; clefts of alveolus and palate are surrounded by only two maxillary bony walls, whereas clefts of only the alveolar portion of the maxilla also have a dorsal supporting wall (Figure 3). On the analogy of periodontal defect classification an interesting feature can be discerned regarding this heterogeneity in alveolar cleft defects. Periodontal bony defects can be restored more predictably when the number of bony walls surrounding the defect increases[17]. This is mainly due to improved stability of the graft within the defect that is to be reconstructed. There is a considerable difference in predictability of restoration between two-bony wall pockets and three-bony wall pockets. If translated to the alveolar cleft situation, this would imply that in patients with cleft lip and alveolus the defect is a three-bony wall pocket, and in patients with cleft lip, alveolus and palate the defect is a two-bony wall pocket.

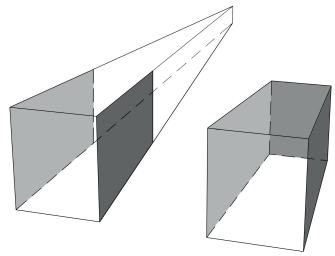

Figure 3: Schematic display of the cleft alveolus and palate defect: on the left containing two bony walls, and the cleft alveolus defect on the right containing three bony walls.

Considering not only the amount of bony walls, but also the bone surface adjacent to the cleft defect a couple of remarks can be made from an osteobiologic perspective: Besides intramembranous bone formation directed by the periosteum of the maxilla, the surrounding revitalized maxillary bone pillars allow direct invasion of capillaries and ectomesenchymal cells from the endosteum and bone marrow into the cleft site[18]. The amount of bony surface adjacent to the cleft defect could therefore play a significant role in regenerative potential of the defect.

Aforementioned theoretic bases would imply that restoration of the defect in cleft lip and alveolus patients will show a more predictable result than restoration of the defect in cleft lip, alveolus and palate patients. However in literature, the difference in regenerative capacity between these two distinct types has never been properly analysed. As morphology is concerned, alveolar cleft grafting studies in the twentieth century have mainly analysed pre- and postoperative data by means of two dimensional radiology in which bony bridging within and tooth eruption into the cleft were assessed. The two-dimensional analysis paradigm kept in mind, the width of the alveolar cleft has often been investigated as predictive factor for outcome for alveolar cleft grafting. A number of studies show positive[19] as well as negative[20, 21] associations. When considering graft volume resorption in studies using three-dimensional radiological analysis, volume does not seem to correlate with the amount of resorption[22].

Bone grafting materials

Throughout the years alveolar cleft grafting by means of an autologous bone graft has become the golden standard for alveolar cleft closure. Most studies published carry out autologous transplantation of bone harvested from the iliac crest or from the mandibular symphysis[23-26]. Even though these methods have proven to be effective and showed a high percentage of success [14, 27], both carry along donor site complication risks and postoperative

comorbidity[28-34]. It is presumable to introduce means to avoid this burden in a vulnerable paediatric population that has to endure multiple surgeries at a young age.

Calcium phosphates and their osteobiological behaviour

Since this thesis focuses on alveolar cleft reconstruction by means of calcium phosphate scaffolds, an introduction into the osteobiologic properties of these biomaterials seems appropriate.

The inorganic fraction of bone exists of calcium phosphate, mostly in the form of hydroxyapatite. It is a logical step to use these scaffolds as bone void fillers and have thus been used in the past decades. The calcium phosphate scaffolds currently available mostly consist out of two fractions, a hydroxyapatite (HA) fraction and a tricalcium phosphate (TCP) fraction. Scaffolds that are mixed in this way are named biphasic calcium phosphate scaffolds (BCP's). HA has a calcium-phosphate ratio of 1.67, equal to the anorganic phase of the crystalline structure of normal bone and TCP has a calcium-phosphate ratio of 1.5, equal to the amorphous predecessor of bone in a mineralization front[35]. Of all calcium phosphate scaffolds, hydroxyapatite is the most stable and least soluble[36], which has clinical implications. In order to provide bony ingrowth into the scaffold, at least part of this scaffold needs to dissolve or to be degraded by cells such as osteoclasts, multinucleated giant cells and macrophages[37]. In the case of hydroxyapatite, this process progresses is significantly slower than in the case of tricalcium phosphate.

In order to be able to form bone, calcium phosphate scaffolds need to adsorb proteins such as growth factors and extracellular matrix proteins. Also, they need to adhere to cells. These qualities depend on surface architecture and surface and micro-environmental charge. Material micro- and macroporosity are topics often discussed in the fabrication of calcium phosphate scaffolds. Microporosity (micropores have a pore size <10 μm) generally facilitates protein adhesion, whereas macroporosity (macropores are >80-100 μm in size) is about the material's capacity to be colonized by cells[38]. To summarize, there is a vast variety in types of calcium phosphate scaffolds, most of them are of the BCP type and vary greatly in granule size, porosity, surface roughness and HA to TCP ratio. The biphasic calcium phosphates used in the clinical trials and in vivo studies in this thesis have a HA/TCP fraction of <10%/>90%. This, because the underlying bone defect needs to be repaired with a rapidly resorbing scaffold in order to facilitate dental eruption.

Aim of the study and thesis outline

The overall aim of this thesis is to investigate a viable alternative for using autologous bone as grafting material for alveolar cleft closure in patients with unilateral cleft lip and palate.

Firstly, a systematic review on bone substitutes for alveolar cleft closure is conducted to find out which types of biomaterials are suitable for further clinical investigation (Chapter two).

A new protocol for accurate volumetric assessment of the reconstructed alveolar cleft is described in Chapter three. This, in order to reliably assess the success of the biomaterial of choice in the following clinical trial.

Chapter four describes the results of both a pilot study and a two-center clinical trial using a beta tricalcium phosphate bone void filler for alveolar cleft repair.

Chapter five reports on an *in vivo* study on Dutch milk goats using the same biomaterial as in the clinical trial, but now the tricalcium phosphate is embedded in a putty matrix. This is done to improve surgical handling and initial volume stability of the scaffold.

A general discussion is carried out and future perspectives are put forth in Chapter six.

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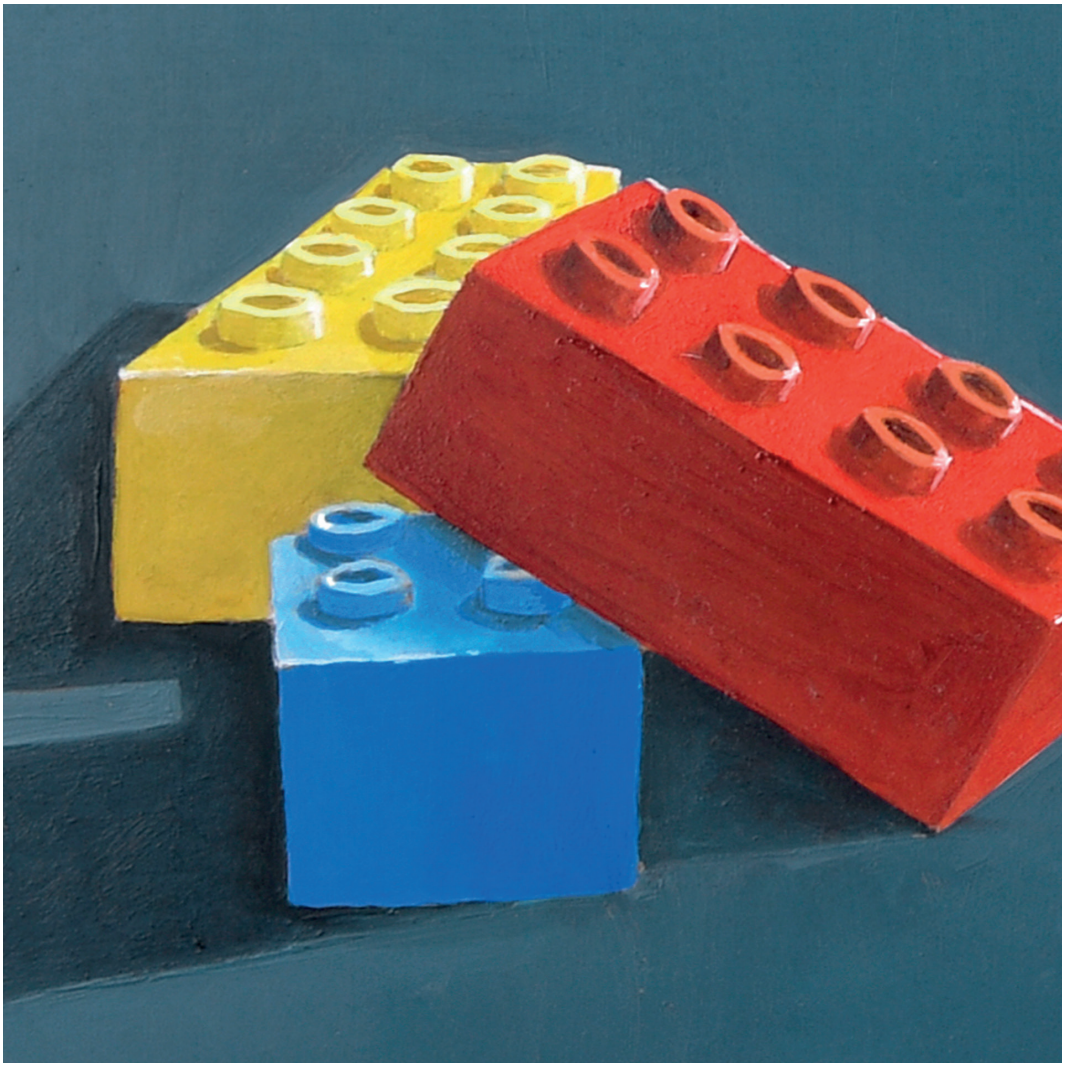

CHAPTER 2

Choosing the Right Biomaterial

Based on the article

Janssen NG, Weijs WL, Koole R, Rosenberg AJ, Meijer GJ. **Tissue engineering strategies for alveolar cleft reconstruction: a systematic review of the literature.** Clin Oral Investig. 2014 Jan;18(1):219-26

Abstract

To date, a great number of tissue engineering strategies have been suggested for alveolar cleft reconstruction, however, autologous bone grafting still remains the golden standard. A systematic review of the literature was conducted in order to evaluate the clinical evidence pertaining to enhancement or replacement of the autologous bone graft in the alveolar cleft by means of tissue engineered substitutes. 16 articles were selected for analysis. Tissue engineering strategies for alveolar cleft grafting include: Enhancing the autologous bone graft by means of platelet-rich-plasma (PRP) addition, the use of barrier membranes and fibrin glue, extension of the autologous graft with calcium phosphate scaffolds and replacement of the graft using Bone Morphogenetic Protein-2 (BMP-2), mesenchymal stem cells or calcium phosphate scaffolds. Selected articles showed a vast heterogeneity in data acquisition and patient selection. Therefore a meta-analysis could not be performed. However, the use of BMP-2 and calcium phosphates show promising results for the future.

Introduction

In patients with an alveolar defect, due to a cleft lip, alveolus and palate bone, grafting is an essential procedure. Primary aims are restoration of the continuity of the maxillary arch, closure of the oronasal fistula, eruption of teeth into the grafted area and support of the affected alar base [1-5].

The gold standard for repair of the alveolar cleft is the use of an autologous bone graft harvested mainly from the iliac crest or from the mandibular symphysis [6-8]. Nevertheless, even though these methods have proven to be effective and showed a high percentage of success [9, 10], both carry along donor site complication risks and postoperative comorbidity. As such, iliac crest harvesting procedures can result in paraesthesia[11], hypersensitivity[12] infection and pelvic instability[13, 14], whereas chin bone grafting may induce paraesthesia and apical root damage[15-17]. In order to avoid these adverse effects, tissue engineering strategies may eliminate donor site morbidity by bypassing the harvesting procedure. Although less spectacular, tissue engineering can also be used to extend the harvested autologous bone volume or to improve the quality of the grafted bone.

Since the mid-seventies, many reports have been written about the reconstruction of alveolar cleft defects in creating or enhancing bone growth by means of non-autologous grafting methods[18], with a publication peak in the last few years. Still today, after almost 40 years of research, autologous grafting remains the golden standard.

This systematic review was conducted in order to evaluate the clinical evidence pertaining to enhancement or replacement of the autologous bone graft in the alveolar cleft by means of tissue engineered substitutes.

Materials and methods

Search protocol and selection of articles

Online and manual search was conducted of Medline and EMBASE databases and the Cochrane library entering the following search terms: “(alveolar) cleft” or “cleft palate” (MeSH term) or “cleft lip” (Mesh term) and “graft” or “repair” or “transplants” (MeSH term) or “transplantation” (MeSH term). All references in the retrieved articles were searched. Results were limited to humans, alveolar cleft, grafting and tissue engineering. All searches were conducted up to January 2012.

Unique search results were checked on basis of title or abstract screening. Each retrieved result was judged by two independent reviewers and any disagreement was resolved by consensus. Articles that met the selection criteria were evaluated by means of full text analysis. In order to properly ascertain outcomes of tissue engineered alveolar cleft reconstructions compared to autologous bone grafting only randomized controlled trials, non-randomized controlled trials and case-control studies were included. Case reports, uncontrolled case series and animal studies were excluded.

Data analysis

Selected articles showed an extensive heterogeneity in age at operation, follow-up methods, interpretation of the performed methods and follow-up time schedules. A reliable meta-analysis on a statistically sound basis could not be performed. Therefore authors decided to analyze the data on a descriptive level.

Results

Three different tissue engineering strategies in alveolar cleft grafting could be discerned (Table 1): Firstly, solutions that enhanced the autologous bone graft, in which the aim was to improve the bone quality of the autologous bone graft[19-27]. Secondly, graft extenders have been used in situations where it is difficult to harvest enough autologous bone[28, 29]. The third category contains studies in which the autologous bone graft has been entirely replaced by a synthetic material[30-34].

Author (year)	Number of patients Cases/Controls	Study type	Study group biomaterial	Control group	Volumetric imaging	Conclusion
Enhancement of the autologous graft						
Duskova (2006)	36/9	Controlled	Bio-Gide and Hypro Sorb membranes with iliac crest bone	iliac crest bone	no	Data on bone formation difference between groups was not published
Lee (2009)	30/30	Retrospectively controlled	PRP/iliac crest bone	iliac crest bone	no	No significant difference in qualitative bone analysis
Luaces-Rey (2010)	10/10	Retrospectively controlled	PRP/iliac crest bone	iliac crest bone	no	No significant difference in bone formation
Marukawa (2010)	14/6	RCT	PRP/iliac crest bone	iliac crest bone	no	Higher bone width and height in the study group
Oyama (2004)	7/5	Retrospectively controlled	PRP/fibrin glue/iliac crest bone	iliac crest bone	yes	Greater bone volume in study group
Clavijo-Alvarez (2010)	15/20	Retrospectively controlled	Acellular dermal matrix/iliac crest bone	iliac crest bone	no	No significant difference in bone graft incorporation
Peled (2005)	10/5	RCT	Resorbable and non-resorbable membranes/iliac crest bone	iliac crest bone	no	Resorbable membrane group showed a significantly greater radiological fill of the defect
Segura-Castillo (2005)	13/14	RCT	Fibrin glue/iliac crest bone	iliac crest bone	yes	Significantly larger bone volume in the study group
Mendez (2006)	Excluded	Retrospectively controlled				
Extension of the autologous graft						
Weijs (2010)	18/29	Retrospectively controlled	B-TCP/chin bone	chin bone	no	No significant difference in bone height
Thuaksuban (2010)	14/13	RCT	Bovine hydroxyapatite/iliac crest bone	iliac crest bone	no	No significant difference in bone density and height
Replacement of the autologous graft						
Alonso (2010)	8/8	RCT	BMP-2/collagen sponge	iliac crest bone	yes	No significant difference in bone volume, significantly less bone height in study group
Herford (2007)	10/2	Retrospectively controlled	BMP-2/collagen sponge	iliac crest bone	yes	No significant difference in postoperative bone volume
Dickinson (2008)	9/12	RCT	BMP-2/collagen sponge	iliac crest bone	yes	Bone mineralization and volume are significantly higher in the study group
Gimbel (2007)	21/48	Prospectively controlled	MSC's/collagen sponge	iliac crest bone	no	Data on bone formation was not published
Benlidayi (2012)	11/12	Retrospectively controlled	Bovine hydroxyapatite	iliac crest bone	no	No significant difference in bone height and density

Enhancing the autologous bone graft

PRP's

Five articles related to the use of PRP's in conjunction with grafting of iliac crest cancellous bone, which met our criteria, were found. In all these studies patients grafted with iliac crest cancellous bone served as a control group. Of them, four were retrospective controlled studies[21, 22, 24, 25] and one a randomized, unblinded, controlled trial[23].

Because patient's inclusion in the articles, as published by Mendez et al. (2006) and Luaces-Rey et al (2010), took place in the same hospital with a significant time-overlap, the article with the smallest study population (Mendez et al.) was excluded due to patient selection bias. Luaces-Rey et al. reported no statistically significant differences in bone formation between the study and control groups. Bone formation was measured by means of conventional radiography up to 6 months postoperatively and analyzed using an observer scoring system. With respect to bone quality also Lee et al. did not find a significant difference between study and control groups using conventional radiography or qualitative computer analysis up to 12 months postoperatively.

In contrast, the randomized controlled trial by Marukawa et al indicated a significant difference in favor of the study group both in width (study group: 26.5±0.71% loss of bone width versus control group: 35.5±2.12% loss of bone width) as in height (study group: 1.42±0.18% loss of bone height versus control group: 2.09±0.36% loss of bone height) of the regenerated bone after one year, whereas bone density did not differ significantly. Bone formation was measured using conventional radiography as also multislice CT-scans up to 1 year postoperatively, however, analysis methods were not described. Also Oyama et al. reported better results when both fibrin glue and PRP's were added to the cancellous iliac crest bone; the residual bone volume in the study group (80.19±6.77%) was significantly higher than for the control group (63.67±13.94%), 5-6 months postoperatively. Volumes were calculated by means of multislice CT-scan analysis.

Membranes and tissue engineered mucosal substitutes

Three articles were identified that reported membranes or acellular dermal matrices to cover the cancellous iliac graft. Of them, one was a randomized controlled trial[26] and two were non- randomized controlled trials[19, 20]. All control groups consisted of patients solely grafted with cancellous iliac crest grafts.

Clavijo Alvarez et al. found no benefit of the use of acellular dermal matrix membranes to cover the cancellous iliac graft; no significant differences were observed on occlusal radiograph evaluation by means of the Chelsea scale between study and control groups 6 and 12 months postoperatively. In contrast, positive results were reported for resorbable membranes[26]. These authors described two study groups: one group in which the cancellous iliac graft was covered with a non-resorbable expanded polytetrafluoroethylene (ePFTE) membrane, which was removed 3-5 months post treatment. In the second group the cancellous iliac graft was covered with a resorbable polylactic-polyglycolic acid membrane. Conventional radiography was performed 2-6 years postoperatively; regenerated bony cleft surfaces were calculated with analysis software. The resorbable membrane group yielded a significantly greater radiological fill of the defect (177.04±30.9 mm^2), as opposed to the ePFTE study group (20.51±57.3 mm^2) and the control group (41.69±31.5 mm^2). Duskova et al. studied cancellous iliac transplants, which were covered by a BioGide® (Geistlich Biomaterials, Wolhuser, Switzerland) or Hypro-Sorb® (Hypro, Otrokovice, Czech Republic) membranes. Conventional radiography was performed 12-14 weeks postoperatively. Since both results and method of analysis were not described, this study was excluded in retrospect.

Fibrin glue

One study, by Segura-Castillo[27] et al., assessed the use of fibrin glue in conjunction with cancellous iliac grafting. The control group consisted of patients grafted with cancellous iliac crest grafts alone. Multislice CT-scans were performed after 3 months. Unclear is how the bone volume was measured, however, bone volume was significantly higher in the study group (64.32±33.70cm^3), as compared to the control group (21.70±21.96cm^3). No significant difference in bone density, as measured according to the classification introduced by Lekholm and Zarb, or bone quality, as scored according to the method of Norton and Gamble, were noted between study and control group.

Extending the autologous bone graft

Calcium phosphates

In two studies calcium phosphates were used in addition to the autologous bone graft. Weijs[29] et al. used a corticocancellous chin bone graft in study and control groups. The graft in the study group was protected with beta-tricalcium phosphate (β-TCP) granules. Care was taken to fill the central part of the alveolar defect with autologous bone. Only at the outer border of the reconstructed defect granules were positioned to serve as a barrier. Alveolar bone height was assessed one year postoperatively through occlusal radiographs. There was no significant difference between study and control groups in alveolar bone height. Thuaksuban et al.[28] performed a randomized controlled trial in which study and control groups were grafted with cancellous iliac bone and the graft in the study group was extended. e.g. mixed with deproteinized bovine bone particles (MTEC, Pathumthani, Thailand). Analysis of the outcome in bone height and density between both groups was performed by means of occlusal radiography up to 24 months postoperatively. No statistically significant difference with respect to bone density and height between study and control groups was scored.

Replacing the autologous bone graft

Bone Morphogenetic Protein-2

Two randomized controlled trials[30, 32] and one retrospective controlled trial[34] investigated the use of bone morphogenetic protein-2 (BMP-2), as a replacement for autologous bone grafting in alveolar cleft repair. All study groups were grafted with recombinant human BMP-2 soaked collagen sponges, all control groups were grafted with an iliac cancellous bone graft. Herford et al. compared bone volume, as calculated in multi slice CT-scans acquired 4 months postoperatively. No significant difference in postoperative residual bone volume between study and control groups was observed. Also after a period of 12 months, no significant difference in residual bone volume as calculated from multislice CT-scans was reported (Alonso et al.). However, bone height remained shorter in a statistically significant level in the study group, compared to the control group (65±1.9% of the bone height at 1 month postoperatively compared to 86±1.4%). Dickinson et al performed CBCT-scans up to 6 months postoperatively and also calculated the residual bone volume. These authors

reported a significantly higher amount of residual bone volume (95% vs. 63%%, no SD's reported) in the study group.

Calcium phosphates

One study, by Benlidayi et al[31], compared bovine-derived hydroxyapatite (Unilab Surgibone, Mississauga, Canada) to cancellous iliac crest bone in the control group. Conventional radiography was performed at a mean postoperative time of 67.82±10.36 months for the study group and 47.33±13.79 months for the control group. Bony ingrowth was evaluated. As measured with the Chelsea scale, no significant difference in filling of the cleft between both groups was noted. CBCT was performed at an unknown time postoperatively to assess bone density at the cleft site. Bone density in the bovine HA group was significantly higher than in the non-cleft site, whereas in the study group this was not the case.

Stem cells

Gimbel et al.[33] performed a prospective controlled clinical trial. The study group was grafted with *in vitro* expanded autologous bone marrow aspirate derived from the iliac crest, where after seeded on a resorbable collagen matrix. The control group was grafted with cancellous iliac crest grafts harvested in two different ways. Data on bone quantity or quality was not provided in this article, instead the author refers to unpublished results presented on a conference[35].

Discussion

The bone defect

The alveolar cleft bone defects comprise a rather heterogeneous group of bone defects with a large variation in volume as well as in surface and in number of bony walls. A vast number of alveolar clefts show spontaneous alveolar bone formation after performing a gingivoperiostoplasty alone[36-38]. As always a gingivoperiostoplasty is conducted, not all alveolar cleft bone defects can be assessed as being of critical size[39]. Whether the grafted bone or other grafting material merely functions as a haematoma stabiliser or has additional osteoinductive or osteogenic effect still remains unclear for this reason.

Timing of surgery

Timing of surgery is a profound confounding factor in alveolar cleft grafting surgery. Therefore, comparing studies with different timing of surgery brings forth serious bias. In addition, mean age published in a great number of studies does not always take into account the dentition stage of the patient, although this is of great importance [40].

One can roughly discern three timing strategies: 1) primary alveolar bone grafting during the first stage of dentition, 2) secondary alveolar bone grafting during the mixed stage of dentition and 3) tertiary bone grafting after the mixed stage of dentition [41]. With respect to alveolar defect restoration, tertiary alveolar grafting often shows less successful results than grafting at earlier stages while primary alveolar bone grafting is considered to put the greatest strain on further maxillary growth[10, 42]. Furthermore, when the grafting procedure is timed within the period of eruption of the lateral incisor or canine into the cleft (secondary alveolar grafting), the erupting tooth will ensure the grafted bone is functionally loaded[43].

Outcome measures

One of the most important reasons a proper meta-analysis could not be performed in this review, was the extreme heterogeneity of outcome measures. Bone regeneration was monitored, either with conventional radiography or computer tomography, where in the last few years an increase in studies is observed using computer tomography. Selected studies showed an enormous spread in postoperative monitoring points and maximum follow up dates.

When comparing two-dimensional radiography with three-dimensional computer tomographic volumetry, two-dimensional radiographs show a clear underestimation of resorption in the transverse plane[44]. Volumetric assessment of residual bone is therefore more accurate in defining residual bone quantities. Furthermore, when using conventional two-dimensional radiography the reliability is even more questionable due to different magnification factors between different radiographs[29]. With the institutionalisation of 3-D analysis of CBCT and CT scans, the authors consider it ethically sound to assess postoperative bone formation with these media on a standard but minimal basis, though radiation dose of each CBCT or low dose CT scan should be carefully evaluated[45]. Taking biopsies for histological purposes in alveolar cleft grafting procedures is considered as non-ethical, except for the cases in which implant placement, or revisional surgery is performed.

Enhancing the graft

To inventory the advantages of adding PRP's to the bone graft, only one article used volumetric analysis[25]. Follow-up periods in all four studies were relatively short; two studies had a maximum follow-up period of six months[22, 25], and the other two 12 months[21, 23]. As firm conclusions cannot be made since the significant amount of heterogeneity, a tendency that PRP-enriched bone substitutes have little or no effect in bone graft enhancement, can be noted.

In the membrane and tissue engineered mucosal substitutes subsection, only one study showed a significantly higher radiological fill of the alveolar cleft defect. Despite the fact that in this study patients were randomized, overall methodology is lacking mostly because of a large spread in follow-up periods (two to six years).

The one article in which fibrin glue was added to the substitute showed a significant decrease of bone resorption, though it must be criticised that these results are calculated already after a three month follow-up period. Until recently, no circumstantial evidence is readily available that autologous alveolar bone grafting can be enhanced in such a way that it improves the overall treatment success.

Extending the graft

Expanding the autologous bone graft can be useful in situations where no sufficient autologous bone can be harvested, for example in the case of chin bone grafting. Another reason for harvesting less bone than needed can be to minimize donor site morbidity.

It must be taken into account that graft expanding needs to be as effective as the autologous bone grafting method. As such, the randomized controlled trial by Thuaksuban et al.[28] showed an equal success rate in bone height and density when mixing the iliac crest graft in a 1:1 volume ratio with bovine hydroxyapatite. Tooth eruption into the cleft was not compromised in the study group.

Weijs et al, showed in a retrospectively controlled study that expansion with a resorbable calcium phosphate matrix (β-TCP) can also be performed successfully by positioning the β-TCP granules at the buccal side of the graft and remaining minor spaces, in such a way that the central part of the cleft region was always filled by autogenous bone forming a bony bridge between the adjacent parts of the maxilla. No disturbance in tooth eruption

was observed. The major point of criticism on these studies pertains to the fact that two-dimensional radiographs were used to analyse a three-dimensional defect. However, bone graft extension with calcium phosphate matrices seems to be a safe and relatively successful method.

Replacing the graft

In all three studies, using rhBMP-2 infused collagen sponges, volumetric analysis for residual bone assessment was used. All reported equal or higher volumes in the study groups compared to the control groups. However, maximum follow-up periods were relatively short; up to 4[34], 6[32] and 12[30] months. Follow-up periods up to 6 months are generally considered too short to reliably ascertain *de novo* osteogenesis. Although above mentioned results sound very promising, one should not let pass by unnoticed the recent increase in reports on adverse effects of rhBMP-2 [46]. The more that in restoring alveolar clefts the use of rhBMP-2 is off-label and most patient are only between 10 and 15 years old.

As for graft replacement by means of bovine hydroxyapatite, the study by Benlidayi[31] et al. shows a 100% success rate and no significant difference in tooth eruption through the scaffold in the study group. However, postoperative radiological assessment through volumetric analysis was not performed. No differences as measured with the Chelsea scale was observed. However, this scale only assesses the position of the bone (or bone substitute) within the cleft in relation to the full length of the root surfaces adjacent to the cleft and cleft midline at eight sites. As such, nothing is reported about the amount of bone ingrowth between the implanted hydroxyapatite-granules. Although CBCT displayed that bone density in the bovine hydroxyapatite group was significantly higher than in the non-cleft site, this is a phenomenon, that is always is seen when the radiopaque hydroxyapatite-material is applied.

On the use of stem cells seeded on a resorbable collagen matrix, no results can be discussed, since the authors did not publish grafting outcomes in the selected article[33].

Conclusion

Articles on tissue engineering solutions for alveolar cleft grafting show a great amount of heterogeneity. Firstly, in selecting patient populations it is important to be informed whether primary, secondary or tertiary alveolar bone grafting procedures are performed. Furthermore, size and shape of the cleft defect play an important role in regeneration of the defect, and therefore should be measured. Although bone regeneration can be assessed by clinical investigation, conventional radiography, multislice CT or CBCT, volumetric imaging by means of CBCT is recommended. Unfortunately, biopsies, to obtain histological information, can only be retrieved if a second operation is indicated, such as is the case in revisional surgery or when implants are indicated and therefore will be an unfulfilled wish, also in the future. Maybe radiological techniques will improve and enable to differentiate between the implanted biomaterials and bone ingrowth itself.

Radiologic follow-up periods should be of sufficient length. Due to vast heterogeneity in confounding factors and lack of statistical data in a number of articles, a meta-analysis on selected articles could not be performed. However, it seems that graft replacing by means of rhBMP-2 soaked collagen sponges and graft extension with calcium phosphate scaffolds show promising results for the future.

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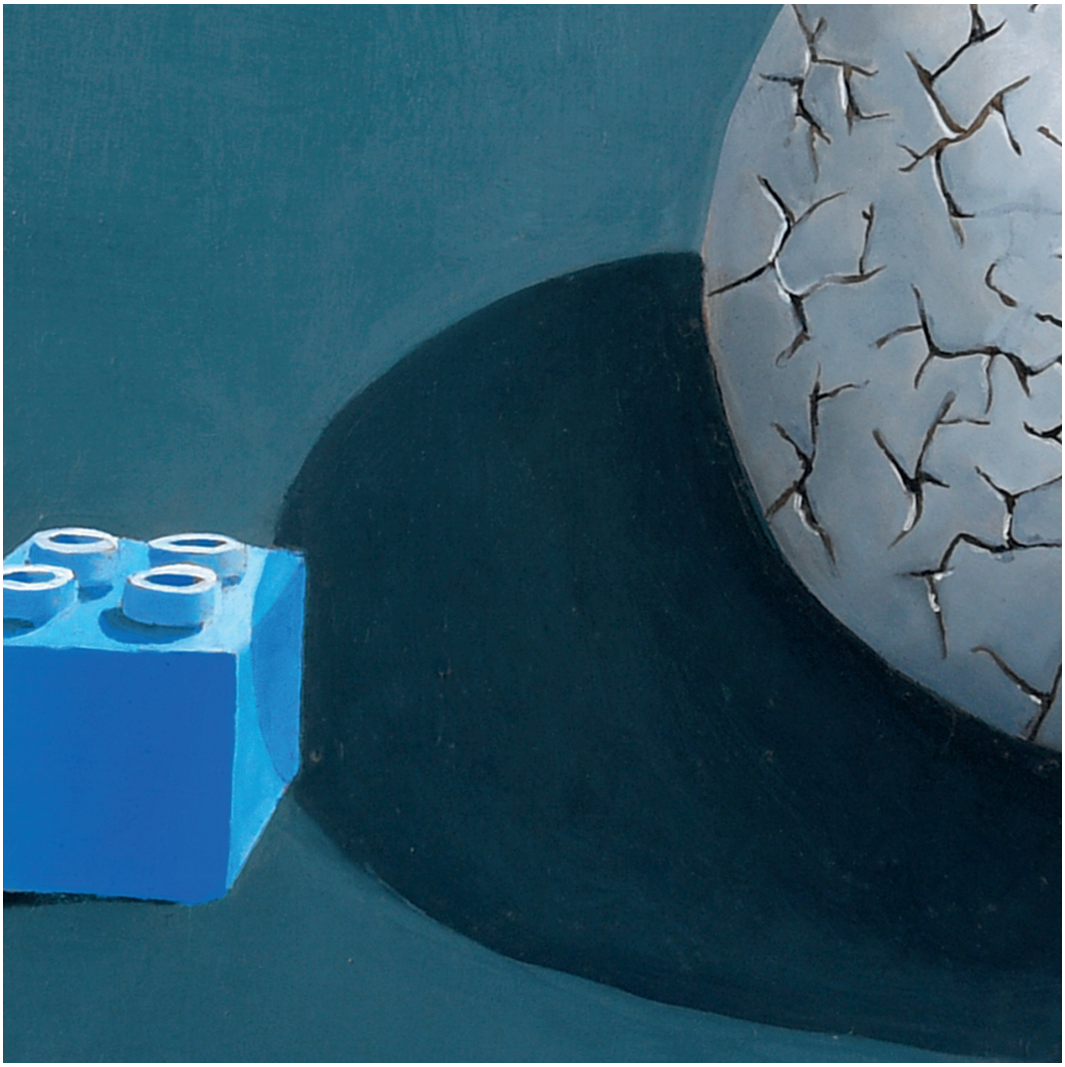

CHAPTER 3

Assessment of the Reconstructed Defect

Based on the article

Janssen NG, Schreurs R, Bittermann GKP, Borstlap WA, Koole R, Meijer GJ, Maal TJJ. **A Novel Semi-Automatic Segmentation Protocol for Volumetric Assessment of Alveolar Cleft Grafting Procedures.** J Craniomaxillofac Surg. 2017 May;45(5):685-689.

Abstract

A novel protocol for volumetric assessment of alveolar cleft grafting procedures is presented. Eleven cone-beam computer tomography (CBCT) datasets of patients who underwent secondary alveolar cleft reconstructive surgery for a unilateral alveolar cleft were evaluated by two investigators. Residual bone volumes one year after surgery were analysed using a semi-automated technique in which preoperative CBCT datasets are superimposed on the postoperative scans using voxel-based registration. To define the correct boundaries of the alveolar cleft defect in the preoperative CBCT dataset, a mirror image of the preoperative CBCT dataset was superimposed on the preoperative CBCT dataset. For the difference in residual bone volume between the two observers an intraclass correlation of 0.98 and a Dice coefficient of 0.89 were found. This study describes a reliable segmentation protocol for volumetric analysis of the alveolar cleft defect in patients with a unilateral alveolar cleft.

Introduction

Secondary alveolar bone grafting in cleft lip and palate patients has a number of qualitative and quantitative outcome parameters. Qualitative parameters consist of closure of the oronasal communication, establishing a continuous maxillary arch, enabling eruption of teeth into the grafted area and providing support of the alar base on the affected side. The need for quantitative analysis of residual bone volume after alveolar bone grafting procedures has been described extensively in modern day literature and has evolved parallel to the development of new radiological techniques. In the 80s and 90s quantitative assessment was mostly performed by means of two-dimensional radiological analysis [1-3]. In the last decade, to evaluate the fate of the transplanted bone, three-dimensional volumetry has gained significantly in popularity. Visualization of the reconstructed alveolar cleft defect in three dimensions makes overestimation of the regenerated bone stock less likely, which is frequently the case in two-dimensional analysis of the defect[4, 5]. For this purpose, cone-beam computed tomography (CBCT) is a widely used modality providing relatively accurate visualization of bone in the maxillofacial region at a favorable radiation dose using short exposure times [6-8].

One of the main problems that arises when using CBCT in bone volumetry is the low contrast, distortion of grayscale values and Hounsfield units[9]. Therefore, due to problems defining a threshold, an adequate assessment of the bone volume present cannot always be performed. Another problem using volumetry on the restored alveolar cleft defect is that, after complete re-ossification of the graft, it remains unclear where the former bony boundaries of the defect were situated. This impedes accurate analysis of the residual volume. Furthermore, when analyzing secondary alveolar bone grafting, radiological analysis is performed in a growing skeleton with eruption of the permanent dentition which also makes it harder to compare postoperative scans with the preoperative situation.

In determining the success of alveolar bone grafting procedures, residual bone volume is an important parameter that effects postoperative orthodontic and dental treatment strategy, as well as the skeletal support of soft tissues. Therefore, a reliable and reproducible approach of volumetric assessment is essential.

This study investigates the reproducibility and accuracy of a novel 3D-protocol for volumetric measurement of the reconstructed alveolar cleft.

Materials and Methods

Patients

CBCT datasets of eleven randomly selected patients (6 males, 5 females) with a unilateral cleft lip, alveolus and/or palate, who underwent secondary alveolar cleft reconstructive surgery between September 2007 and February 2012 were evaluated. Surgery was performed by a single surgeon (WB). The mean age at surgery was 10.0 years (sd 0.5 years). The surgical procedure was timed when one-quarter to one-half of the final root length of the canine adjacent to the alveolar cleft was formed, as indicated by the radiographic appearance of the root length equal to that of the crown [10]. During surgery, alveolar cleft closure was performed by creating nasal, buccal and palatal layers by means of watertight suturing with Vicryl™ (Ethicon, Brussels, Belgium). For the grafting procedure, a bicortical bone block was harvested from the mandibular symphysis.

Data acquisition

CBCT scans were acquired one week preoperatively (T0) and one year postoperatively (T1) using the iCat 3D Imaging System (Imaging Sciences International Inc, Hatfield, PA, USA). A standard CBCT scanning protocol was applied (iCAT™, 3D Imaging System, Imaging Sciences, International Inc., Hatfield, PA, USA). Scans were performed at 120 kV and 3-8 mA pulse mode. The field of view was 13 cm, scan time was 20 seconds and the voxel size was 0.4mm. Patients were scanned in natural head position. The image data were exported in DICOM format. The T0 image volume was imported in Matlab™ (v2012b, The Mathworks Inc., Natick, MA, USA), mirrored ($T0_{mirror}$) and subsequently exported in DICOM format.

Three-dimensional virtual head models were reconstructed from the T0, $T0_{mirror}$ and T1 image volumes in Maxilim™ software (version 2.3.0, Medicim NV, Mechelen, Belgium). The $T0_{mirror}$ image volume was superimposed on the T0 volume using voxel-based registration using the posterior part of the maxilla as a reference, since this region was unaffected in both the original and mirror image [11]. Subsequently, using the same reference, also the T1 volume was superimposed on the T0 volume through voxel-based matching. This resulted in three aligned image volumes (T0, $T0_{mirror,aligned}$ and $T1_{aligned}$ as shown in 1) [12].

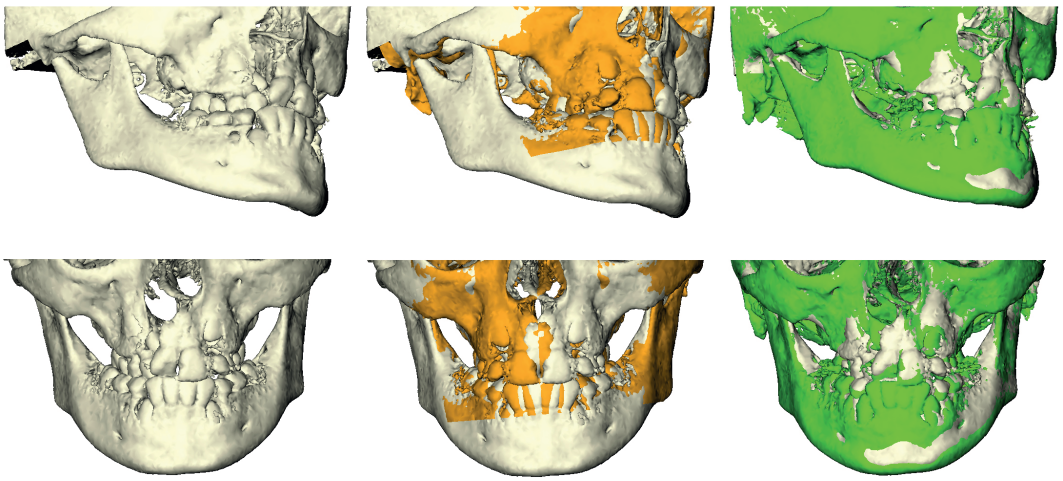

Figure 1. Three dimensional virtual head models. In white T0 (preoperative situation) is depicted. The orange superimposition represents $T0_{mirror,aligned}$ (the mirror image of the preoperative situation). The green superimposition represents $T1_{aligned}$ (the one year postoperative situation).

Data segmentation

The image volumes were imported in a Graphical User Interface in Matlab™ for further image processing. Segmentation of the residual bone volume after secondary alveolar reconstruction was generated by two observers (NJ, consultant maxillofacial surgeon and GB, resident maxillofacial surgery), applying the following protocol. A semi-automatic segmentation protocol was used to acquire a segmentation of the maxillary border adjoining the alveolar cleft defect (S0) [13]. Segmentation of the maxilla was also generated in the mirrored image volume ($T0_{mirror}$), using the same segmentation protocol ($S0_{mirror}$). In the T1 image volume, the residual alveolar cleft bone volume one year postoperative to surgery was delineated (S1). Semi-automatic post-processing and manual postprocessing was performed to correct small deviations in the segmentation (Figure 2).

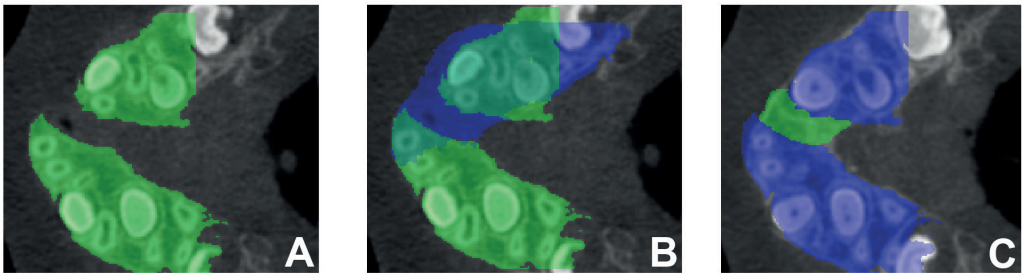

Figure 2. Segmentation of the preoperative and postoperative scans. A. Segmentation of S0, shown in green: Maxillary bone adjoining the alveolar cleft in the T0 (preoperative) scan. B. Superimposition of $S0_{mirror, aligned}$ (segmentation of bone in the mirror image of the preoperative situation), shown in blue, on the T0 scan with segmentation of bone (S0) shown in green in order to assist in reliably assessing the alveolar cleft defect's buccal and palatal borders. C. Superimposition of the S0 (preoperative) bone segmentation shown in blue on the T1 scan (one year postoperative). The residual bone volume is successively segmented in green (S1).

Selection of the region of interest

The preoperative alveolar cleft defect was defined as the segmented region in $S0_{mirror}$. It is confined laterally by the maxillary borders delineated in S0 (set difference: ($S0_{mirror}$\S0)). Anteriorly and posteriorly, the defect size was limited by the hard-tissue boundary of the aligned unaffected mirrored side of the maxilla ($S0_{mirror}$). Cranially, the defect was restricted by the lowest part of the piriform aperture at the non-affected side and caudally by the cemento-enamel junction of the central incisor most proximal to the cleft region. Laterally, the defect was limited by a plane parallel to the median plane, through the piriform aperture of the affected side. Dorsally, a plane perpendicular to the median plane, through the bucco-mesial cusps of the first molars limited the defect. The regenerated bone volume one year postoperatively was defined as the bony region within the preoperative alveolar cleft defect [set union: (S1 U ($S0_{mirror}$\S0))]; residual bone outside the original defect was not included in the measurements. Residual bone volume was calculated for each reconstructed alveolar cleft segmentation.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows (version 21, IBM Corp., Armonk, NY, USA). For descriptive statistics of the population, the mean and standard deviation of the preoperative defect sizes and residual bone volumes were calculated.
The intraclass correlation coefficient (ICC) was calculated for the defect sizes and residual

bone volumes of both observers as a measure of conformity between the observers. A 2-way mixed model (absolute agreement) was used; an ICC score <0.40 was considered as poor agreement, 0.40-0.59 as fair agreement, 0.60-0.74 as good agreement and 0.75-1.00 as excellent agreement. Inter-observer reliability of the segmentation protocol was assessed by calculation of the Dice-coefficient in all patients. The formula for calculation of the Dice-coefficient is given below [14]. The coefficient calculates the overlap between two segmentations and ranges from zero to one, where a Dice-coefficient of 0 indicates 'no overlap' between the segmentations of the observers and 1 indicates 'perfect overlap' between the segmentations. A Dice-coefficient >0.7 is generally considered as good [15]. A visual explanation of the Dice coefficient and the relation to the ICC score is provided in 3. The formula for the dice coefficient is:

$$Dice = \frac{2\times|A\cap B|}{|A|+|B|}$$

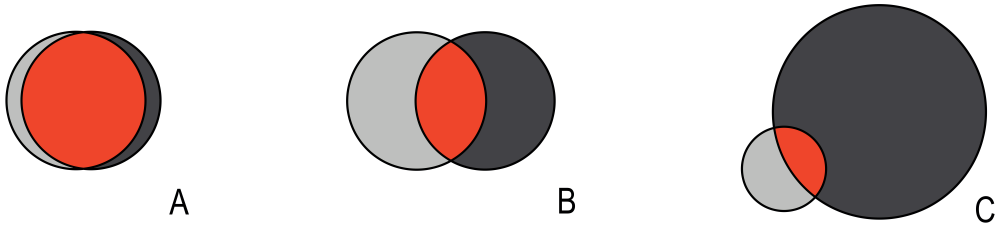

Figure 3. Difference between intraclass correlation (ICC) and dice correlation. Grey circles represent two surface area measurements. Regions with spatial overlap are shown in red. A. A high dice score (large percentage of overlap and equal surface area) and a high ICC. B. A low dice score with a high ICC value (only surface area is equal). C. A low dice score and a low ICC value.

Results

Reliability of the method used

The Dice-coefficient for segmentation of the preoperative defect size between the two observers was 0.93 (sd 0.02). The mean volumetric difference between observers was 0.05 cm3 (sd 0.05), which represents a mean difference of 5.1% (sd 3.9%) of the defect volume. The ICC between the measured volumes of the observers was 0.98 (95% ci [0.91-0.99]).

For the regenerated bone volumes, a Dice-coefficient of 0.89 (sd 0.03) was found. The mean volumetric difference was 0.03 cm3 (sd 0.02), which represents 6.1% (sd 3.7%) of the regenerated bone volume. The ICC between the observers was 0.98 (95% ci [0.91-1.00]).

Bone volume

The mean preoperative defect size was 0.83cm3 (sd 0.28). The postoperative mean residual bone volume was 0.47 cm^3, with a standard deviation of 0.17 cm^3. These findings show that one year after surgery still 61.6% on average (sd 23%) of the original defect volume was filled with transplanted bone.

Discussion

Three-dimensional analysis of the reconstructed alveolar cleft defect has become the method of choice for quantitative assessment of the postoperative bone stock. With the introduction of biomaterials to replace the autologous bone graft, such as calcium phosphate scaffolds and the use of rhBMP-2 , the need to compare the effect of the various grafting methods has risen significantly [7, 8, 16]. However, defining regenerated alveolar bone volume is subject to a certain amount of inaccuracy, depending on the diagnostic method used.

Quantification of residual bone volume in the reconstructed alveolar cleft is difficult for two reasons. Firstly, on a CBCT scan it is challenging to discriminate between pre-existing bone and regenerated bone. The second contest lies in defining the borders of the pre-existing alveolar cleft defect, since cranio-caudal and bucco-palatal boundaries are also often difficult to discriminate. The method presented overcomes these two problems by 1) voxel based matching of the T0 and T1 scans and 2) by mirroring the contralateral, unaffected side over the alveolar cleft defect to delineate the original defect's bony boundaries. An excellent intraclass correlation (ICC) of 0.97 was found for measurement of the preoperative defect size; even a higher ICC (0.98) was noted for the measurement of the regenerated bone volume. The ICC relates the amount of variation between observers to the amount of variation between patients; the high variation in cleft size and regenerated bone volume between patients has a positive effect on the found ICC. The Dice-coefficient does not suffer from this positive bias: it measures the amount of overlap of the segmentation on voxel level, and is thus better suited to truly evaluate observer differences within the protocol . A mean Dice-score of 0.93 (sd 0.02) was found for preoperative defect size, whereas a Dice-score of 0.89 (sd 0.03) was found for regenerated bone volume; although lower than the ICC values, both indicate good overlap in the segmentations for all patients.

Previous studies already described volumetry as method to assess the restored alveolar cleft defect [7, 8, 17, 18]. However, the reliability of the methods used in these studies is debatable. Moreover, in some studies reproducibility was not mentioned at all. If reproducibility was reported, correlation was determined between the measured volumes; to the best of our knowledge, no other studies reported overlap measures of the segmentations. When analyzing samples that have high individual variations in size, such as the alveolar cleft defect, standard inter- and intra-observer correlation tend to end up higher than those truly are, as was explained before. Some authors reported methods delineating the defect preoperatively and postoperatively on individual axial CBCT slices. However, taking into account the current development of image superimposition, older methods that discerned between newly formed bone and preexisting bone without this technique make delineation of the postoperative reconstructed alveolar cleft arbitrary. Dickinson et al [8]. reported an interrater error of their method of 1.9%; Alonso et al [7] only stated that their method *'demonstrated no statistically significant difference in intra-operator measures'*. The same method was used by our study group in 2015 (De Ruiter et. al., 2015). Canan et al. [17] did not report any intraobserver or interobserver statistics. [18] used manual segmentation for calculation of preoperative defect size and residual cleft defect volume postoperatively; however, no intra-observer and inter-observer statistics were reported.

A semi-automatic segmentation protocol, different from the one used in this study, was introduced by Linderup et al. [6] The intra-observer and inter-observer variability were assessed by means of a Pearson correlation coefficient; values of 0.99 and 0.88 were found for the intra-observer and inter-observer measurements of the residual bone volume, respectively. The bucco-palatal defect size was estimated by the observers based on the bucco-palatal dimensions of the contralateral side. This may introduce observer variability. Moreover, accurate estimation of the bucco-palatal dimensions from the contralateral side on an axial slice may be hampered if a patient has not been positioned perfectly during CBCT acquisition, which could result in visualization of contralateral structures at a different height level in the same axial slice. Proper alignment of left and right-sided structures can only be achieved through image superimposition of a mirrored image volume, as presented in this study. Image superimposition of the contralateral side over the defect could also reduce observer dependency in identifying the bucco-palatal dimensions, since only minimal user interaction is required in the voxel-based matching protocol.

This study showed that even when a semi-automated segmentation protocol is used, accurate assessment of postoperative alveolar bone volume on CBCT images in a reproducible manner is difficult. A number of possible explanations can be discerned: firstly,

acquisition of the T0 and T1 CBCT scans is performed at different time points in a growing craniofacial skeleton. Accurate superimposition is more difficult if the time interval between T0 and T1 is larger, as the migrating and erupting dentition may present difficulties in comparing preoperative and postoperative image volumes.

Compared to Multislice CT (MsCT), the use of CBCT imaging provides a few obstacles; the relatively low contrast of CBCT images could make it extra difficult to delineate the border between bony and non-bony structures. Furthermore, distortion of Hounsfield units in CBCT scans adds up to this problem [9]. Multislice CT (MsCT) scans do not suffer from these problems. However, the increment in radiation dose associated with MsCT imaging presents a great drawback, especially for follow-up imaging in the paediatric population.

In our opinion, clinical outcome parameters are as important, if not more important, than the results of volumetric analysis. In the end, success of the alveolar bone grafting procedure is determined by establishing a continuous maxillary arch that facilitates eruption and bony support of the lateral incisor and canine, a closed oro-nasal communication and adequate bony support of the alar base through volume increase of the piriform aperture. However, since volumetric analysis is a quantitative modality, it is very well suited to compare differences in alveolar cleft closure methods such as technique, surgical timing, and the choice for a specific grafting material.

Conclusion

In the past decade, volumetric analysis has become the method of choice to quantify the success of alveolar cleft grafting procedures. This study describes a reliable segmentation protocol for volumetric analysis of CBCT scans. The two major issues that affect its reliability are the relatively low resolution of the CBCT scans and the fact that the two scans that are superimposed are performed in a growing craniofacial skeleton in the mixed dentition stage with a time interval of one year.

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CHAPTER 4

Performing a Clinical Trial

CHAPTER 4a

Pilot Study

Based on the article

De Ruiter AP, Janssen NG, van Es RJJ, Frank MH, Meijer GJ, Koole R, Rosenberg AJWP. **Micro-structured Beta-Tricalcium Phosphate for Repair of the Alveolar Cleft in Cleft Lip and Palate Patients: A Pilot Study.** Cleft Palate Craniofac J. 2015 May;52(3):336-40.

Abstract

Can a synthetic bone substitute be used to repair the alveolar cleft to bypass donor site morbidity as well as to shorten the operating time? In earlier experimental studies, micro-structured beta-tricalcium phosphate (β-TCP) provided similar bone healing when compared with grafting with iliac crest bone. This justifies the clinical evaluation of this bone substitute in the human alveolar cleft situation. Seven patients, all with unilateral alveolar cleft, were randomly included for alveolar cleft repair with β-TCP in 2010 and 2011. In all patients the alveolar cleft was repaired by micro-structured β-TCP grafting. Our assessments were distilled from cone beam computed tomography scans taken preoperatively, 1 week postoperatively, and 6 months postoperatively. A volumetric outcome could be realized. Six months after the operative grafting of micro-structured β-TCP into the alveolar cleft, the bone volume thus acquired was satisfactory. We found an average bone volume percentage of 73% ± 6% compared with the original cleft volume. Previous experimental and clinical studies and the initial findings of this pilot study now elucidate a path toward the clinical use of micro-structured β-TCP bone substitute for repair of the alveolar cleft.

Introduction

The incidence of congenital facial clefts varies throughout the world and between ethnic groups[1]. In Europe, the occurrence of clefts of lip, alveolus, and palate among Caucasians has been reported to be between 0.69 and 2.35 per 1000 births[2]. Repair of the alveolar cleft by creating continuity of the alveolar process comprises more than the treatment of a local bony defect only[3]. It helps restore dental arch continuity, stabilizes the maxilla complex, and provides support to soft and cartilaginous tissue structures such as the alar base and upper lip[4,5]. The current gold standard for repair of the alveolar cleft is autologous bone graft harvested either from the iliac crest or from the mandibular symphysis[6-9]. Both grafting procedures carry potential risks for donor site morbidity, such as postoperative pain, hypersensitivity, pelvic instability, meralgia paresthetica, lateral femoral cutaneous nerve injury, infection, paraesthesia, apical root damage, and visible cutaneous scarring[10-17]. To prevent donor site morbidity, as well as to shorten surgery time, a synthetic bone substitute could be used to close the alveolar cleft. An in vivo study in a goat model has shown that grafting created alveolar clefts with pure-phase beta-tricalcium phosphate (β-TCP) provides similar bone healing to grafting with iliac crest bone[18]. In addition, when β-TCP was used in this model, orthodontic tooth movement proved to be similar. Although the exact mechanism of osteogenesis stimulated by β-TCP is still not completely understood, the aforementioned study justifies clinical evaluation in the human alveolar cleft situation. The aim of this pilot study (n = 7) was to generally evaluate the repair of the alveolar cleft after grafting with microstructured β-TCP.

Materials and methods

Patients

Permission to carry out this study was granted by the Medical Ethics Committee, University Medical Centre, Utrecht, the Netherlands, protocol No. 09-129.

Seven patients, all with unilateral alveolar cleft were randomly included for alveolar cleft repair with β-TCP in 2010 and 2011. The male-female ratio was 5:2, and the mean age of the patients was 11.16 years (SD, ±1.83 years). The timing of the surgical procedure was on orthodontic indication (i.e., the point at which formation of two-thirds of the root of the tooth (canine or lateral incisor) bound to erupt into the repaired graft was visible on radiograph). In all patients, the alveolar cleft was repaired with micro-structured β-TCP grafting.

Surgical Procedure

Alveolar cleft repair was performed under general anesthesia with nasoendotracheal intubation. Antibiotic prophylaxis was given intravenously for 3 days (clindamycin, Hameln Pharmaceuticals, Hameln, Germany; 10 mg/kg per 24 hours). The surgical technique as described by Koole in 1994[6] and used until now was carried out. All patients had a persisting oronasal communication. The alveolar cleft region was exposed by means of a marginal incision and a releasing incision in the buccal sulcus. After the nasal lining was prepared and closed, the alveolar cleft was filled with β-TCP mixed with autologous blood (Figs. 1 and 2). No membranes were used.

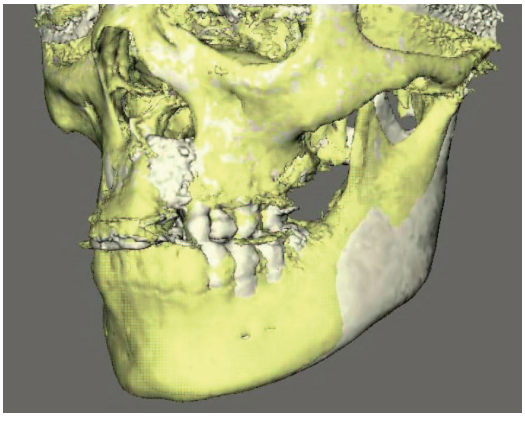

Figure 1: Micro-structured β-TCP granules inserted for repair of the alveolar cleft (CBCT scan).

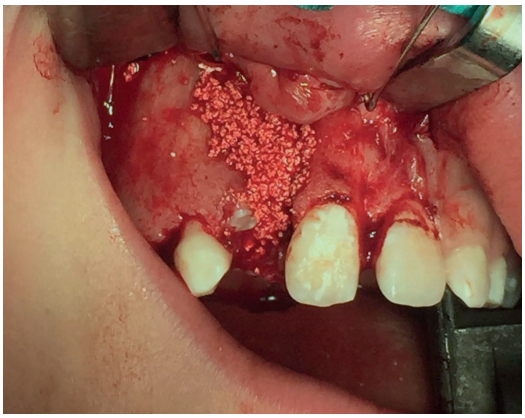

Figure 2: Micro-structured β-TCP granules inserted preoperatively for repair of the alveolar cleft.

The palatal mucosa was then sutured, and a vestibular mucoperiosteal layer was constructed with transpositional mucoperiosteal flaps. All sutures used were resorbable (Vicryl 4.0, Ethicon, Brussels, Belgium).

Orthodontic Procedure

Three months postoperatively, orthodontic therapy was started without actively engaging erupting teeth adjacent to the cleft. No orthodontic opening of the cleft gap was carried out.

Bone Substitute

The micro-structured β-TCP used in this study is a 65% ± 15% porous and >90% pure phase β-TCP graft material (X-Pand Biotechnology BV, Bilthoven, the Netherlands). It is synthetic, osteoconductive, osteoinductive, and resorbable[19].

Radiographic Assessment

In the study group, a cone beam computed tomography (CBCT) scan was carried out directly preoperatively, 1 week postoperatively, and after 6 months. Radiation hazards restrained us from using conventional CT scanning. Parallel to this new radiographic protocol, conventional postoperative radiology by means of orthopantomography, occlusal radiography, and lateral cephalometry was also carried out. The CBCT scan analysis was performed by two independent investigators using Osirix Dicom Viewer[20] (Apple Inc., Cupertino, CA). Each investigator performed the analysis three times. Axial images were processed by demarcating the alveolar cleft using a free-form tool. The measured cleft region was assessed in the coronal plane from the lowest part of the non-affected piriform

aperture to the most caudal interdental alveolar bone level of the two proximal teeth in the large cleft segment. Thereafter, a three-dimensional model was calculated. The volume of the pre-existing bone defect was compared in percentages with the total graft volume and with the residual bone volume 6 months postoperatively. We also looked at the vertical eruption of adjacent teeth into the bone substitute graft from the transversal images of the scans.

Statistical Analysis

Repeated-measures analysis of variance (SPSS 15.0, SPSS, Chicago, IL) was used to determine the influence of the within-subject factors moment of measurement, examiner, and repetition. Subsequently, contrasts were determined to study the levels of the within-subject factors. $P < .05$ was considered significant.

Results

Bone Volume

The 6-month postoperative examinations showed progressive alveolar bone union in all but one patient, in whom there was total graft loss. We excluded this case from further analysis, as shown in Tables 1 and 2. Eruption of the adjacent canine or lateral incisor into the repaired cleft occurred spontaneously in all patients.

TABLE 1: Cleft Volumes Preoperatively, Postoperatively, and 6 Months After Operation			
	Cleft Volume Preop (cm^3)	Graft Volume Postop (cm3)	Bone Volume 6 Months Postop (cm3)
Patient 1	1.06	1.42	0.81
Patient 2	0.40	0.66	0.29
Patient 3	0.84	0.89	0.65
Patient 4	0.58	0.77	0.36
Patient 5	0.53	0.68	0.41
Patient 6	0.93	1.15	0.67
Patient 7	1.41-nc *	1.83-nc *	Loss of graft
Mean	0.72	0.93	0.53
SD	0.26	0.30	0.21

**nc: not calculated*

TABLE 2: Percentages of Cleft Volumes Preoperatively, Postoperatively, and 6 Months After Operation			
	Cleft Volume Preop (%)	Graft Volume Postop (%)	Bone Volume 6 Months Postop (%)
Patient 1	100	134	76
Patient 2	100	165	73
Patient 3	100	106	77
Patient 4	100	133	62
Patient 5	100	128	77
Patient 6	100	124	72
Patient 7	100	130-nc *	Loss of graft
Mean	100	132	73
SD		19	6

**nc: not calculated*

Statistical Analysis

Examiners did not have a significant influence on the determination of the bone volume (P = .58). Also, repetition of the determination did not significantly influence the outcomes (P = .21). The moment of the measurement, however, significantly influenced the bone volume outcomes (P < .001).

In Tables 1 and 2, a significant increase in bone volume was observed directly after operation (P < .005). The volume increased from on average 0.72 cm3 to 0.93 cm3, an increase of 32%. A significant decrease in volume occurred in the following 6-month period, from 0.93 cm3 to 0.53 cm3 (P < .001). The bone volume after 6 months was significantly lower than the initial value, 73% (P < .001). The results are depicted in Tables 3 and 4 with the data including the lost graft case.

TABLE 3 Cleft Volumes Preoperatively, Postoperatively, and 6 Months After Operation, Including the Lost Graft

	Cleft Volume Preop (cm^3)	Graft Volume Postop (cm3)	Bone Volume 6 Months Postop (cm3)
Patient 1	1.06	1.42	0.81
Patient 2	0.40	0.66	0.29
Patient 3	0.84	0.89	0.65
Patient 4	0.58	0.77	0.36
Patient 5	0.53	0.68	0.41
Patient 6	0.93	1.15	0.67
Patient 7	1.41	1.83*	Loss of graft = 0.00
Mean	0.82	1.06	0.46
SD	0.35	0.44	0.28

TABLE 4 Percentages of Cleft Volumes Preoperatively, Postoperatively, and 6 Months After Operation, Including the Lost Graft

	Cleft Volume Preop (%)	Graft Volume Postop (%)	Bone Volume 6 Months Postop (%)
Patient 1	100	134	76
Patient 2	100	165	73
Patient 3	100	106	77
Patient 4	100	133	62
Patient 5	100	128	77
Patient 6	100	124	72
Patient 7	100	130	Loss of graft = 0
Mean	100	131	62
SD		18	28

Discussion

Six months after the operative grafting of microstructured β-TCP into the alveolar cleft, the bone volume thus acquired was satisfactory.

This leads to the assumption that the bone formation in the alveolar cleft after grafting it with micro-structured β-TCP was successful. We pixel-counted and compared the outcome as volumetric percentages, bearing in mind that the number of patients (n = 7) characterizes this study as a pilot study.

As low as reasonably achievable (ALARA) is an accepted principle in medicine and dentistry that dictates that health care professionals should use the smallest amount of radiation required to produce the information needed to diagnose and treat the patient[21]. Conventional medical CT scanning of a maxilla subjects the patient to 200 to 300 times the amount of radiation required for a panoramic radiography. Cone beam scanners use a narrow, collimated cone beam of radiation that can scan the maxilla only. This requires only 2 to 8 times the amount of radiation used in a panoramic radiograph. Compared with the two-dimensional data delivered by panoramic radiographs, the three-dimensional data generated by a CBCT provide outstanding information about alveolar cleft repair, thereby justifying its higher radiation load.

Overfill repair of the alveolar cleft with micro-structured β-TCP showed a mean fill percentage of 131.5% with regard to the original cleft. The percentages of acquired new bone volume with regard to the original cleft showed a mean percentage of 73%. This means a considerable resorption has occurred as also occurs when grafting the cleft with autologous bone. In general, these findings are corroborated in the literature[22-27].

Repairing an alveolar cleft does obviously not mean that the alveolar process needs to be completely restored. Restoration of only 73% of the original cleft size bone volume apparently satisfies the functional load-bearing requirements of the roots of the teeth in terms of width and height of the alveolar process.

On the issue of filling the alveolar cleft defect, it is paramount to first assess which part of the defect needs to be filled with bone to obtain a successful result. The cranial part of the cleft site, which does not contribute to the facilitation of tooth eruption, is in most cases a bony site that is not subject to functional loading. The primary reason for this is the lack of paranasal musculature due to the cleft deformity. Resorption rates will therefore presumably be higher in the cranial part of the graft.

Overfilling the alveolar cleft defect is a method that most surgeons, whether using autologous or synthetic grafts, are inclined to. In an overfilled defect, there is a great deal of graft material that is not functionally loaded. When calculating the resorption rates of overfilled defects with regard to the original cleft volume, higher resorption rates will be found than when calculating resorption rates in non-overfilled defects.

In one patient in this pilot study, there was total loss of the graft. The reason for this was surgery related and could not be attributed to the β-TCP scaffold. In this patient, a lateral incisor had erupted through the palatal side of the alveolar cleft defect, which made water-tight closure of the mucosa of the defect virtually impossible and caused salivary contamination and leakage of the granules. We therefore excluded this case from further analysis in Tables 1 and 2. These tables depict the more realistic outcome of the actual effect of grafting with β-TCP.

The authors heavily stress the importance of water-tight closure of the mucoperiosteal flaps when using β-TCP granules. It is even more important than when using bone blocks or spongious bone, since granules may leak from the restored cleft site, as was the case in this patient. Currently, the application of a putty-like micro-structured β-TCP is investigated in order to get around the problem of leaking graft material.

The spontaneous eruption that was scored for all six patients was considered as proof of the presence of bone. Histological data could not be provided since in the Netherlands, taking biopsies in humans for research purposes is strictly forbidden.

Conclusion

Worldwide, congenital facial clefts are considered to be a burden[1]. To date, repair of the alveolar process in patients with cleft of lip, alveolus, and palate means the harvesting of autologous bone with its associated donor site morbidity. Previous experimental and clinical studies and the initial findings of this pilot study now elucidate a path toward the clinical use of microstructured β-TCP bone substitute for repair of the alveolar cleft.

Because of the considerable amount of bone resorption after overfilling the alveolar cleft, only recontouring the alveolar process with graft material will be sufficient for the bone volume needed for the functional loading of the bone by the teeth present.

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CHAPTER 4b

Two-Center Clinical Study

Based on the article

Janssen NG, Schreurs R, De Ruiter AP, Sylvester-Jensen HC, Blindheim G, Meijer GJ, Koole R, Vindenes H. **Micro-structured β-TCP for alveolar cleft reconstruction: a two-center study**
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Abstract

The current standard of care in alveolar cleft repair is timing the procedure in the mixed dentition stage and making use of autologous bone to restore the maxillary defect. Using a synthetic bone substitute bypasses the risk of donor site morbidity and reduces operation time. The authors investigated the outcome of alveolar cleft repair using microporous β-TCP in patients with unilateral cleft lip and palate. 20 patients were prospectively enrolled in this study, divided between two centers. One year postoperatively the authors evaluated continuity of the alveolar process, recurrence of oronasal fistulas and eruption of teeth into the repaired cleft. Also, cone beam computer tomography (CBCT) scans were analyzed using a volume based semi-automatic segmentation protocol. No adverse events were reported. Mean residual bone volume in the repaired cleft one year postoperatively was 65%. There was no recurrence of oronasal fistulas, 90% of the teeth adjacent to the cleft erupted spontaneously and all patients showed a continuous alveolar process. Secondary alveolar grafting using microporous β-TCP can be safely used in a clinical situation. Residual calcified tissue, canine eruption and complication rates in the acceptor region are comparable to autologous grafts.

Introduction

Reconstruction of the alveolar cleft in patients with cleft lip and palate serves a number of goals. It provides a continuous maxillary arch in which the erupting dentition can be aligned. It remedies persistent oronasal fistulas. Finally, it stabilizes the maxillary complex and provides support to the alar base and adjacent soft tissue structures. The current gold standard for alveolar cleft repair is the use of autologous bone harvested from the iliac crest or the mandibular symphysis[1-3]. Though these methods proved to be effective and showed a high percentage of success, these grafting procedures carry a risk of comorbidity such as; postoperative pain, hypersensitivity, paresthesia, infection, apical root damage and visible scarring[4-12]. Using a synthetic bone substitute circumvents this risk of donor site morbidity and reduces operation time.

In the past decades, a number of biomaterials have been clinically tested as an alternative for autologous bone grafting in alveolar cleft repair. The most promising results so far have been obtained using rhBMP-2[13, 14]. However, after serious adverse events have been reported for maxillofacial surgery applications, its use has lost popularity in the past years[15].

Our study group researched the use of microstructured β-TCP as a bone substitute for alveolar cleft reconstruction both *in vitro*[16], *in vivo*[17, 18] and also in a human pilot study[19]. The aim of this study is to evaluate the use of microstructured β-TCP in a prospective two-center clinical trial.

Materials and methods

Patients

At the University hospitals of Bergen, Norway (Haukeland University Hospital) and Utrecht, the Netherlands (Utrecht University Medical Center) a total of 20 patients, 10 subjects at each hospital, with a unilateral alveolar cleft were prospectively enrolled in the study. Permission to carry out this study was granted by the Medical Ethics Committee of the Utrecht University Medical Center (protocol number 09-129/K) and Haukeland University Hospital (protocol number 2013/732).

All patients underwent early secondary alveolar cleft closure. Surgery was performed when radiological data showed at least 50% development of the root of the canine (or in selected cases the lateral incisor, dependent of the opinion of the orthodontist) adjacent to the cleft.

If needed, deciduous teeth located around the alveolar cleft were removed at least 8 weeks preoperatively to facilitate watertight closure of the mucoperiosteal layers. If this goal could not be reached, the patient was excluded from the study (20). Mean age at time of surgery and gender distribution are depicted in table 1.

	Bergen Group	Utrecht Group	Groups Combined
Number of patients	10	10	20
Mean age at surgery	8,3 years	9,6 years	8,9 years
Gender distribution	8 males, 2 females	6 males, 4 females	14 males, 6 females

Table 1: Age and gender distribution of the Utrecht and Bergen groups, and the two groups combined.

All patients were grafted with microstructured β-TCP (OsOpia, Regedent, Zurich, Switzerland), 10 in Bergen and 10 in Utrecht. One surgeon in Bergen (HV) and one surgeon in Utrecht (RK) performed the alveolar cleft grafting surgeries.

Bone substitute

The calcium phosphate-based scaffold used is a biphasic calcium phosphate with a hydroxyapatite fraction of <10% and a tricalcium phosphate fraction of >90% (OsOpia©, Regedent, Zurich, Switzerland). Since the hydroxyapatite fraction is smaller than 10 percent, we will refer to the scaffold in this article as β-TCP. The scaffold has a porosity of 70% and a granule size of 250-1000 µm. Due to its unique micro-architecture and macro-architecture both osteoconductive and osteoinductive properties are attributed to this bone substitute [16]. Furthermore, *in vivo* studies showed a near complete resorption of the scaffold, which is essential in order to facilitate eruption of the lateral incisor or canine into the grafted region [17, 18].

Surgery

Alveolar cleft reconstruction was carried out using general anaesthesia with naso-endotracheal intubation. Antibiotic prophylaxis was prescribed for three days (Clindamycin© 10mg/kg/day, Hameln Pharmaceuticals, 31789 Hameln, Germany). Surgery was conducted by means of creating three mucoperiosteal layers (nasal, buccal and palatal). Nasal and palatal layers were prepared and closed with resorbable sutures (3-0 Vicryl and 4-0 Ethicon, Brussels). Following closure of the nasal and palatal layers, the cleft defect was filled with microstructured Beta-TCP. The graft was covered with the buccal layer and closed with resorbable sutures (3-0 Vicryl and 4-0 Ethicon, Brussels).

Data acquisition

Cone beam CT scans of the maxillary region were acquired preoperatively and one year postoperatively. In Utrecht the iCat© 3D imaging system (Imaging Sciences International, Hatfield, PA, USA) was used. Scans were performed at 120 kV and 3- to 8-mA pulse mode. Field of view was 13x6cm, scan time was 8.9 seconds and voxel size was 0.4mm. In Bergen the 3D Accuitomo system was used (Morita©, Irvine, CA, USA). These scans were executed at 85 kV and 9 mA with a field of view of 4x4cm, scan time was 9 seconds and voxel size was 0.08mm. Using a recently described semi-automatic segmentation protocol [20] the preoperative and one-year postoperative data sets were superimposed and residual volumes of calcified material were calculated. Because the Bergen scans were executed at a significantly smaller horizontal and vertical field of view, it was not possible to match the

preoperative and postoperative scans according to the protocol. These scans were manually matched using the Image Fusion module in iPlan Cranial (BrainLab AG, Feldkirchen, Germany) and subsequently segmented using the Smart Brush module in BrainLab Elements.

Results

All 20 patients underwent uneventful surgery and without postoperative complications (Figure 1). Loss of a very small amount of granules was rarely seen. Wound dehiscence, postoperative infection or significant loss of granules did not occur.

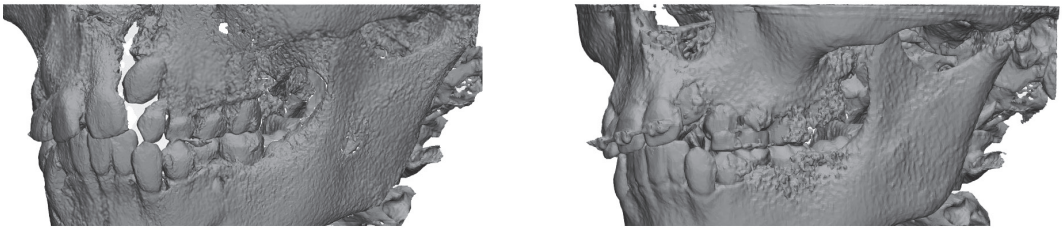

Figure 1: 3D-rendering of a CBCT-scan preoperatively (left) and the same patient 1 year postoperatively (right). A continuous alveolar process and eruption of the permanent canine into the alveolar cleft region can be clearly discerned.

One year postoperatively, no residual oronasal fistulas were present. All patients had a continuous alveolar process on the affected side. The canine adjacent to the alveolar cleft did not erupt spontaneoulsly in two cases. One case was grafted in Bergen and the other case was grafted in Utrecht. Percentages of residual calcified tissue were normally distributed (as confirmed by Shapiro-Wilk test: p= 0,839) for the Bergen group, the Utrecht group, and both groups combined. Mean residual calcified material of the reconstructed alveolar cleft defect one year postoperatively was 61% (SD 14%) for the Bergen group and 69% (SD 12%) for the Utrecht group. Mean residual calcified material for both groups combined was 65% (SD 14%) (Table 2).

	Bergen Group	Utrecht Group	Groups Combined
Residual calcified tissue	61% (SD 14%)	69% (SD 12%)	65% (SD 14%)
Spontaneous eruption of canine/lateral incisor	9 (90%)	9 (90%)	18 (90%)
Continuous alveolar process	10 (100%)	10 (100%)	20 (100)%
Residual oronasal fistula	None	None	None

Table 2: Postoperative outcome parameters 1 year after alveolar cleft closure

Discussion

An average residual calcified tissue volume in the 20 operated patients of 65 percent matches with the 61,6 percent reported one year after filling the alveolar cleft with autologous chin bone grafts using the same surgical procedure, surgical timing and segmentation protocol [20].

As described in earlier studies, the largest part of resorption took place in the cranial part of the graft [19]. This resorption may be related to poor functional loading of the scaffold in the region of the piriform aperture, because paranasal muscular function is of lower quality on the cleft side.

Since histological examination of the reconstructed alveolar bone is not possible, we have to rely on radiological and clinical evidence to prove the presence of bone in the reconstructed alveolar cleft. The calcified tissue, as measured in the reconstructed alveolar cleft is supposed to be nearly 100% bone. Histopathological examination in previous goat model studies on alveolar cleft grafts showed an almost 100% conversion of the grafted β-TCP after six months[17, 21]. Furthermore, in radiology one can discern newly formed corticocancellous bone after at least one year after alveolar cleft reconstruction. In addition, since a dental follicle only can erupt when vital bone is present, eruption can be seen as evidence for bony transformation of the scaffold; in 18 out of 20 cases, the canine or lateral incisor (dependent on orthodontic indication) adjacent to the cleft erupted completely through the scaffold. This 90% eruption of the canine or lateral incisor through the alveolar cleft concurs with the percentage of 87,5% described by Vellone et al, for 24 patients who underwent secondary alveolar bone grafting with autologous iliac crest bone grafts[22].

The advantage of the application of a using a bone substitute is that no co-morbidity arises. In a retrospective study performed in Utrecht, cleft patients reconstructed with β-TCP (n=31) were compared to cleft patients reconstructed with autologous chin bone (n=45). Pain was scored one day postoperatively using a visual analogue scale (VAS) ranging from 0-10. Also the number of days in the hospital were registered. Both VAS one day postoperatively and duration of hospital stay were dramatically lower in the group of patients in which β-TCP was used (unpublished data).

No cost effectiveness analysis has been performed in this study. The price of a single vial of 1cc of β-TCP is approximately 70 euros, and in almost all procedures only one vial is needed. Obviously, the cost difference using β-TCP instead of an autologous bone graft is dependent on the clinic where it is carried out. In Utrecht, chin bone grafts are harvested by the resident and this takes up to 20 minutes of extra operating time. In Bergen, iliac crest bone is harvested simultaneously with the alveolar cleft closure procedure by another surgeon or resident. Although it takes the same amount of time, a second surgeon is needed in the surgical theatre.

The importance of watertight closure is indisputable in order to prevent the bone substitute from leaking and to avoid infection. The same applies to the presence of deciduous teeth; an alveolar cleft that is not cleared of deciduous dentition is more prone for these adverse effects as was shown in our previous pilot study [19]. Care needs to be taken not to overfill the defect and to leave no granules at the cemento-enamel junction of teeth adjacent to the cleft since these factors will keep the closure from being watertight.

The method of analysis, however accurate for measuring the regeneration of a bone defect in a growing skeleton with permanent teeth erupting, is rather cumbersome and takes a lot of time. For research purposes it can very well be realized, but for daily use it is impractical.

Conclusion

Secondary alveolar grafting using microporous β-TCP can be safely used in a clinical situation. Residual calcified tissue, canine eruption and complication rates in the acceptor region are comparable to autologous grafts. Furthermore, it is an inexpensive procedure that, compared to autologous bone grafting, reduces postoperative pain, hospital stay and operating time.

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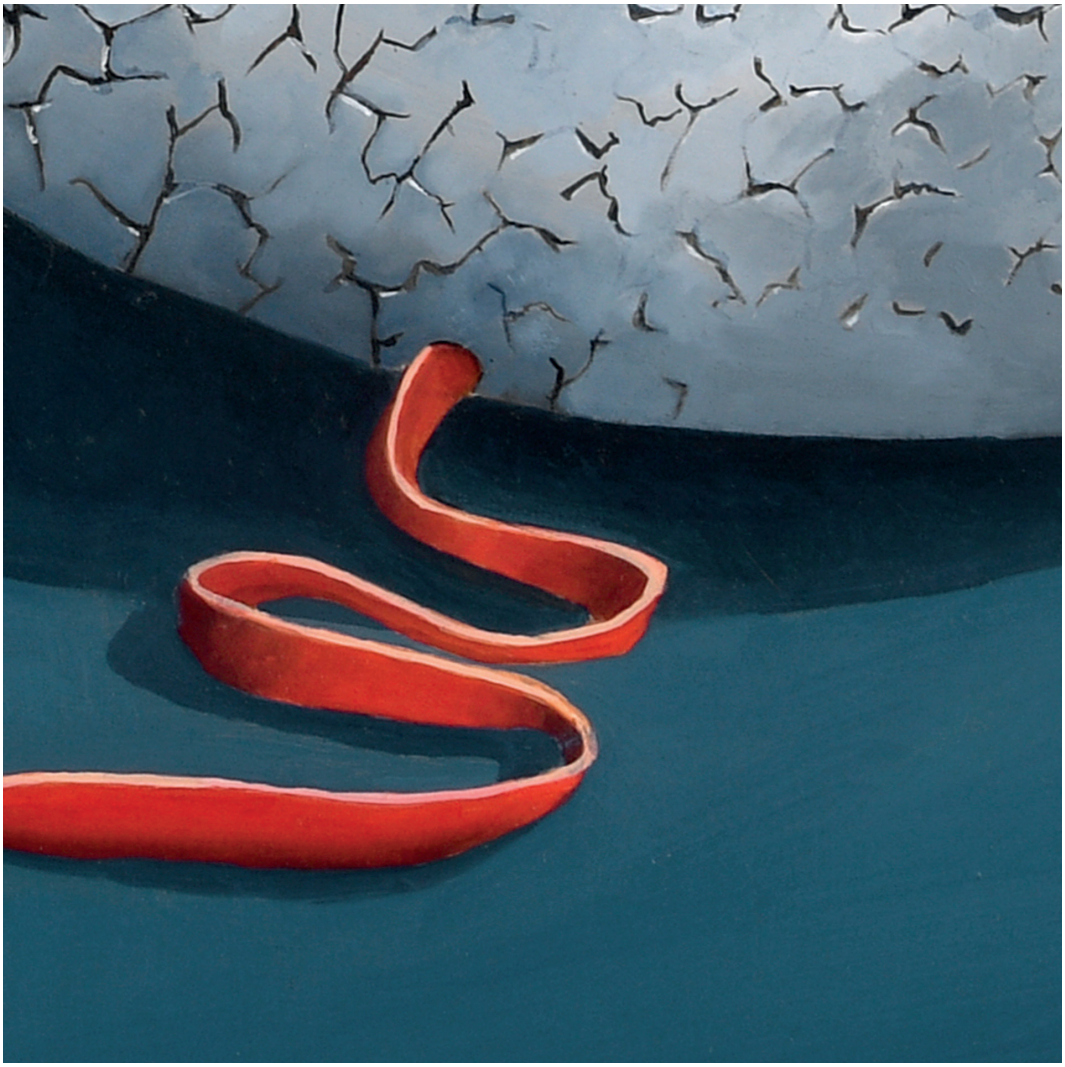

CHAPTER 5

Fine Tuning the Scaffold

Based on the article

Janssen NG, de Ruiter AP, van Hout WMMT, van Miegem V, Gawlitta D, Groot FB, Meijer GJ, Rosenberg AJWP, Koole R. **Microstructured β-Tricalcium Phosphate Putty Versus Autologous Bone for Repair of Alveolar Clefts in a Goat Model.** Cleft Palate Craniofac J. 2017 Nov;54(6):699-706.

Abstract

For the first time, it was demonstrated that an osteoinductive calcium phosphate-based putty is effective in restoration of complex maxillofacial defects. In these defects, adequate mechanical confinement by multiple bony walls and osteoconduction from multiple surfaces are usually lacking. This study compares the efficacy of a microstructured beta-tricalcium phosphate (β -TCP) putty with autologous bone for repair of alveolar cleft defects. Ten Dutch milk goats were operated on in a split-mouth study design in which two-wall bony alveolar clefts were created and successively repaired with autologous bone (the gold standard) at one side and β –TCP putty at the other. After 24 weeks of implantation, histomorphometric and micro-CT analysis proved that β-TCP putty group showed equal bone quality and volume to clefts reconstructed with autologous bone In addition, surgical handling of the putty is superior to the use of calcium phosphates in a granular form. Therefore, the results of this study open a clear trajectory for the clinical use of β-TCP-putty in reconstruction of the alveolar cleft and other challenging two-wall bony defects.

Introduction

In patients with cleft lip and palate alveolar bone grafting is paramount. It provides continuity of the dental arch, closure of the oronasal communication, it facilitates tooth eruption of teeth adjacent to the cleft and successive orthodontic tooth movement, it restores the support of the nasal base and provides stability of the maxillary arch[1, 2]. Until now, for the repair of alveolar cleft defects, autologous bone grafts are the gold standard[3]. As these are harvested from either the iliac crest or the mandibular symphysis, donor site morbidity is introduced[4, 5]. To bypass this cause for postoperative pain, and also to reduce operating time, the use of synthetic bone substitutes is a logical next step.

To be suitable for alveolar cleft repair, the selected bone substitute should be resorbable, meaning that it should be replaced by vital bone in time. Bone formation is accomplished by means of creeping substitution, eruption of the dental follicle and the periodontal ligament of the erupting tooth. In the presence of bone, tooth eruption, orthodontic tooth movement through the grafted area, and root stability of the erupted tooth, are guaranteed. These requirements have strict implications on the degradation kinetics of the bone substitute selected. For example, if resorption is too slow, orthodontic tooth movement will be blocked. On the other hand, if resorption is too fast, insufficient volume of scaffold will be provided to the growing osteoblasts, as such frustrating bone formation, leaving an underfilled or unfilled alveolar cleft.

As an alternative for autologous bone grafts, osteoinductive calcium phosphate scaffolds (OCPSs) seem to be a promising candidate for alveolar cleft repair. OCPSs are biomaterials able to induce bone formation when implanted at heterotopic sites, an ability known as osteoinduction. This phenomenon has been recognized for 30 years[6] and the superiority of OCPSs has been demonstrated above osteoconductive calcium phosphate ceramics in clinically relevant models [7]. Although the osteoinduction mechanism is not yet completely unraveled, the relationship between the physical and chemical features of the OCPS material and the osteogenic differentiation of human mesenchymal stem cells has been demonstrated *in vitro* [8, 9] .Furthermore, osteoinductive calcium phosphates do not have the drawbacks of the existing osteoinductive alternatives, i.e. autograft (two surgical procedures, donor site morbidity, prolonged operating time) and bone morphogenetic proteins (BMPs) (systemic effects leading to severe side effects, risks even higher in the pediatric population[10], expensive procedure[11]).

In two recent clinical studies alveolar defects in patients with unilateral cleft lip and palate were successfully reconstructed with β-TCP granules[12] (and yet unpublished results in Chapter 4 of this thesis). However, the dimensional stability of the reconstructed sites was found to be suboptimal because of the free motion of the granules. The alveolar cleft defect typically is a two-wall bony defect in which mucoperiosteal flaps are sutured in two layers to create both a new nasal floor and a continuous oral mucosa. It is very challenging to create a watertight closure of these mucoperiosteal flaps to retain the graft within the allocated area and to prevent microorganisms from infecting the graft. In the alveolar cleft defect, it is of paramount importance that the applied bone graft or bone substitute remains in place. The addition of these β -TCP granules with a binder into a putty is a significant improvement for both the surgical handling and the initial dimensional stability of the reconstructed area, provided that the binder does not hinder bone formation[13, 14]. Davison et al.[15] showed that a blend of carboxymethyl cellulose in glycerol (CMCG) is a suitable binder formulation in combination with osteoinductive b- TCP granules. The CMCG binder is inert, biocompatible, and ensures the initial containment of the b-TCP granules in the defect. With a rapid clearance kinetics (<4 hours in vitro, <48 hours in vivo), the CMCG binder allows the rapid exposure of the b-TCP microstructure surface for optimal bone growth. Furthermore, the water-free nature of CMCG prevents the alteration of the microstructured surface upon storage. Therefore, the resulting putty can be readily applied at the surgical site.

Materials and methods

Study design

The goat model was used that has been previously described by the authors[17]. The study and its protocol were approved by the Dutch Animal Care and Use Committee (DEC-UMC 2013.III.02.013). A split mouth study was conducted on 10 female adult Dutch milk goats (*capra hircus*, Heythuysen, the Netherlands) in which bilaterally created alveolar clefts were repaired using β-TCP-CMCG on one side and autologous bone harvested from the iliac crest on the other side. Appointment of the β-TCP-CMCG grafting side was randomized. All goats were aged between 36 and 38 months ensuring no deciduous teeth remained and to prevent sample bias due to age.

Surgical procedure

Surgery was performed under general intravenous and inhalation anesthesia. For extraoral disinfection a 1% iodine in 70% ethanol solution was used. Intraoral disinfection was achieved with 0.12% chlorohexidine. After extraction of the left and right maxillary second premolars, buccal and palatal mucoperiosteal flaps were raised. Consecutively, two –wall bony defects of approximately 1cm^3 were created in the maxilla by removing all buccal, palatal and nasal bone. The nasal mucosal layer was left intact. The bone defect on one side was filled with β-TCP-CMCG. The defect on the other side was repaired with autologous cancellous iliac crest bone chips. Mucoperiosteal flaps were closed in a tension free manner using resorbable sutures (Vicryl 3-0, Ethicon, Brussels, Belgium)(Figures 1 and 2).

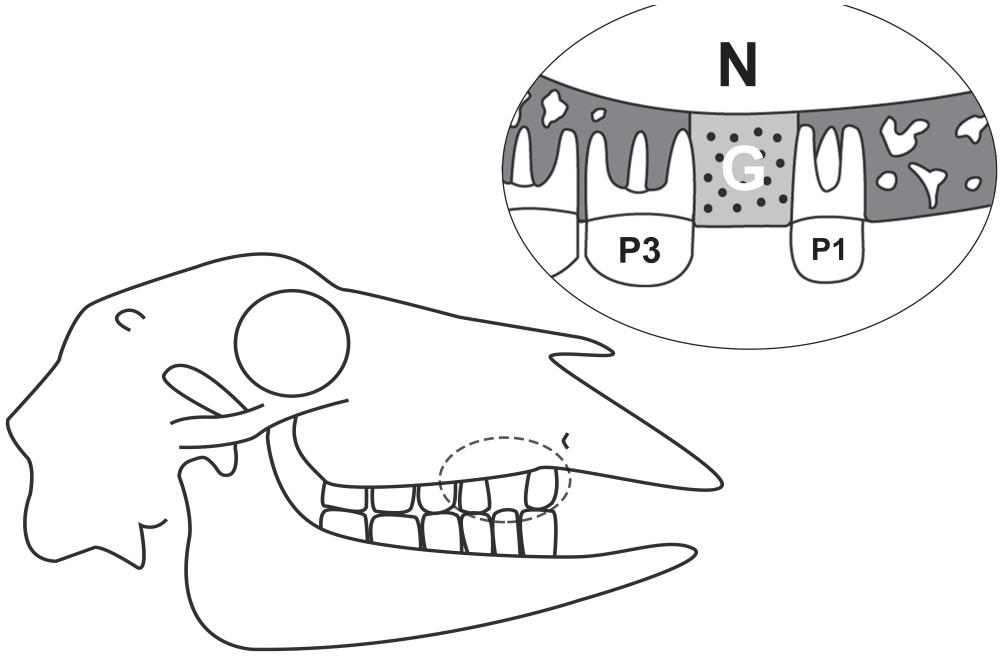

Figure 1. Schematic drawing of the surgical procedure: Region of interest in the goat maxilla is indicated with a dotted line. The defect site is located between the first (P1) and third (P3) premolar and is cranially confined by the nasal floor (N). Successively, the defect is repaired with grafting material (G).

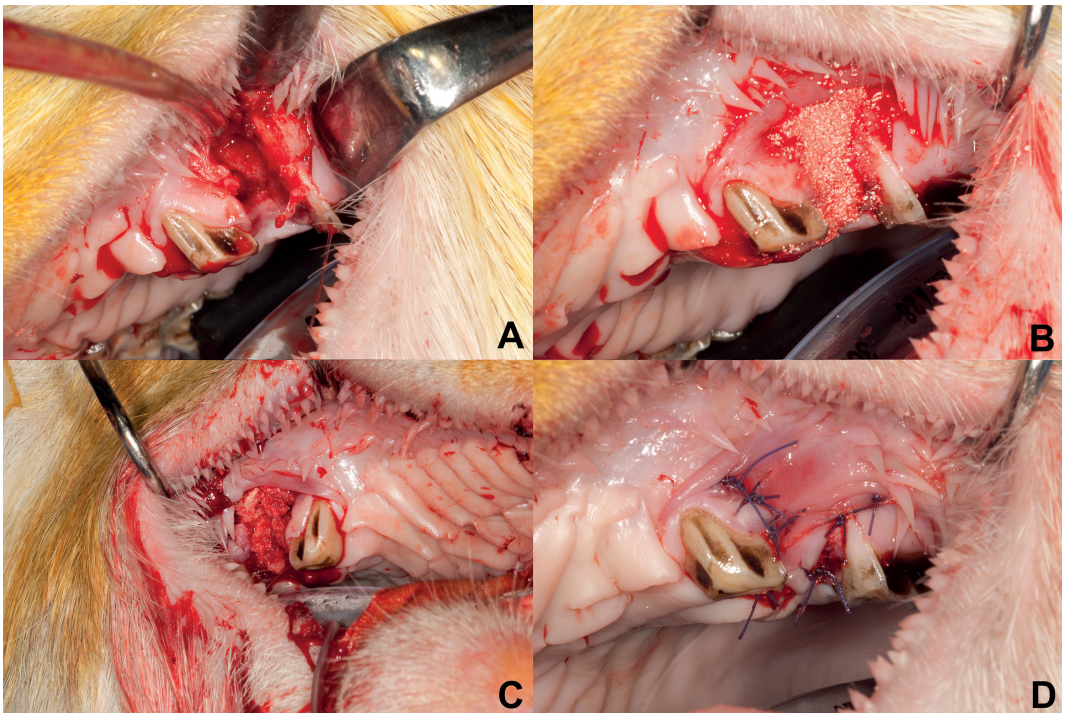

Figure 2. Intraoperative view: Surgical creation of the alveolar cleft defect between the first and third premolar after extraction of the second premolar and removal of bone up to the nasal mucosa (A). One defect site was filled with β-TCP-CMCG putty (B) and the contralateral with autologous iliac crest bone chips (C). Mucoperiosteal flaps were raised and the defect sites were closed in a tension-free manner (D).

Bone substitute preparation

The putty was a kind gift from Xpand Biotechnology BV (Bilthoven, The Netherlands). The microstructured β-TCP (composition: 98% β-TCP, 2% Hydroxyapatite) ceramic particles were made by wet precipitation of apatite powder (Ca/P ratio 1.5), followed by green body H_2O_2 foaming and sintering at 1050 °C, as previously described[16]. The desired TCP granule fraction 150–500μm was collected after sieving (Retsch, Haan, Germany), and cleaned with ethanol, acetone and deionized water. Total porosity was found to be 70% by mercury intrusion testing (Micromeritics, Aachen, Germany).

The granules were physically blended at 80°C with a 4% carboxymethyl cellulose (CMC) in glycerol matrix (CMC, Cekol, CPKelco, Atlanta, Georgia; and glycerol, Sigma-Aldrich, St Louis, Missouri at a weight ratio 1:1. The putty was sterilized by E-beam at 25kGy (Synergyhealth, Radeberg, Germany). In volume, the granules occupied >90% of the putty volume so that the bone defect is essentially filled with the microstructured β-TCP after CMCG clearance.

Animal care

Starting one week preoperatively to three weeks postoperatively, all goats were fed with pre-moistened ground chunks of beet pulp mixed with lucerne pulp (De Heus Diervoeders, Rijsbosch, Beusichem, The Netherlands). This was precured in order to prevent wound dehiscence. During the remainder of the pre- and postoperative periods, all goats were fed with hay and regular ground chunks (Arie Blok Diervoeding, Woerden, The Netherlands). Six months postoperatively, all goats were sacrificed by means of an overdose of pentobarbital (Euthesaat, Organon, Oss, The Netherlands).

Radiographic assessment

The specimens retrieved from the sacrificed animals were scanned using a micro-CT scanner (Quantum FX micro-CT, PerkinElmer, Waltham, Massachusetts). Samples were scanned at a 120μm voxel size (90 kV, 180μA current, 1.0 mm Al/0.25 mm Cu filter, and 0.5° rotation step, 20 min scan). All scans were blinded and residual bone volumes were calculated using a DICOM viewer (Osirix, Pixmeo, Geneva, Switzerland). The region of interest consisted of the area between the first and third premolar in the anteroposterior plane and mediolateral plane. The defect was measured up to the nasal floor in the craniocaudal plane.

Residual bone volume was quantified after segmentation of calcified tissue from non-calcified tissue using a global threshold. This global threshold was determined based on visual inspection and was kept constant for all scans. Segmentation of residual bone volume was carried out manually using a free form tool. After segmentation of each slide within the region of interest total residual bone volume was calculated.

Histological procedure and assessment

The specimens were first fixed for one week in 4% formalin solution (VWR-Prolabo); thereafter dehydrated using the Milestone TT Mega histoprocessing microwave in 80% ethanol (VWR), JFC solution (Milestone, Klinipath, Duiven, The Netherlands) and two steps of 100% ethanol. They were embedded in K-plast MMA (L.T.I. Bilthoven, The Netherlands), sectioned with a slice thickness of 50 µm using the Leica SP1600 saw (Leica Microsystems, Rijswijk, The Netherlands) with a diamond saw blade (Saint-Gobain Diamantwerkzeuge Norderstedt, Germany) and stained with methylene blue and basic fuchsin (Sigma-Aldrich, St Louis, Missouri). Sections were digitalized using the Konica Minolta Dimage II slide scanner and histomorphometry was performed to determine the amount of bone in the available area. Using Photoshop Elements software (Adobe Systems, San Jose, California) a region of interest (ROI) was selected between the two premolars, the nasal and palatal mucosa, representing the defect. In the ROI, the total amount of pixels was determined as well as the amount of bone and residual material (if applicable) by pseudocoloring.

Statistical analysis

A paired-samples t-test was conducted to compare differences in residual bone volumes between β-TCP-CMCG and iliac crest graft as assessed from the micro-CT scans (SPSS, version 21, IBM, Chicago, Illinois). A second paired-samples t-test was conducted to compare the percentages of bone formation on the histological sections. A p-value <0.05 was considered as statistically significant. All data are represented as means ± standard deviation.

Results

Clinical observations

Surgical handling of the putty showed to be very effective. Even though the surgically introduced maxillary defects were difficult to reach as the goats were in a prone position, the putty was easily administered with a syringe and remained in place. All intraoral operation sites healed successfully without any signs of wound dehiscence or infection. After sacrificing all goats, one of the animals (goat number 6, β-TCP-CMCG side) appeared to suffer from a mobile first premolar showing signs of local periodontitis due to an impacted hay splinter, but was not excluded from the study.

Micro-CT scans

Volumes of the restored bone defects on both sides of the maxilla are depicted in figure 3.

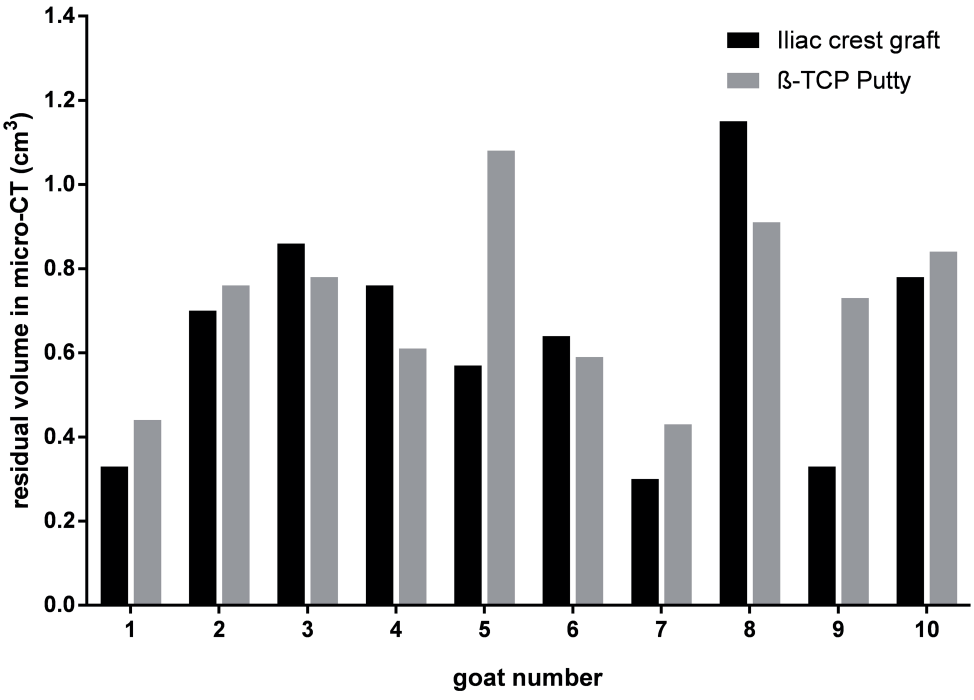

Figure 3. Residual volumes of the reconstructed defects are shown for all animals at six months postoperatively. Per animal the β-TCP-CMCG group and the iliac crest control group are depicted.

A paired-samples t-test did not show any statistically significant difference between cleft sites grafted with β-TCP-CMCG or autologous bone graft (p=0.34). All samples showed continuity of the maxillary bone at the defect site and formation of normal cortical and cancellous bone. The mean residual volume was 0.71±0.2 cm^3 for the β-TCP-CMCG group and 0.64±0.27 cm^3 for the iliac crest group

Histological sections

At histological evaluation after 6 months, the tissue responses were similar for all implanted graft materials: all tissues surrounding the implants were normal, and no severe inflammatory reaction was observed. Microscopically, no cytotoxic effects were detected in the host tissue surface in contact with the implanted material. There were no significant differences in terms of biological response of tissues surrounding the implants between the β-TCP-CMCG group and autograft groups, indicating the biocompatible nature of the CMCG binder. All samples showed bone regeneration yielding bone tissue with an architecture resembling that of natural bone (4).

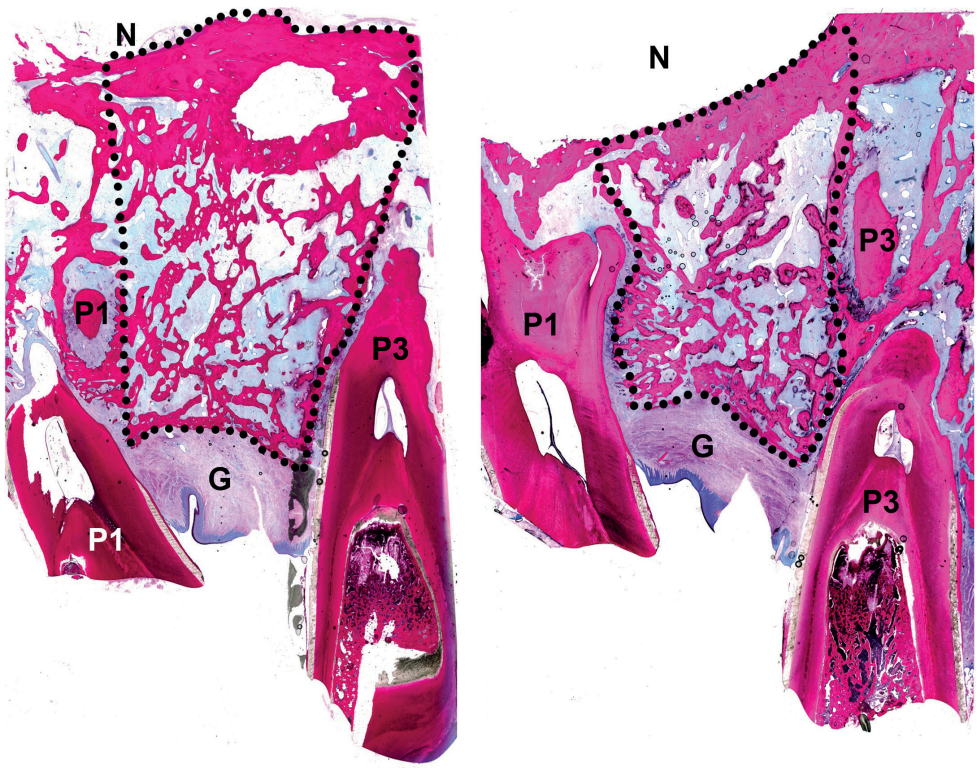

Figure 4. Bone formation in an alveolar cleft grafted with β-TCP-CMCG (left figure) and in the contralateral alveolar cleft defect repaired with autologous iliac bone graft (right figure). Both sections show a trabecular bone architecture resembling natural maxillary bone structure. P1: First premolar. P3: Third premolar. N: Nasal floor. G: Gingiva. Dotted line represents the region of interest.

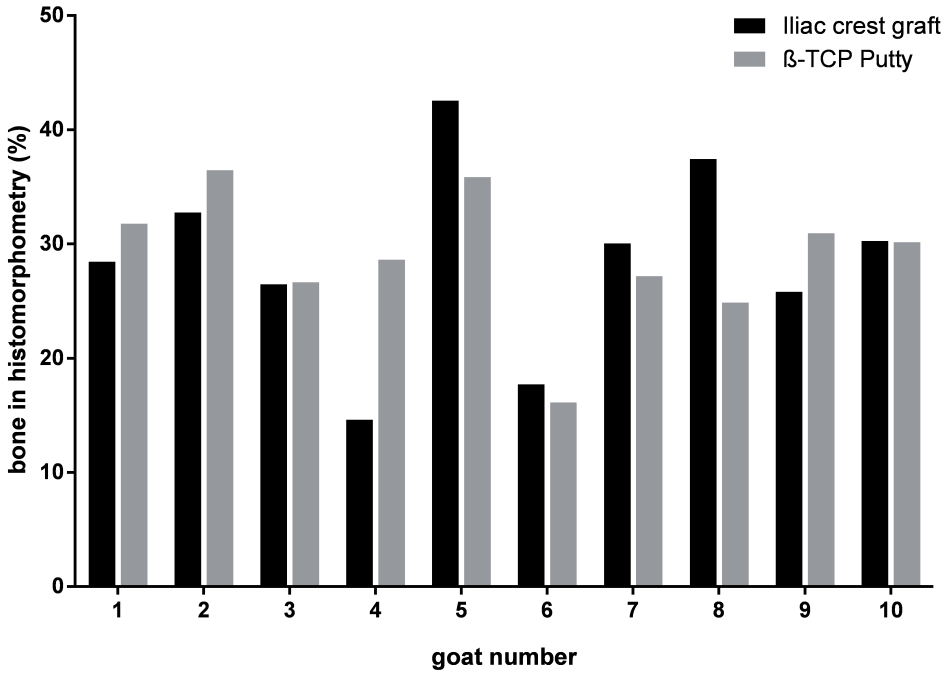

Figure 5. Area percentage of newly formed bone in the defect site from histomorphometric analysis. Per animal the β-TCP-CMCG group and the iliac crest control group are depicted.

Histomorphometric analysis of the area percentages of newly formed trabecular and cortical bone structures revealed an average of 28.9±5.5% in the β-TCP-CMCG group and an average of 28.6±7.9% in the autologous bone group (Figure 5). There was no significant difference in the bone area percentages between the two groups (p=0.91). On average only 0.1% of biomaterial was discernible in the β-TCP-CMCG group, with a maximum percentage of 0.6% in goat 1.

Discussion

Newly formed bone with an architecture resembling natural maxillary bone was found in all reconstructed defects repaired with both autologous iliac crest bone and β-TCP-CMCG. The two grafting materials showed comparable results. The surgical handling of the putty was excellent and remained in place adequately.

Animal model considerations

In a previous study, it was shown that the goat model is suitable to study alveolar cleft repair[17]. The created defect is similar in size as in alveolar cleft patients and the goat metabolism is more equivalent to the human situation than phylogenetically less kindred species such as rodents in which bone metabolism is higher and defect sizes are much smaller[18, 19].

It has been extensively shown in the human analogy of the alveolar cleft defect that the bony defect is not always of critical size[20]. In over 50% of human cleft patients a very small bony bridge is established when the alveolar cleft is closed using only the surrounding mucosal tissue without implanting a bone graft. Therefore, in this study, a sham group has not been studied. Thus, since we are not trying to repair a critical size bone defect, we have not considered it worthwhile to create a sham group, taking into account the reduction in use of laboratory animals. Accordingly, Koole et al. showed that in a sheep cleft defect of similar size and locus the sham group (in which no bone graft was used) showed regeneration of the defect[21]. However, this occurred in a severely delayed way, with insufficient bone volume compared to the grafted subjects. As a critical note to the study design, carry-over effects from one operated site to the contralateral one and vice versa may induce bias in split-mouth studies[22].

Surgical considerations

Surgery consisted of a one-stage operation in which the defects were bilaterally created and immediately restored. Furthermore, in the human situation, a complete alveolar cleft is also compromised by the presence of an oronasal fistula, an unwanted communication between the oral and nasal cavity. Due to the one-stage character of the surgery, whilst creating the alveolar cleft defect, the nasal mucosa was left intact, since piercing and suturing it in the same session through the created alveolar defect is surgically far more unreliable than in the human situation. A remark on the translatability of the surgical model can be made

on these two issues, since closure of a chronic oronasal fistula in the human situation is a more challenging matter than healing of an alveolar wound that did not suffer from a nasal communication, as was created in the animal model. However, due to animal ethical standards in the Netherlands, a model in which two consecutive surgeries are performed for this procedure is not allowed.

The putty-like structure of the β-TCP-CMCG provided an optimal surgical handling. CMCG was chosen as a carrier for the β-TCP because Davison et al. described 100% granule retention and orthotopic bone bridging, as well as ample ectopic bone formation, in an *in vivo* dog's femur model[15]. However, contrary to the bony defect that was investigated in our study, the defect was only monocortical, consisted of five bony walls and was only 5 millimeters in diameter. In Davison's study β-TCP-CMCG showed a slightly delayed onset of bone formation compared to β-TCP granules only. However, after 12 weeks the amounts of bone formation were equal. CMCG is a rapidly dissolvable carrier with a dissolution time of <4 hours *in vitro*, and <48 hours *in vivo*. Barbieri et al. showed that carriers dissolving rapidly (i.e. within 48 hours) do not inhibit the osteoinductive potential of the β-TCP granules[23]. The choice for a non-aqueous carrier has been made because of the prolonged shelf-life compared to aqueous carriers[24].

Histological and radiographic considerations

Volumetric and histomorphometric analyses showed no significant differences between β-TCP-CMCG and autologous bone harvested from the iliac crest. All samples showed bone regeneration resulting in a normal bony architecture. In the β-TCP-CMCG samples, scarcely any β-TCP remnants were visible. When sparse remnants of the β-TCP were studied, osteoinductive properties of the scaffold can be seen (Figure 6).

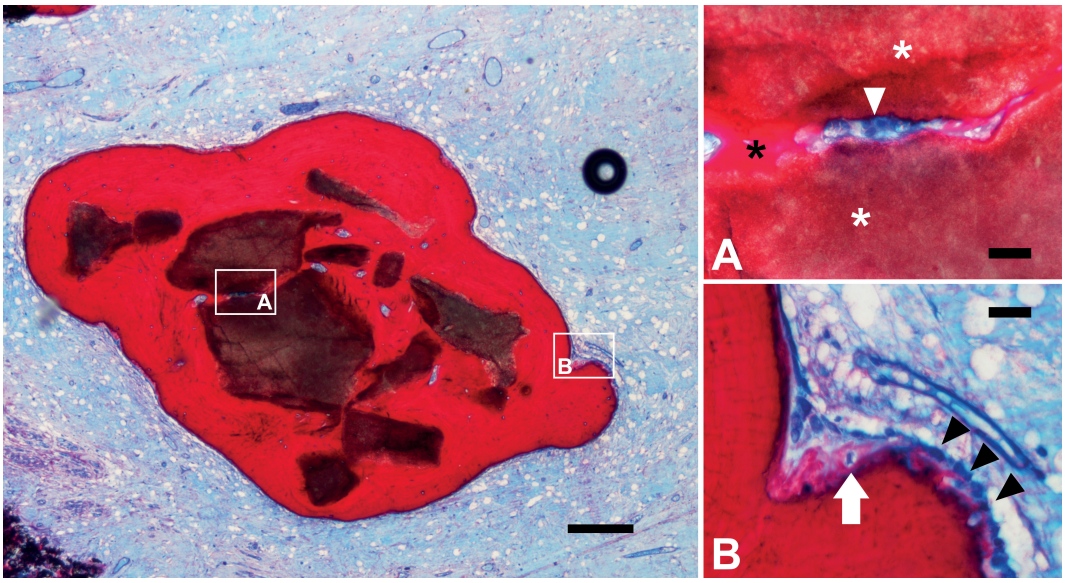

Figure 6. Osteoinduction by β-TCP-CMCG putty in the maxillary cleft of goats at 6 months. At low magnification (left), induced bone tissue (red) surrounds partially degraded TCP particles (brown) in the soft tissue (light blue) of the goat maxilla (scale bar left = 250 µm). At higher magnification, (A) a multinucleated osteoclast-like cell (white arrowhead) resorbs the material (white stars) adjacent to newly formed bone (black star). Elsewhere (B), cuboidal osteoblasts (black arrow heads) lay down new bone (pink) adjacent to an osteocyte (white arrow) in its lacuna (scale bars A,B = 25 µm).

These findings concur with our earlier published data on osteoinductive properties of similar microstructured TCP's[8]. In most samples there was a considerable amount of decrease in volume of the restored defect six months postoperatively, regardless of the grafting substance. A, the grafted sites with either β-TCP-CMCG putty or autologous bone show a continuous maxillary arch with normal bone architecture. Hardly any remnants of β-TCP are discernible in the histological specimens. Results of this study open a clear trajectory for clinical use regarding repair of the human alveolar cleft with β-TCP-CMCG putty.

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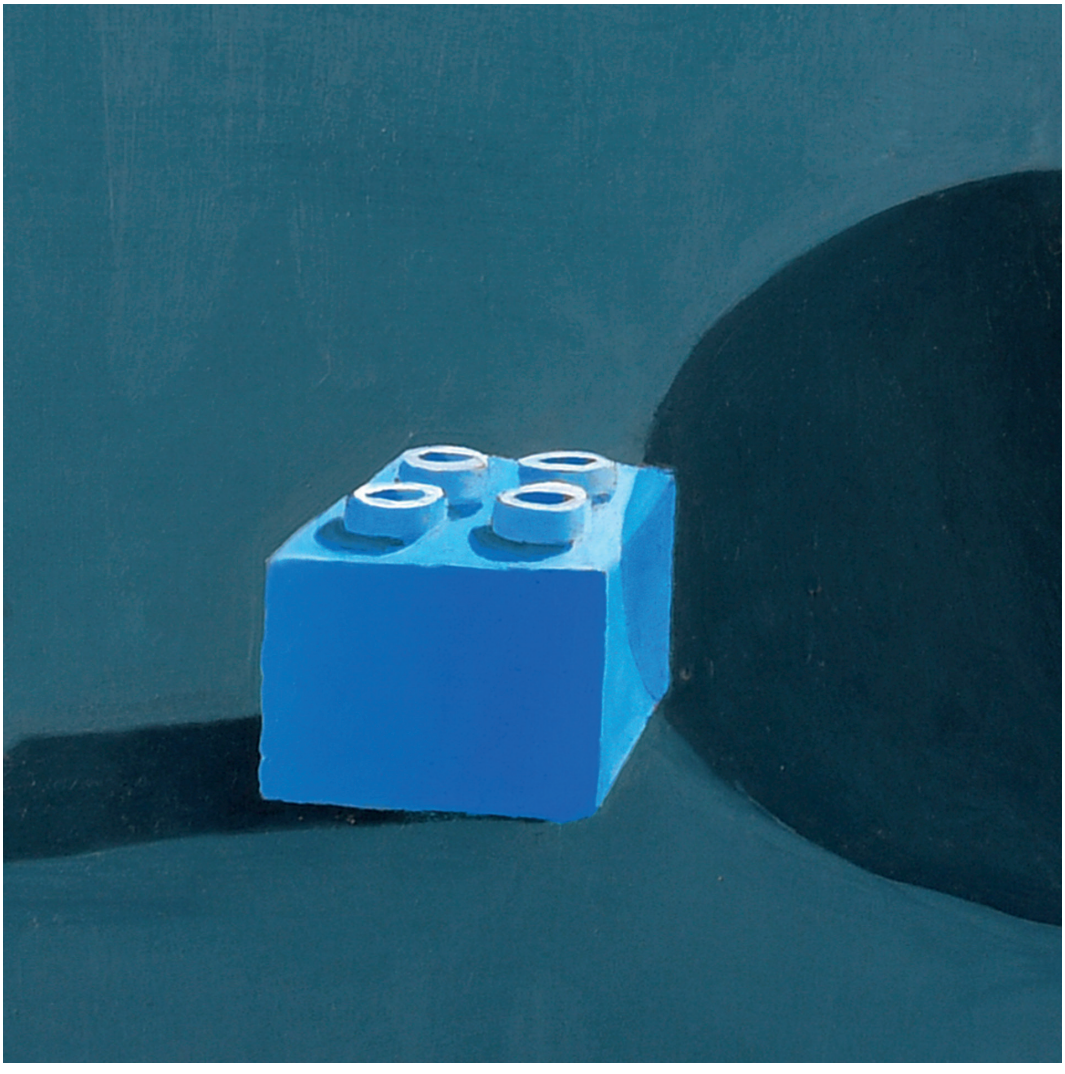

CHAPTER 6

General Discussion and Future Perspectives

Introduction

In cleft lip and palate patients the surgical burden is high. Patients undergo multiple surgeries starting in early infancy. When they reach the point of alveolar cleft closure, the majority already underwent at least two previous operations (closure of cleft lip and of cleft soft/hard palate), but often more than two (phased closure of soft and hard palate, speech improvement surgery). To reduce this burden, one should not only think in terms of reducing the number of surgeries (as in combined closure of the hard and soft palate or combining speech improvement surgery with alveolar cleft closure), but also in terms of reducing postoperative morbidity, hospital stay and duration of surgery. This thesis focuses on improving the latter three parameters, simply by skipping autologous bone grafting in alveolar cleft repair and thereby bypassing donor site morbidity .

Address to aims

Choosing the right biomaterial for alveolar cleft repair

In the systematic review (this thesis; chapter two) it was emphasized that the included studies could not be compared with each other. A great amount of heterogeneity is present in the studies conducted on replacing or enhancing autologous bone grafting in alveolar cleft repair. Outcome measurements of conducted studies are either poorly defined or inaccurate. When comparing the use of a biomaterial to autologous bone grafting, information should be presented about clinical details such as: the presence of continuity of the maxillary arch, the way of closure of the oronasal fistula, the grade of eruption of the canine and last but not least, the amount of the residual bone volume in the transplanted region. None of the included studies fully met these prerequisites. Mostly, this could be attributed to the fact that three-dimensional radiological evaluation of the alveolar cleft region was still in an early developmental phase at time of publication of these studies. This formed the incentive of creating more reliable means of assessing the residual bone volume in the repaired alveolar cleft, as will be discussed in the next paragraph. On the matter of biomaterial selection, when fully replacing the autologous graft, the use of rhBMP-2 soaked collagen sponges showed the most convincing results. However, this thesis investigates the use of a calcium phosphate based scaffold. The choice not to further delve into reconstruction with growth factor based scaffolds is due to a number of reasons. Firstly, at the start of conducting our research, the first cracks in the good reputation of clinical use of rhBMP-2 became visible

when Woo et al. reported ectopic bone formation and extensive soft tissue swelling after the use of rhBMP-2 in the maxilla[1]. Popularity of the clinical use of rhBMP-2 plummeted as Medtronic, the manufacturer of commercially available rhBMP-2, was accused of paying for slanted research to aid sales of their product. In addition, numerous patient claims were filed against Medtronic because of alleged complications, deceptive marketing and unethical practices. Up to this day, clinical and preclinical research on rhBMP is indecisive on exact doses and optimal dose-release curves [2]. Furthermore, the cost of for alveolar cleft closure is high and reported to be around $2600 per surgery[3]. As the potential use of rhBMP-2 raises more questions than answers, our research group focused on the use of calcium phosphates only.

Parallel to the decreasing reputation of rhBMP-2 our research department's collaboration with Xpand Biomaterials (Bilthoven, The Netherlands), a developer of calcium phosphate based bone void fillers, provided a logical base for preclinical and clinical investigation of a calcium phosphate based scaffold. The β-TCP used in this thesis actually is a synthetic biphasic calcium phosphate that consists for more than 90% out of β-TCP and less than 10% out of hydroxyapatite. It has a porosity of 70% and the clinical study makes use of a granule size of 250-1000 µm (figure 1).

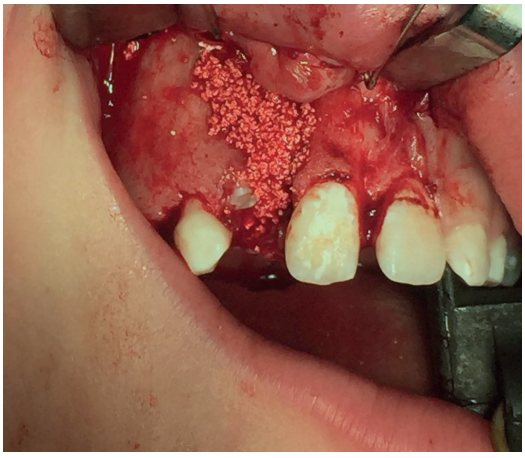

Figure 1: Direct view on the applied β-TCP in the alveolar cleft. The buccal mucoperiosteal layer is retracted cranially in order to apply the scaffold.

The cost of 1cc of β-TCP in our study is around €70. The high β-TCP content in the scaffold used is of paramount importance, since the majority of the scaffold needs to resorb in a relatively short timeframe, forming bone by creeping substitution and thereby facilitating eruption of the canine (or lateral incisor) into the alveolar cleft. High hydroxyapatite

percentages would inhibit such dental eruption and, would successively hinder functional loading of newly formed bone due to absence of the canine root (or the root of the lateral incisor) in the reconstructed defect. Already, before the start of this thesis the findings of an *in vivo* study conducted on Dutch milk goats with aforementioned β-TCP granules (though with a granule size of 1-2mm) was published. Specimens, taken 6 months after implantation in an artificially created alveolar cleft, defect showed an almost 100% resorption of the β-TCP and excellent new bone formation. Furthermore, it was feasible to orthodontically move tooth roots through the newly formed bone. These two findings supported our hypothesis that this scaffold could also induce bone formation in the human alveolar cleft, as well as facilitate eruption of teeth through the repaired alveolar cleft.

Assessment of the reconstructed defect

Evaluating the success of alveolar cleft repair, realizing the importance of outcome parameters in the physical oral examination is imperative. In literature, factors such as a continuous alveolar process, closure of the residual oronasal communication, adequate support of the alar base and eruption of the canine or lateral incisor into the cleft and periodontal status of teeth in the cleft region are often overlooked. A parallel can be made with the clinical adagium *'one does not treat radiographs, one treats patients'*. As such, assessment of the recreated bone volume after alveolar cleft repair is essential in comparing results between different methods of repair. Ergo, in scientific literature when the effect of treatment methods should be objectified, volumetric assessment plays an important role, whereas in everyday practice the center of gravity is located around thorough physical examination.

With the introduction of both multislice and cone beam computed tomography the formerly broadly accepted two dimensional radiography of assessment of the amount of the residual bone, such as used in the Bergland index[4], has been rapidly losing ground. The two dimensional methods mainly focused on interradicular alveolar cleft bone height. One of the advantages of (CB)CT analysis, besides the addition of an anteroposterior dimension, is that also the reconstructed nasal part of the nasal-alveolar-palatal defect can be assessed.

There are a number of difficulties when calculating alveolar cleft volumes. Firstly, the alveolar cleft defect has arbitrary boundaries, especially at the palatal side. This inaccuracy can be reduced by superimposing a mirror image of the non-affected side in unilateral cleft patients

as we have illustrated in our segmentation method (chapter 3). Drawback of using a mirror image is that often transversal compression of the maxillary fragment lateral to the alveolar cleft is present, resulting in an asymmetric maxillary arch, obstructing mirroring. Also, the alveolar process most medial to the alveolar cleft often protrudes more than a continuous maxillary arch would do, also resulting in a certain amount of asymmetry. Secondly, our study in chapter 3, as well as most other studies evaluating volumetric assessment methods, make use of CBCT as opposed to multislice CT. When using the latter, bony tissue can be reliable discerned by means of selecting the correct spectrum of Hounsfield units. When using CBCT scans, segmentation of the bony portion gives more problems since it is difficult to define a threshold due to low contrast, distortion of grayscale values and Hounsfield units[5]. Studies using multislice CT mainly originate from the Asian world, in Europe there seems to be more emphasis on keeping radiation levels lower. Studies using multislice CT have the advantage of a superior and far easier segmentation procedure. A third obstacle in reliably assessing the repaired alveolar cleft bone volume is that grafting takes place in a growing craniofacial skeleton in the mixed dentition stage. Erupting teeth hinder alveolar cleft volumetry. Additionally, growth of the facial skeleton makes superimposition of preoperative and postoperative scans challenging.

When evaluating the postoperative residual bone volume in the alveolar cleft, it is essential that the preoperative scan can be superimposed over the postoperative scan. This is the only way to reliably assess which part of the bony surface belongs to the newly reconstructed maxilla and which part fits to the maxillary skeleton in the preoperative situation. Many studies performed segmentation procedures in postoperative (CB)CT scans without superimposition of the preoperative situation[6-9]. Also in our pilot study[10] this erroneous method was used. Authors are often misguided due to the fact that these methods give high inter- and intra-observer values. Unfortunately, these high inter- and intra-observer correlations are due to the high variation in alveolar cleft defect sizes that mask the inaccuracy of the method used.

Calculating residual alveolar cleft bone volumes is a challenging task, since many difficulties need to be overcome as shown in this section (and in Chapter 3). Furthermore, it takes a large amount of time and technical skills to perform such calculations. The present procedure is impractical for daily use or examination of large patient cohorts. Making it easier and faster to perform is, therefore, the next goal. With beam collimation in current multislice CT scan devices one can ponder whether multislice CT will become the medium of choice for measurement of alveolar cleft bone defects, since bony tissue can be discerned far easier, more reliable and faster using this medium compared to CBCT.

Clinical trials with β-TCP

After the pilot study mentioned in chapter 4 a couple of lessons were learned, which were unknown during the *in vivo* study conducted in 2011[11]. The foremost is that, when performing alveolar bone grafting with calcium phosphate granules, one must meticulously deliver a watertight closure of the mucoperiosteal layers. Alveolar clefts often have deciduous teeth adjacent to, or sometimes even protruding into the alveolar cleft, making watertight closure of especially the rugged palatal mucosa very difficult. In one patient, in whom the alveolar cleft was not sanitized before alveolar cleft repair, inaccurate wound closure caused a total loss of the applied granules. Furthermore, extra care should be taken to prevent exposure of granules into the nasal cavity. Especially, in case of a narrow alveolar cleft, closure of the nasal layer can be very difficult. In these cases, the application of a resorbable barrier membrane may ascertain proper confinement of the applied scaffold.

In the pilot study, radiological examination of CBCT scans 6 months after alveolar cleft closure gave difficulties in discerning new bony boundaries, since radiologically not in all cases the grafted region showed signs of bone formation. This finding was contrary to the previously mentioned goat study in which all histological specimens showed a near complete bony turnover after 6 months. This difference in rate of bone formation can be declared by the fact that the remodeling speed in goats is higher than in humans. In the two-center trial, therefore, radiologic evaluation was performed one year after alveolar cleft closure.

After the 20 cases included in the two-center trial, in Utrecht another 21 unilateral cleft cases were operated, which adds up to a total of 31 patients operated on in Utrecht with the use of β-TCP. In the same period, also in Utrecht, 45 patients were operated on using autologous chin bone as graft material. Since this study has a prospective character with an in advance fixed amount of patients, these patients were not included in the aforementioned trial. In 69 of these 76 patients pain scores one day postoperatively were reported (visual analogue scale of 1 to 10). Also duration of hospital stay was evaluated (Table 1). Both VAS-score one day postoperatively and duration of hospital stay were dramatically lower for the group of patients in whom β-TCP was used.

	β-TCP	Chin bone	
Mean VAS-score one day postoperatively	0.53 (N=30)	2.46 (N=39)	P 0.015 (χ2test)
Mean no. days of hospital stay (including day of surgery)	1.25 (N=31)	2.73 (N=45)	P0.000 (χ2 test)

Table 1: Mean VAS-score one day postoperatively and days of hospital stay in 2 groups of alveolar cleft patients, grafted with either β-TCP or autologous chin bone

To conclude, the use of β-TCP for unilateral alveolar cleft repair is safe and effective. It provides a continuous maxillary arch in which eruption of the canine (or lateral incisor) is possible (Figure 2). Costs of the material are very low (70 euro's per 1cc vial) and postoperative pain and hospital stay are significantly lower than when using autologous chin bone grafts.

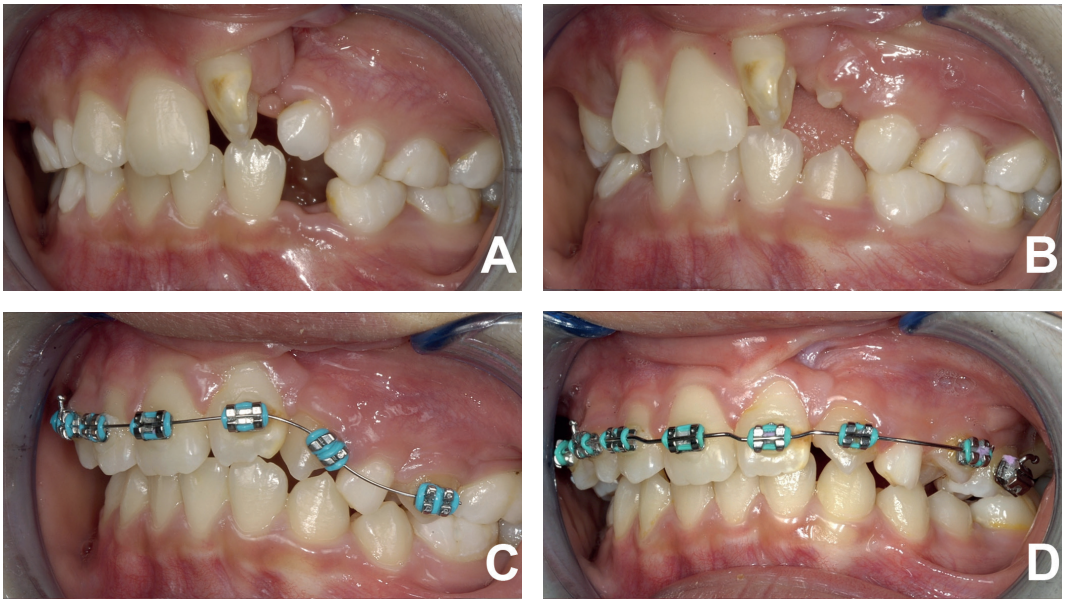

Figure 2: Light photographs of canine eruption into the repaired alveolar cleft, grafted with β-TCP : A: Preoperative situation, B: 3 months after surgery, C: 12 months after surgery, D: 24 months after surgery. Note the absence of the lateral incisor adjacent to the cleft and the deciduous canine that is kept in place temporarily due to aplasia of the left first premolar.

Enhancing the graft

The most important drawback of the β-TCP granules is that the surgical handling and initial form stability are suboptimal. Despite the fact that the graft initially is not loaded and is kept in place by two or three bony walls and three mucoperiosteal layers, CBCT scans performed one week postoperatively sometimes show displacement of the applied granules (Figure 3).

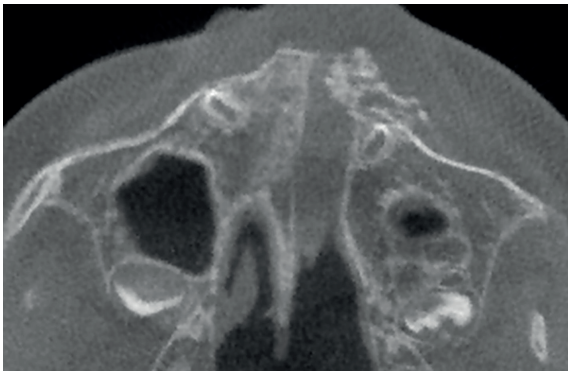

Figure 3: Axial slice of a subject 1 week after grafting with β-TCP. The slice depicts the cranial part of the alveolar cleft. Anterolateral displacement of the graft can be clearly seen. The cleft area is almost devoid of granules.

The *in vivo* study in Dutch milk goats (chapter 5) focuses on the application of the same granules used in the clinical trial, although now embedded in a putty matrix. Radiological and histopathological analysis 24 weeks after implantation showed excellent bone quantity and quality in the repaired alveolar cleft defects. Application of a putty bypasses the drawback of early particle migration and enhances its handling properties. This study advocates the clinical use of β-TCP putty for alveolar cleft closure.

Future perspectives

This thesis shows that calcium phosphate based scaffolds are cheap, effective and safe in repairing alveolar clefts. Moreover, the need for harvesting autologous grafts has disappeared. Also, toxic effects induced by the use of growth factors, such as BMP, are avoided. Cell-based bone tissue engineering strategies, though currently not well described for alveolar cleft grafting, is also costly and claims more preparation time, for example in culturing patient specific autologous bone cells. Using calcium phosphate based scaffolds agrees excellently with the ruling paradigm of keeping procedures safe and simple, now widely established in the surgical world.

In the near future clinical investigation of the β-TCP putty will be a logical next step in the improvement of alveolar cleft grafting procedures. It is to be expected that similar results can be achieved as in our clinical trial using granules only.

Still we will be confronted with the phenomenon that a great amount of the scaffold in the nasal part of the graft is resorbed. This is explained by the fact that the upper part of the β-TCP granules is not functionally loaded by a dental root as is the case in the lower part of the grafted region. In order to overcome this massive cranial resorption that occurs both in patients grafted with β-TCP and autologous bone transplants, one can increase the hydroxyapatite fraction of the scaffold. The problem that will successively arise is that with rising hydroxyapatite percentages eruption of teeth through the graft will be slowed down or even hindered, . Keeping this in mind, this problem could be tackled by creating a hybrid scaffold in which the two distinct parts exhibit different osteobiologic properties. Most alveolar clefts show a craniocaudal length of 15 to 20 millimeters and the canine follicle erupts only into the lower 2/3rd portion of the alveolar cleft. If the cranial (say 1/3rd) part of the alveolar cleft is reconstructed with a Biphasic Calcium Phosphate containing a substantial higher HA fraction, this part of the graft will not succumb to significant resorption. The lower part can successively be grafted with Biphasic Calcium Phosphate containing a very low HA fraction, thus facilitating resorption and bone formation, thereby allowing tooth eruption. Prerequisite for maintaining a watershed between two types of calcium phosphate after application is that both are putty-based to avoid blending during closure of the mucoperiosteal layers and after surgery.

In the era of patient specific implants one can contemplate what the advantages are of creating a 3D-printed patient specific implant for alveolar cleft grafting. Rapid prototyping of patient specific alveolar cleft scaffolds may provide enhanced volume stability of the newly reconstructed maxilla, provided there are no insurmountable undercuts in the implant design. Furthermore, when segmented accurately from a preoperative (CB)CT scan, the patient specific implant could provide improved contact with the defect's bony walls and therefore optimize osteoconduction. Also, 3-D printing can facilitate implementing a hybrid scaffold with different HA-TCP ratios as discussed above. This can be created in low-temperature 3D printed implants[12], as also in scaffolds which are assembled three dimensionally and successively sintered at high temperatures[13]. Though the exact mechanism remains unclear, microporosity of the scaffold is of importance. In the beginning of *3D calcium phosphate scaffold printing* it was a problem to create microporosity. However, recent publications show that 3D printed calcium phosphate scaffolds can be manufactured with similar microporosity as their non-printed equivalents[14]. A great

advantage in the clinical development of patient specific implants is that in alveolar cleft grafting the scaffold is not exposed to large forces. Therefore the brittle nature of printed calcium phosphate scaffolds does not have to be physically enhanced with polymer fibers to prevent the scaffold from fracturing.

Altogether, in the near future, regenerative medicine in alveolar cleft surgery can not only provide an alternative that is equally as good as autologous bone grafting, but also can provide the surgeon with an option that is superior to the current gold standard.

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Addendum

Summary

Repairing the alveolar cleft in patients with cleft lip and palate is an essential procedure. It provides maxillary stability and facilitates eruption of teeth adjacent to the cleft. Furthermore, it finalizes closure of the congenital oronasal communication and provides support to the alar base. In alveolar cleft repair, the use of an autologous bone graft has become the golden standard. Most studies carry out transplantation of bone harvested from the iliac crest or from the mandibular symphysis. Even though these methods have proven to be effective showing a high percentage of success, both carry along donor site complication risks and postoperative comorbidity. It is presumable to introduce means to avoid this burden in a vulnerable paediatric population that has to endure multiple surgeries at a young age. In this thesis a viable alternative is investigated for the use of autologous bone as grafting material for alveolar cleft closure in patients with unilateral cleft lip and palate. A further introduction to timing, technique and materials of choice for alveolar cleft repair is given in **chapter 1**.

In **chapter 2** a systematic review on bone substitutes for alveolar cleft closure is conducted to elucidate which types of biomaterials are suitable for further clinical investigation. A great number of strategies making use of regenerative medicine have been suggested for alveolar cleft reconstruction; however, autologous bone grafting seems to remain the gold standard. In total 16 articles were selected for analysis. Strategies using regenerative medicine for alveolar cleft grafting included enhancing the autologous bone graft by means of platelet-rich plasma addition, the use of barrier membranes and fibrin glue, extension of the autologous graft with calcium phosphate scaffolds, and replacement of the graft using bone morphogenetic protein-2, mesenchymal stem cells, or calcium phosphate scaffolds. Selected articles showed a vast heterogeneity in data acquisition and patient selection. Therefore, a meta-analysis could not be performed. Bypassing or enhancing autologous bone grafting by means of tissue engineering solutions has become an important topic in alveolar cleft grafting. Graft replacement by means of rhBMP-2 soaked collagen sponges and graft extension with calcium phosphate scaffolds showed promising results.

In **chapter 3** we investigated how to reliably measure the repaired alveolar cleft defect. Older methods of radiologic analysis only use two dimensions for analysis. In case of reports of three dimensional analysis, often the method used was not reliably in assessing the exact dimensions of the postoperative reconstructed bony tissue. In chapter 3, a novel protocol for volumetric assessment of alveolar cleft grafting procedures is presented. In total 11 cone-beam computed tomography (CBCT) datasets of patients who underwent secondary alveolar cleft reconstructive surgery were evaluated by two investigators. Residual bone

volumes one year after surgery were measured using a semi-automated technique in which preoperative CBCT datasets were superimposed on the postoperative scans using voxel-based registration. To define the correct boundaries of the alveolar cleft defect in the preoperative CBCT dataset, a mirror image of the preoperative CBCT dataset was superimposed on preoperative CBCT dataset. For the difference in residual bone volume between the two observers, an intraclass correlation of 0.98 and a Dice coefficient of 0.89 were found. In this chapter a reliable segmentation protocol for volumetric analysis of the alveolar cleft defect in patients with a unilateral alveolar cleft is presented.

In **chapter 4** a pilot study and a clinical trial are described on alveolar cleft closure using beta-tricalcium phosphate (β-TCP), a rapidly resorbable calcium phosphate scaffold with osteoinductive properties. The pilot study showed results that legitimized the succesive clinical trial. In the clinical trial, 20 patients were prospectively enrolled by two centers, being the Wilhelmina Children's Hospital, Utrecht, The Netherlands and the Haukeland University Hospital, Bergen, Norway. One year postoperatively we evaluated the continuity of the alveolar process, recurrence of oronasal fistulas and eruption of teeth into the repaired cleft. Also, CBCT scans were analysed using the volume based semi-automatic segmentation protocol described in chapter 3. No adverse events were reported. One year postoperatively, the mean residual bone volume in the repaired cleft was 65%. There was no recurrence of oronasal fistulas, 90% of the teeth adjacent to the cleft erupted spontaneously and all patients showed a continuous alveolar process. It was concluded, that secondary alveolar grafting using microporous β-TCP can be safely used in a clinical situation. Residual calcified tissue, canine eruption and complication rates in the acceptor region were comparable when autologous grafts would have been used. Furthermore, it is an inexpensive procedure that, compared to autologous bone grafting, reduces postoperative pain, hospital stay and operating time.

The biomaterial that is used in chapter 4 provides a number of drawbacks. First and foremost it lacks initial stability, resulting occasionally in early displacement of parts of the grafted material. Furthermore the surgical handling of a granulate is suboptimal for alveolar cleft closure. An *in vivo* study is described in **chapter 5** in which the same granules were used, however, now embedded in a putty matrix made of a blend of carboxymethyl cellulose in glycerol (CMCG). A total of 10 Dutch milk goats were operated on in a split-mouth study design in which two-wall bony alveolar clefts were created and successively repaired with autologous bone (the gold standard) at one side and β-TCP putty at the other. After 24 weeks of implantation, histomorphometric and micro-computer tomography analyses proved that clefts reconstructed with β-TCP putty showed equal bone quality and volume

compared to clefts reconstructed with autologous bone. It was concluded that surgical handling of the putty is superior to the use of calcium phosphates in a granular form. Therefore, the results of this study open a clear trajectory for the clinical use of β-TCP putty in the reconstruction of the alveolar cleft and other challenging two-wall bony defects.

A summarizing discussion is held in **chapter 6**, in which we conclude that the use of β-TCP for unilateral alveolar cleft repair is safe and effective. Moreover, costs of the material are very low and postoperative pain and hospital stay are significantly lower than when using autologous chin bone grafts. Future perspectives are plotted in the same chapter, pertaining to enhancing results of the procedure by means of scaffold consistency (granules versus putty), scaffold composition (different hydroxyapatite/tricalcium phosphate ratios) and the creation of patient specific implants.

Samenvatting

Sluiting van de gnathoschisis (kaakspleet) bij patiënten met een cheilognathopalatoschisis (lip-, kaak- gehemeltespleet) of een cheilognathoschisis (lip-kaakspleet) is een cruciale ingreep. Hierdoor wordt continuïteit van de maxilla (bovenkaak) verkregen en wordt het voor de naast de kaakspleet gelegen gebitselementen mogelijk door te breken in de mondholte. Tegelijkertijd wordt de resterende oronasale communicatie gesloten en de basis van de neusvleugel beter ondersteund. Gebruik van een autoloog bottransplantaat tijdens sluiting van de gnathoschisis is momenteel de gouden standaard. De twee meest onderzochte locaties voor het oogsten van het autologe bottransplantaat zijn de crista iliaca (bekkenkam) en de kin. Deze methodes hebben hun succes uitvoerig bewezen. Echter, bij het oogsten van een autoloog bottransplantaat bestaat het risico op postoperatieve complicaties ter plaatse van de donorsite, alsmede een zekere mate van postoperatieve comorbiditeit. Daarom ligt het voor de hand om naar alternatieven voor het oogsten van autoloog bot te zoeken voor een toch al kwetsbare patiëntenpopulatie die een groot aantal operaties op jonge leeftijd moet doorstaan. In dit proefschrift wordt gezocht naar een goed functionerend alternatief voor het gebruik van een autoloog bottransplantaat ten behoeve van sluiting van de gnathoschisis bij kinderen met een enkelzijdige cheilognathopalatoschisis of een enkelzijdige cheilognathoschisis. Een verdere inleiding in de timing, techniek en keuze van het transplantaat bij sluiting van de gnathoschisis wordt gegeven in **hoofdstuk 1**.

Hoofdstuk 2 beschrijft een systematisch literatuuroverzicht van het gebruik van botvervangers bij sluiting van de gnathoschisis. Een veelheid aan methoden die gebruik maken van regeneratieve technieken (tissue engineering) voor sluiting van de gnathoschisis wordt beschreven in de hedendaagse wetenschappelijke literatuur. Echter, het gebruik van een autoloog bottransplantaat blijft nog altijd de gouden standaard. In totaal 16 artikelen werden geselecteerd voor nadere analyse. Strategieën voor optimalisatie van het autoloog bottransplantaat zijn onder andere het additioneel gebruik van platelet-rich-plasma, het gebruik van barrière-membranen en fibrinelijm. Om een geoogst autoloog bottransplantaat in volume te vergroten wordt gebruik gemaakt van het toevoegen van calciumfosfaat matrices. Volledige vervanging van een autoloog bottransplantaat wordt bereikt met ‘in recombinant humaan BMP-2 gedrenkte collageen sponzen’, mesenchymale stamcellen en calciumfosfaat matrices. Conclusie van het gepresenteerde literatuuroverzicht is dat zowel vervanging van een autoloog bot transplantaat door middel van ’in recombinant humaan BMP-2 gedrenkte collageen sponzen’, alsmede uitbreiding van het autoloog transplantaat met calciumfosfaat matrices veelbelovende resultaten toont.

In **hoofdstuk 3** wordt beschreven hoe een kaakspleet op een betrouwbare manier gemeten kan worden. Eerdere methoden maken veelal gebruik van tweedimensionale röntgenanalyse. Als al gebruik werd gemaakt om röntgenologisch driedimensionaal de kaakspleet vast te leggen, dan betrof het vaak een onnauwkeurige meetmethode. In dit hoofdstuk wordt een nieuw protocol voor volumetrische analyse van de gnathoschisis gepresenteerd, dat zowel te gebruiken is vòòr sluiting, als ook ná sluiting van de kaakspleet. Door twee onderzoekers werden elf cone beam computertomografie (CBCT) datasets onderzocht van patiënten die sluiting van een unilaterale gnathoschisis ondergingen. In deze studie werd het botvolume één jaar na sluiting van de gnathoschisis met autoloog kinbot geanalyseerd. Gebruik werd gemaakt van een semi-geautomatiseerde techniek waarbij de 1-jaar-postoperatieve CBCT-scans gesuperponeerd werden op de preoperatieve CBCT-scans middels voxel-based matching. Om de correcte begrenzingen van het preoperatieve gnathoschisis defect te bepalen in de preoperatieve datasets, werd gebruik gemaakt van superpositie van de gespiegelde preoperatieve dataset waarbij derhalve de gezonde zijde van de maxilla werd geprojecteerd over de aangedane zijde. Voor het verschil in bepaling van residuaal botvolume tussen de twee verschillende onderzoekers, werd een ‘intraclass coëfficiënt’ van 0.98 en een ‘Dice-coëfficiënt’ van 0.89 gevonden. Geconcludeerd werd dat in hoofdstuk 3 een betrouwbare segmentatiemethode voor volumetrische analyse van de gnathoschisis werd gepresenteerd.

In **hoofdstuk 4** wordt een klinische studie beschreven, voorafgegaan door een pilotstudie, waarbij de gnathoschisis werd gesloten met korrels vervaardigd uit bèta-tricalcium fosfaat (β-TCP), een snel resorbeerbare calciumfosfaat matrix waaraan osteoinductieve eigenschappen worden toegekend. De resultaten van de pilot-studie legitimeerden verder onderzoek in een grotere klinische studie. In de klinische studie werden 20 patiënten prospectief geïncludeerd verdeeld over twee ziekenhuizen, te weten het Wilhelmina Kinderziekenhuis te Utrecht en het Haukeland Universiteitsziekenhuis te Bergen (Noorwegen). Alle patiënten vertoonden een enkelzijdige gnathoschisis. Eén jaar na de ingreep werd de mate van continuïteit van de processus alveolaris superior, het herstel van de oronasale communicatie en de eruptie van de in de gnathoschisis gelegen gebitselementen klinisch geëvalueerd. Op de CBCT-scans werd de mate van kaakspleetsluiting gemeten met behulp van de segmentatiemethode beschreven in hoofdstuk 3. Bij de 20 patiënten deden zich geen complicaties voor. Postoperatief na 1 jaar bedroeg het gemiddelde residuale botvolume in de gereconstrueerde gnathoschisis 65%. Er deden zich geen residuale oronasale fistels voor, alle patiënten vertoonden een continue processus alveolaris superior en 90% van de naast de gnathoschisis gelegen ongeërupteerde gebitselementen brak alsnog spontaan door in de gnathoschisis. β-TCP

kan op een veilige manier gebruikt worden bij secundaire sluiting van de (unilaterale) gnathoschisis. De klinische en radiologische uitkomsten zijn vergelijkbaar met de resultaten na het gebruik van autologe bottransplantaten. Verder zijn de materiaalkosten van β-TCP erg laag en is sprake van minder postoperatieve pijn, alsmede een reductie van de opname- en operatieduur dan wanneer gebruik wordt gemaakt van een autoloog bottransplantaat.

Het materiaal dat in hoofdstuk 4 wordt gebruikt heeft een aantal nadelen. Het belangrijkste minpunt is dat de β-TCP als granulaat, zoals gebruikt in de klinische studie, initiële stabiliteit ontbeert. Dit resulteert soms in vroegtijdige verplaatsing van het aangebrachte materiaal. Verder is bij sluiting van de gnathoschisis een granulaat lastig te hanteren. In **hoofdstuk 5** wordt een in vivo studie beschreven, waarbij gebruik wordt gemaakt van hetzelfde β-TCP granulaat als in de klinische studie, echter nu ingebed in een putty-matrix, gemaakt van een mengsel van carboxymethyl cellulose in glycerol (CMCG). In totaal werden tien Hollandse melkgeiten geopereerd in een split-mouth studie waarbij twee-wandige kaakspleetdefecten in de bovenkaak werden gecreëerd. In dezelfde sessie werden deze hersteld met autoloog bot (uit de crista iliaca) aan de ene zijde en β-TCP-putty aan de andere zijde. Na 24 weken werden histomorfometrisch onderzoek verricht, alsmede een micro-computertomografie analyse. Hieruit bleek dat de gnathoschisis defecten gereconstrueerd met β-TCP-putty een vergelijkbare botkwaliteit en botvolume toonden als de defecten gerepareerd met autoloog bot. Verder was de chirurgische hanteerbaarheid van een putty superieur aan die van een granulaat. Deze resultaten bieden een goede opstap voor het gebruik van β-TCP-putty bij sluiting van de gnathoschisis en andere uitdagende tweewandige botdefecten in een klinische setting.

In **hoofdstuk 6** worden de belangrijkste resultaten van de eerdere hoofdstukken bediscussieerd. Hier wordt de conclusie getrokken dat het gebruik van β-TCP voor het sluiten van de unilaterale gnathoschisis veilig en effectief is. Verder zijn de materiaalkosten laag en is postoperatieve pijn en ongemak, alsmede ziekenhuisverblijf significant lager dan wanneer gebruik wordt gemaakt van autologe kinbot-transplantaten. Verder worden in dit hoofdstuk toekomstperspectieven behandeld, waarbij de nadruk ligt op het verbeteren van het botsubstituut door het aanpassen van de compositie (β-TCP/Hydroxyapatiet ratio), de consistentie (granulaat versus putty) en het vervaardigen van patiënt specifieke implantaten.

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Allereerst ben ik dank verschuldigd aan alle patiënten en hun ouders die meegewerkt hebben aan de klinische studie. Zij hebben de weg geplaveid voor deze alhier nu regulier aangeboden toepassing.

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