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Micro-structured β-TCP for repair of the alveolar cleft in patients with cleft lip, alveolus and palate

Ad de Ruiter

Colofon

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- His individuality freed him from the collective energy. He did not dwell upon or live in the past; instead - full of expectation - he always looked to the future, while building on his own individual world of ideas of phantasms and colour. -

- Zijn individualiteit bevrijdde hem van de collectieve energie. Hij leefde niet in of vanuit het verleden, maar keek steeds vol verwachting vooruit, bouwend aan zijn eigen individuele ideeënwereld van droombeelden en kleur. -

Micro-structured β-TCP for repair of the alveolar cleft in patients with cleft lip, alveolus and palate

Microstructuur-β-TCP voor het sluiten van de gnathoschisis in patiënten met schisis van lip, kaak en gehemelte

(with a summary in English, met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 november 2012 des ochtends te 10.30 uur

door

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geboren op 17 september 1951

te Heerlen

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PREFACE

The Department of Oral and Maxillofacial Surgery at the UMC Utrecht has inherited the mantel of Dutch history in the treatment of patients with cleft lip, alveolus and palate. Even before the Second World War, Prof. J.W.A. Tjebbes had already established central and total treatment of babies and children with cleft lip, alveolus and palate (CLP). Until 1988 the primary lip closure, palatal closure and palatopharyngeal lengthening procedures carried out on speech therapy indication were executed at the Department of Oral and Maxillofacial Surgery by Prof. P. Egyedi, Dr. H. Müller and Dr. M. F. Noorman van der Dussen. At that time CLP children were also treated by the Department of Plastic and Reconstructive Surgery at the former Academic Hospital in Utrecht; later this was continued at the Wilhelmina Children's Hospital. In 1984 the Oral and Maxillofacial Surgery Department was integrated into the CLP team at the Wilhelmina Children's Hospital. Primary cleft lip and palate surgery and oral and maxillo-facial surgical procedures such as grafting autologous bone to the alveolar cleft at the age of 11-12 and osteotomies of the maxilla were carried out in CLP team approach.

Another important aspect of the treatment of the CLP patient - orthodontics - was not always accorded the attention it deserved by the former Dental Institute of the University of Utrecht. The multidisciplinary CLP team of professor of plastic and reconstructive surgery Prof. C. A. Honig and professor of orthodontics Prof. H.J.L. Smeets did not provide for the requirement of extensive pre- and post-surgical orthodontic treatment. The Utrecht Department of Orthodontics did not take the opportunity to put the CLP heritage on to the orthodontic map. Later on when the Dental Faculty training institute in Utrecht was closed, this aspect was described both nationally and internationally and established by the efforts of Prof. B. Prahl-Andersen and Prof. A.M. Kuijpers-Jagtman (Eurocleft).

The patient material discussed in Chapter 2 has come from the CLP team at the Wilhelmina Children's Hospital in Utrecht. Drs. G.A. Elema and E. Etty, orthodontists of the CLP team of the Wilhelmina Children's Hospital provided the orthodontic treatment of the CLP children. It is the extension and expansion of this patient material that has led to the studies in this doctoral thesis.

VOORWOORD

De afdeling Mondziekten, Kaak- en Aangezichtschirurgie van het UMC Utrecht is erfgenaam van Nederlandse historie op gebied de behandeling van de cheilo-gnatho-palatoschisis (CLP). Al voor de tweede wereldoorlog werd door Prof. J.W.A. Tjebbes een aanvang gemaakt met het centraal en totaal behandelen van schisisbaby's en -kinderen. Tot 1988 werden de primaire lipsluitingen, palatumplastieken en de palatopharyngeale verlengingsplastieken -op logopedische indicatie- uitgevoerd op de afdeling Kaakchirurgie door Prof. Dr P. Egyedi, Dr H. Müller en Dr M.F. Noorman van der Dussen. Eveneens werd die behandeling van de schisiskinderen uitgevoerd door de afdeling Plastische en Reconstructieve Chirurgie van het toenmalige Academisch Ziekenhuis Utrecht; later werd dit voortgezet in het Wilhelmina Kinderziekenhuis. In 1984 werd de afdeling Mondziekten, Kaak- en Aangezichtschirurgie geïntegreerd in het schisisteam van het Wilhelmina Kinderziekenhuis. De primaire schisischirurgie en de kaakchirurgische ingrepen zoals de bottransplantatie in de gnathoschisis op 10-12-jarige leeftijd, kaakosteotomieën van de maxilla en secundaire wekedelencorrecties werden tot op heden in CLP teamverband voortgezet.

Een ander belangrijk onderdeel van behandeling voor de schisispatient -de orthodontie- kreeg op het Tandheelkundig Instituut van de Rijksuniversiteit Utrecht niet altijd de aandacht die het vereiste. Een gezamenlijk schisisspreekuur van de hoogleraar plastische en reconstructieve chirurgie Prof. Dr C.A. Honig en de hoogleraar orthodontie Prof. H.J.L. Smeets, voorzag niet volledig in de behoefte aan uitgebreide pre- en postchirurgische orthodontie. De afdeling nam niet de kans te baat om het schisis-erfgoed op de orthodontische kaart te zetten. Later, toen Utrecht als opleidingsinstituut gesloten was, werd dit aspect nationaal en internationaal beschreven en vastgelegd door de inspanningen van Prof. Dr B. Prahl-Andersen en Prof. Dr A.M. Kuijpers-Jagtman (Eurocleft).

Vanuit het schisisteam in het Wilhelmina Kinderziekenhuis Utrecht kwam het materiaal naar voren zoals beschreven in hoofdstuk 2. Drs G.A. Elema en Drs E. Etty, als orthodontisten verbonden aan het schisisteam van het WKZ, voerden de orthodontische behandelingen uit van de in hoofdstuk 2 beschreven schisiskinderen. Daarop voortbordurend volgde het onderzoek waarover in dit proefschrift wordt gerapporteerd.

Chapter 1

Introduction

INTRODUCTION

Status quo

The orthodontist is co-responsible for a large part of the total treatment of the cleft lip, alveolus and palate (CLP) patient[1,2]. A stable, uninterrupted maxillary arch with a good relationship to the mandibular arch is the aim of the orthodontic treatment of cleft patients[3]. As the development of the mandible is identical in cleft and non-cleft children the final orthodontic treatment begins with the creation of a good lower dental arch[4] with fixed appliances between the ages of nine and eleven dependent on the timing of the grafting operation. The timing of operation is based on radiographic images of the unerupted canine or lateral incisor adjacent to the cleft, whereby the root has to be 50-75% formed[5-9].

The alveolar process cleft needs to be filled with autologous bone in order to enable eruption[10-12] and orthodontic movement of teeth[13,14].

Fresh autologous cancellous bone is ideal for alveolar cleft bone grafting because it supplies immunocompatible bone cells and matrix that can incorporate fully into the maxillary bone and are essential for osteogenesis. In the surgical repair of the alveolar cleft in children with cleft lip, alveolar process and palate attention has been focused on bone grafting[10,15,16], the origin of the grafting material[17-26], and on the results of closing the oronasal fistula[27,28].

Until recently, the traditional sites for harvesting were mainly the iliac crest[10] or the mandibular symphysis[21-30]. Harvesting autologous bone has several disadvantages, such as prolonged operation time and the occurrence of co-morbidity. The risk of complications at the donor site ranges from 10 to 30 percent and comprises postoperative pain, hypersensitivity, pelvic instability, infection, meralgia paresthetica caused by lateral femoral cutaneous nerve injury, visible cutaneous scarring and apical root damage[31-33]. Therefore in order to avoid co-morbidity the use of a bone substitute for repair of the alveolar cleft seems to be the next logical step [34,35].

Bone substitute

Biomaterials used as osteoinductive-like materials for clinical application in bone regenerative surgery can be endued with biologically instructive properties by changing basic parameters such as elasticity and surface texture[36,37]. This thesis describes the application of a micro-structured calcium phosphate ceramic with varying physicochemical and structural characteristics. Micro-porosity correlated to affinity stimulates osteogenic differentiation of stem cells *in vitro* and bone induction *in vivo*. It is based on surface structured ceramic biomaterial scaffolds that have shown the ability to attract stem cells after implantation. It comes from a novel family of ß-TCP granules that have demonstrated osteoinductive-like properties in preclinical models[38,39].

Up to now ceramics have been used to treat small bone defects but in general do not induce stem cell differentiation, which is essential for regenerating larger bone defects[40]. As translation from the *in vitro* proof of concept to clinical application is missing it was decided to put the micro-structured calcium phosphate to the test in a goat model.

Experimental goat model

We postulated that by using a bone substitute instead of autologous bone, the extent, duration and complication rate of cleft alveolus defect-filling procedures could be reduced. This principle itself had to be put to the test using an appropriate defect model in a suitable animal species[41].

As the Joint Animal Laboratory Institute (GDL, Utrecht, The Netherlands) was familiar with the use of goats as an experimental animal model for tissue engineering purposes[42,43], and as a literature search revealed no other appropriate animal model for our research purposes, we decided to use the goat as a model for the creation and the repair of bilateral alveolar clefts, and to examine the subsequent orthodontic tooth movement into an alveolar cleft repaired with either autologous bone or a bone substitute. As such the caprine model for repair of the alveolar cleft and subsequent orthodontic movement was introduced[44].

Chin donor site repair

The step from the experimental animal study to carrying out this procedure in humans is a large one. The use of bone substitute only, i.e. not using autologous bone and/or cell material or growth factors such as Bone Morphogenetic Proteins (BMPs), for the repair of alveolar clefts in children is a novel and challenging approach. We wanted to minimize the risk of complications accompanying the use of artificial grafting material for filling clefts in CLP patients. To this end we chose to first investigate micro-structured resorbable calcium phosphate in an alternative model[45]. As in our Utrecht cleft repair protocol chin bone is used, it was decided to repair the chin donor site with ß-TCP granules and to compare it with a biologically resorbable gelatine sponge (Spongostan®), that is routinely used to restrict bleeding, to cause platelet rupture and to support fibrin threads[46].

Aims of the study

the overall aim of this series of studies was to improve the treatment of repair of the alveolar cleft in children with cleft of lip, alveolus and palate.

First of all we wanted to analyze our results of mandibular bone grafting in the alveolar cleft and subsequent orthodontic treatment (Chapter 2).

We aimed at introducing an osteoinductive bone substitute as an alternative to the autologous bone graft so harvesting procedures, co-morbidity, prolonged operation time and probable extra cost could be avoided (Chapter 3).

Our study in goats aimed at introducing and describing an animal model to be used in the surgical and orthodontic aspects of alveolar-cleft-repair studies. In addition we wanted to test the hypothesis that the novel synthetic bone substitute could be used equally as well as autologous bone harvested from the iliac crest for grafting and repair of alveolar clefts (Chapter 4).

Our next aim was to evaluate and quantify the residual bony defect in the mandibular symphysis when filled with Spongostan® (Chapter 5) or ß-TCP (Chapter 6) and to inventory the effects on the soft tissue contour at a minimum of one year after harvesting chin bone.

The final aim of this study was to evaluate the effectiveness of the calcium phosphate-based micro-structured resorbable bone substitute in children. In a pilot study (N=6) we grafted 6 unilateral alveolar clefts and compared our results with those of an iliac crest graft study[47] (Chapter7).

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Chapter 2

Orthodontic treatment results following grafting autologous mandibular bone to the alveolar cleft in patients with a complete unilateral cleft

Ad de Ruiter, Andries van der Bilt, Gert Meijer, Ronald Koole

Cleft Palate Craniofacial Journal 2010; 47: 35-43.

INTRODUCTION

For repair of the alveolar cleft attention has been largely focused on bone grafting[1-3], the origin of the grafting material[4-13], and on the results of closing the oronasal fistula[14,15]. Subsequent orthodontic treatment and its outcome have been poorly described in the literature. For this reason, we decided to carry out an orthodontic analysis and assessment of study models from patients who underwent bone grafting with autologous mandibular symphysis bone and postoperative orthodontic treatment between 1990 and 2008.

PATIENTS AND METHODS

Sample size

Data from 445 patients treated at the University Medical Centre, Utrecht, The Netherlands and at the Wilhelmina Children's Hospital, Utrecht, The Netherlands for unilateral or bilateral clefts of lip, alveolus and palate, included study models, made both pre- and postoperatively and pre- and post-orthodontic treatment. We excluded 93 patients with bilateral cleft of lip, alveolus and palate and 44 patients with lip cleft only. Also incompletely filed patients were not taken into consideration. So the research patient group included complete unilateral cleft patients, 202 male and 106 female (Table 1).

The male patient group consisted 128 left clefts, 74 right clefts – a left/right ratio of approximately 5:3. The female patient group consisted 79 left clefts, 27 right clefts– a

TABLE 1: UCLP patients with unilateral cleft grafted in UMC / WKZ Utrecht, The Netherlands, between 1990 and 2008

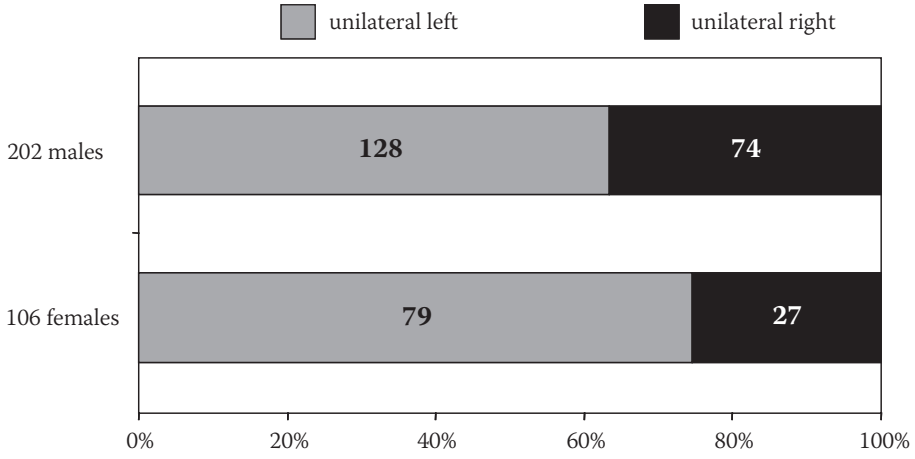
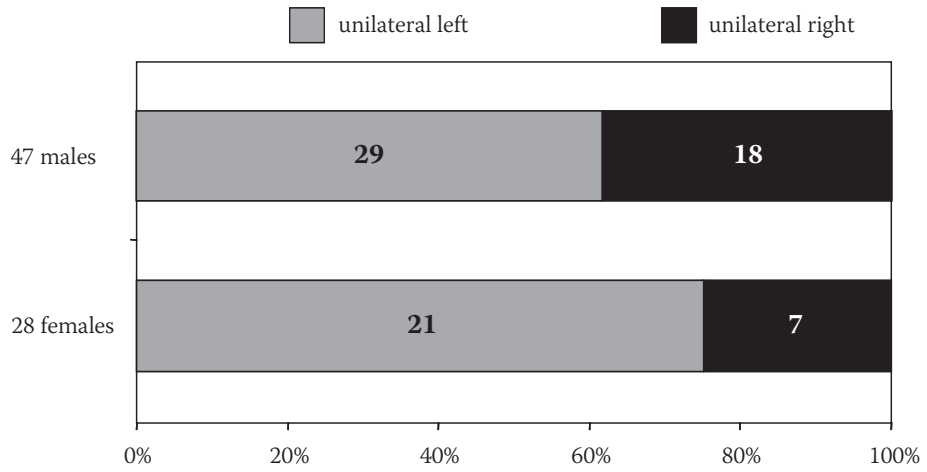


TABLE 2: At random selected CLP patients with unilateral cleft (total number of patients: 75).



left/right ratio of approximately 3:1. Seventy-five completely filed patients with unilateral cleft were selected by means of a random table from the patient group (Table 2).
The average age at operation of the 47 male patients was 11 years and 9 months. The average age at operation of the 28 female patients was 11 years and 3 months.

Surgical treatment protocol

The secondary bone graft to the alveolar cleft in patients with a cleft of lip, alveolus and palate is not only local treatment of a bone defect[16]. It is a reconstruction of the floor of the nose[17,18] and the alveolar process[19]. The continuity of the mucosa and bone in the nose, the support of its alar base and the continuity of the maxillary alveolus are restored and the opening between the nasal and oral cavities is closed by means of a 3-layer closure[20,21]. The alveolar process is filled with autologous bone in order to enable eruption[22,23] and orthodontic movement of teeth[24,25].
The time at which surgical bone grafting to the alveolar cleft takes place is very important and depends on maxillofacial growth and on dental development at the site of the alveolar cleft[26,27]. The timing of operation is based on radiographic images of the unerupted cuspid adjacent to the cleft, whereby the root has to be 50-75% formed[28-32].

Orthodontic treatment protocol

The orthodontist is responsible for a large part of the total treatment time of a cleft patient[33,34]. A stable, uninterrupted maxillary arch with good relationships to the mandibular arch is the aim of the orthodontic treatment of patients with a complete cleft of lip, alveolus and palate[35]. As the development of the mandible is identical in cleft and non-cleft children the final orthodontic treatment begins with the creation

of a good lower dental arch[36] by means of fixed appliances between the ages of nine and eleven dependent on the timing of the grafting operation. Prior to the grafting operation, a short, interceptive orthodontic expansion/rotation procedure of the upper jaw sometimes takes place with Quad-Helix devices[37,38]. This expansion is necessary not only to enlarge the operating area and facilitate access to it, but also to determine the future intermaxillary transverse relationship. Twelve weeks after the grafting operation a final long-term active orthodontic treatment was performed to restore dental intra- and interarch relationships. This orthodontic treatment was carried out by one orthodontist with full fixed appliances and mean treatment time was two years. The bone in the cleft was functionally loaded to ensure that the newly-created bone continuity of the alveolar process was not reduced in volume due to resorption[39,40]. Special notice was given to the uprighting of the roots of the teeth in the middle of the new bone. The functional load is then evenly spread and the interdental and labiolingual alveolar bone height and width remained at the desired level[41,42]. The upper and lower front teeth from cuspid to cuspid were retained with fixed retainers. The transversal expansion of the alveolar process in the upper jaw was also retained with a removable appliance, to be worn at night lifetime.

Study outcome

The aim of this study is to assess the upper dental arch at dental and basal bone level in relation to the mandibular arch after grafting and subsequent orthodontic treatment. Ideally an uninterrupted maxillary arch should have been obtained and the results should be stable.

The pre-operative study models and the post orthodontic treatment study models were collected directly before grafting surgery and at least one year after orthodontic treatment respectively. From these models, using an analogue of the GOSLON Yardstick[43-44], the upper dental arch and the intermaxillary relationships were assessed. The assessments were carried out twice with an interval of three months by one orthodontist who was not involved in the orthodontic treatment. This orthodontist has 25 year experience in the treatment of cleft lip, alveolus and palate patients and was not calibrated in the GOSLON Yardstick. The models were unpaired and assessed randomly.

The GOSLON Yardstick

There are numerous possible variables that may be used to measure the results of surgical and/or orthodontic treatment of cleft dentition very accurately[45-49]. However, there was a need for a method of assessment which was not difficult to carry out and furthermore provided insight into the results of treatment. For this reason, Mars et al. introduced a clinical tool known as the 'GOSLON (Great Ormond Street LONdon and OSLO) Yardstick' [43,50-53]. The GOSLON Yardstick is a clinical visual assessment to view the upper dental arch and its relationships with the mandibular dental arch from dental plaster models. It is a reliable and valid outcome measure for the dental arch relationship

but does not reflect the individual contribution of the skeletal and dental components to the malocclusion or the success/failure of the bone graft.
The dental arch relationships in unilateral cleft patients are divided into five categories in a sliding scale according to the severity of the anomaly. The cleft occlusion/malocclusion is allocated to one of these five categories and the extent of the occlusion/malocclusion and the type of orthodontic and/or corrective surgical procedure that may be necessary in the future is indicated.
Occlusions that require no, or very little, orthodontic treatment are allocated to group1. Cases that will always need combined orthodontic-surgical treatment in order to correct skeletal malrelationships are allocated to group 5. Although this may appear to be a rather coarse method of distinguishing malocclusions, this is intentional: finer discrimination would distract attention from the basics of the degree of severity of the cases in groups 1 to 5. In our study, plaster models were made prior to bone grafting and rated according to the GOSLON Yardstick method and categorized into groups 1 to 5. After bone grafting and orthodontic treatment we used the GOSLON Yardstick again as an applied method to illustrate the dental intra- and intermaxillary changes by re-categorizing the same patients into GOSLON Yardstick groups 1 to 5.

Statistical analysis

Cohen's kappa coefficient was calculated to determine agreement between the results of the two assessments (intra-rater reliability). The distribution of patients over the GOSLON Yardstick groups before and after treatment was analysed with cross tabulation. Possible differences in the distributions were evaluated with the Fisher's exact test.

RESULTS

A large Cohen's kappa was obtained (0.963), which indicates a strong significant agreement ($p < 0.001$) between the two assessments carried out with an interval of three months. The distribution of patients over the GOSLON Yardstick groups before and after treatment is shown in Table 3.

The results of the first assessment are shown. A horizontal line indicates how patients classified in a given GOSLON Yardstick group before treatment moved to other groups following operation and orthodontic treatment (improvement/deterioration). Numbers on the diagonal (bold and highlighted) indicate numbers of patients with the same classification before and after treatment. The numbers below the diagonal (italic) indicate improvement. Fisher's exact test showed a highly significant difference in the distributions before and after treatment ($X^2 = 38$ and 35 for allocation 1 and 2; $p < 0.001$).

TABLE 3: Cross tabulation of patients over Goslon Yardstick groups. A horizontal line indicates how patients classified in a given group before treatment moved to other groups after treatment. Numbers on the diagonal (bold and highlighted) indicate numbers of patients with the same classification before and after treatment. Italic numbers indicate improvement.

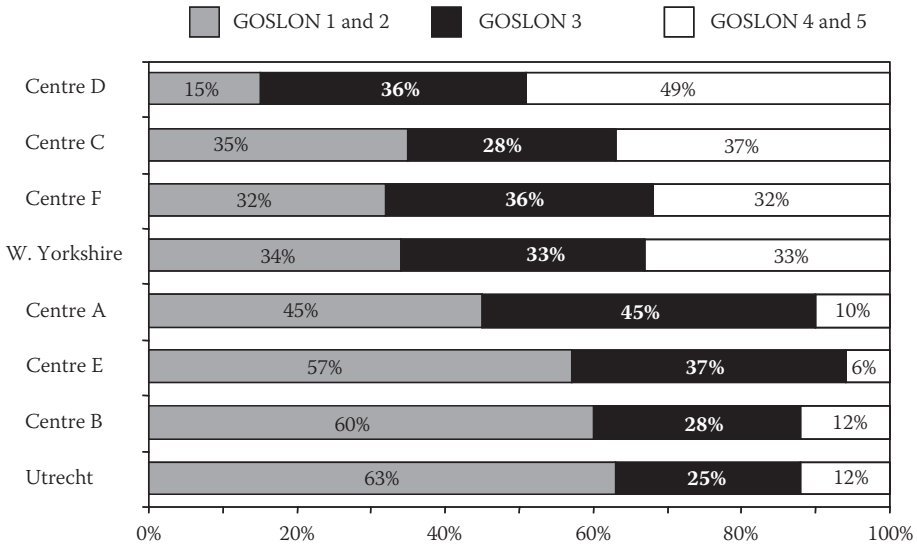
	total (before)		group 1	group 2	group 3	group 4	group 5
group 1	8	→	**7**	1	0	0	0
group 2	39	→	*27*	**3**	9	0	0
group 3	17	→	*5*	*3*	**5**	3	1
group 4	9	→	*1*	*1*	*3*	**4**	0
group 5	2	→	*0*	*1*	*0*	*0*	**1**
total (after)	75		40	9	17	7	2

DISCUSSION

The first GOSLON Yardstick group categorization of our study models prior to bone graft corresponded with the studies carried out by Mars and Morris[50,54]. The percentage of 'successful' (GOSLON groups 1 and 2) cleft occlusions, 62.7% (Table 4), at an average age of eleven following closure of the hard palate is high. In our opinion, this is related to the epiperiostal closure of the hard palate which results in less scar tissue and thus less transversal contraction of the upper maxillary arch.

The second categorization using the GOSLON Yardstick dental models was carried out following bone grafting and orthodontic treatment. From a total of 75 patients, 41 were moved up to a higher group (Table 3, sum of bold numbers). Of the eight patients in

TABLE 4: Comparison of the GOSLON Yardstick – category allocation before grafting between the UMC / WKZ - Utrecht group, the West Yorkshire group[50,54] and six European centres[50].



group 1, seven remained in group 1 (Table 3; first line). We regarded the final result of treatment as being excellent if the patient finished in group 1. Group 2 was regarded as a very acceptable result, with only a single small diastema or some limited rotation. This means that in 49 patients (= group 1 + group 2 = 65.3%) the aim of treatment had been achieved by the time of the second assessment i.e. after operation and orthodontic treatment. In some cases growth had not yet stopped and this may still affect the results. However, in September 1999, Levitt et al concluded "*when evaluated longitudinally, maxillary growth in patients having received secondary alveolar bone grafting did not differ from a group of matched results*" [55].

We also looked at the ways in which improvement or deterioration came about.

After operation and orthodontic treatment, the malocclusion of one patient from group 1 worsened and the patient was reclassified into group 2 (Table 3; first line). This was probably related to relapse combined with inhibition of growth caused by scar tissue. The same was seen in group 2 where nine patients moved down to group 3 (Table 3; second line). In contrast however, 27 patients improved and moved up to group 1. Eight group 3 patients moved up to groups 1 and 2 (Table 3; line 3), after orthodontic treatment which included the use of implants or prostheses. Four patients from group 3 worsened and moved down to groups 4 and 5. This too was probably the result of unfavourable growth and relapse of the orthodontic treatment result. Following bone graft and orthodontic treatment group 4 consisted seven patients (Table 3). Three of these patients came from group 3, and four remained in group 4. Four of the seven patients in group 4 underwent a le Fort I osteotomy after growth had stopped. This indicates that due to inhibited maxillary growth, patients who prior to bone graft were in GOSLON Yardstick group 4, and sometimes in group 3, are more likely to require combined orthodontic-surgical treatment in the form of an osteotomy of the upper and/or lower jaw after growth is completed. The treatment of the two patients who finished up in group 5 (Table 3) can be regarded as a failure. As well as very unfavourable growth, there was also bone graft resorption as a result of orthodontic treatment being started too late. Ultimately, despite orthodontic treatment and a le Fort I osteotomy, relapse occurred.

The statistical analyses showed high significance ($p<0.001$) in both the allocations of the outcome of the results before/after operation and following orthodontic treatment. Of course large improvements of both surgical and orthodontic efforts may be expected, but statistical proof adds to this assumption.

The contributing factors to these results are, the epiperiostal approach in the closure of the hard palate, choosing the correct time to carry out the bone grafting procedure and following it up at approximately three months by the start of orthodontic treatment and also regular follow-up to monitor and maintain the results that were achieved[56-58]. Mars et al.[53] and Morris[54] also add that the experience of the treating professionals and the following of strict treatment protocols point the way to success[59]. However, no

orthodontic parameter only is suitable to give a verdict on the merit of the origin of the bone graft[60].

In the Wilhelmina Children's Hospital (Utrecht, The Netherlands) we use mandibular symphysis bone for grafting. Mostly the surgery is performed by one oral maxillofacial surgeon. After Bosker and van Dijk published their results on grafting bone from the chin to the alveolar cleft in 1980[61], Bosker and Koole[62] produced a number of articles concerning the transplantation of autologous ectomesenchymal mandibular symphysis bone from the chin to the alveolar cleft[5,60,62]. The particular suitability of using the mandibular symphysis bone for grafting to the alveolar cleft was also described by several authors[8,63-67]. They all preferred working at one operation site and they also described the advantage of low incidence of morbidity.

The graft operation using autologous ectomesenchymal mandibular symphysis bone resulted in an alveolar process of sufficient volume[8]. The teeth on either side of the grafted cleft were able to erupt normally (66.70%) or needed surgical assistance(33.3%) (Table 5).

TABLE 5: Cuspid status and eruption after Alveolar Cleft Repair in UCLP patients

Cuspid status	Impacted pre-op	Spontaneous eruption	Surgical assistance
	50%	66.7 %	33.3 %

The orthodontist had sufficient bone to be able to move teeth into the new bone for functional loading and to upright them in overcorrection. This functional loading occurs either when teeth erupt spontaneously or when the teeth are guided orthodontically into the new bone once erupted. Although the assessments of the radiography are beyond the limits of this study, there was no doubt about supporting bone and interradicular bone height. The mandibular symphysis bone necessary for the graft was harvested from one donor site i.e. the same operating field in which the grafting takes place. The amount obtained was often enough to fill even a bilateral cleft defect[68,69] and the operation itself did not result in extraoral scarring and morbidity[13,70,71]. At only one donor site one incisor root canal was obliterated.

Depending on whether elements erupt spontaneously into the newly transplanted bone or not, as well as pattern of exfoliation and age at which exfoliation takes place, there will almost always be a need for orthodontic treatment. After orthodontic treatment, it is of the utmost importance that the result that has been achieved should be maintained. The upper and lower front teeth from cuspid to cuspid will require permanent 'lifetime' retention with fixed retainers[72]. No recurrence of rotation or diastema is illusive. The transversal expansion of the alveolar process in the upper jaw will also require lifetime retention with a removable appliance, worn at night[37]. Scarring of the palate will occur following the closure of the hard palate and this will cause constant traction towards the midline[17,72].

None of these methods of retention, however, can exclude the possibility that the result of treatment may be negatively affected by growth[73,74]. If this occurs then an osteotomy of the upper and/or lower jaw is unavoidable[75,76].
For all mentioned reasons it is our opinion that the use of autologous mandibular bone is a reliable method of filling the alveolar cleft in patients with cleft of lip, alveolus and palate and that correctly timed subsequent orthodontic treatment adds to the excellence of the overall treatment result.

CONCLUSIONS

On comparison with a multicentre international study on the results of closure of the hard palate, the WKZ epiperiostal closure group scores high.
Bosker, van Dijk, Koole, Freihofer et al. and Enemark[8,60-65], all show a preference for using autologous intramembranous ectomesenchymal mandibular symphysis bone for grafting the cleft. Our study corroborates the assumption that orthodontic treatment of patients with complete cleft of lip, alveolus and palate after bone grafting with chin bone shows excellent results.
Patients, who prior to bone graft are in GOSLON Yardstick group 4, and sometimes in group 3, are more likely to require combined orthodontic-surgical treatment in the form of an osteotomy of the upper and/or lower jaw after growth has completed.
In the Netherlands current best practice involves harvesting bone for grafting intraorally, thus avoiding the necessity of a potentially detrimental intervention at a second extraoral site. In the future, however, resorbable bone substitute is likely be the treatment of choice[77-81]. The results of a current study on the use of a resorbable osteoconductive Bèta-TriCalcium Phosphate (β-TCP) as a bone substitute will provide more information on this subject at short notice.

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Chapter 3

Osteoinductive ceramics as a synthetic alternative to autologous bone grafting

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INTRODUCTION

The role of biomaterials as medical devices is changing from a biologically passive, structural role to one in which the properties of the material will orchestrate the process of tissue regeneration. The change is fed by an increasing number of reports demonstrating that cellular behaviour can be modulated by material properties such as surface texture, elasticity, and chemistry[1-5].

For instance, in the area of biomaterials for the restoration of bone defects, it has been reported that surface topography influences osteogenesis and proliferation of bone marrow-derived multipotent mesenchymal stromal cells (MSCs) *in vitro*[6,7]. Although tissue instructive materials hold great potential as off-the-shelf bioactive medical devices, so far the concept has not progressed beyond the proof-of-concept phase in which *in vitro* assays demonstrate an effect on cellular differentiation or proliferation[8].

Consequently, transplantation of autologous bone is still the gold standard in bone repair strategies. Auto-grafts guide the in-growth of osteoblasts -the primary cell type responsible for bone matrix apposition- from the adjacent tissues into the defect, a process referred to as osteoconduction. In addition, autologous bone induces de novo bone formation by triggering the differentiation of undifferentiated progenitor cells into the osteogenic lineage, referred to as osteoinduction[9,10]. This phenomenon is essential for the repair of large critical-size bone defects.

Drawbacks of auto grafting are the limited availability of autologous bone and the negative side effects of bone harvesting, which make the search for bone graft substitutes an area of intense research[11].

The discovery that osteoinduction can be accomplished by devitalized demineralized bone matrix (DBM) and the subsequent identification of bone morphogenetic proteins (BMPs) provided an alternative to bone auto grafts[9]. Both DBM and BMPs are broadly applied in clinical practice, but their biological nature has implications for the production process leading to rather high batch variability and high production cost[12-15]. Moreover, the *in vivo* delivery of soluble molecules such as BMPs is inefficient. An alternative to the biological approach to bone regeneration would be the development of a synthetic material with intrinsic osteoinductive capacity. Winter and Simpson reported bone formation upon implantation of a polyhydroxyethylmethacrylate sponge under the skin of pigs, an experiment performed in an attempt to explain incidences of hard tissue formation upon implantation of synthetic breast implants[16]. Over the last 30 years, several porous calcium phosphate biomaterials as well as some metals have been reported to possess osteoinductive capacity[17].

The underlying mechanism leading to bone induction by synthetic materials remains largely unknown; however, the osteoinductive potential of biomaterials can be controlled by tailoring material characteristics such as chemical composition, surface topography, and geometry, which in turn affect resorption rate and cell-material interactions.

The aim of this study was to correlate the osteogenic potential of a family of porous ceramic materials *in vitro* to ectopic bone formation *in vivo* and to demonstrate that synthetic materials present a bona fide alternative to auto graft and BMP therapy with equal performance in the healing of a critical-size bone defect.

RESULTS

Synthesis and Characterization of Calcium Phosphate Ceramics

In order to produce porous calcium phosphate ceramics with varying biological activities, we either used calcium phosphate powder with different chemical compositions (hydroxyapatite [HA], tricalcium phosphate [TCP], or a mixture thereof [biphasic calcium phosphate, BCP]) or we exposed the ceramics to different post synthesis sintering temperatures (BCP1150 sintered at 1150 °C and BCP1300 sintered at 1300 °C) to obtain materials with equal chemistry but varying microstructure. Using X-ray diffraction(XRD) analysis, we observed the presence of β-TCP in BCP and a trace of HA (<10 wt%) in TCP, whereas HA was phase pure (Fig. 1A). Image analysis on cross-section

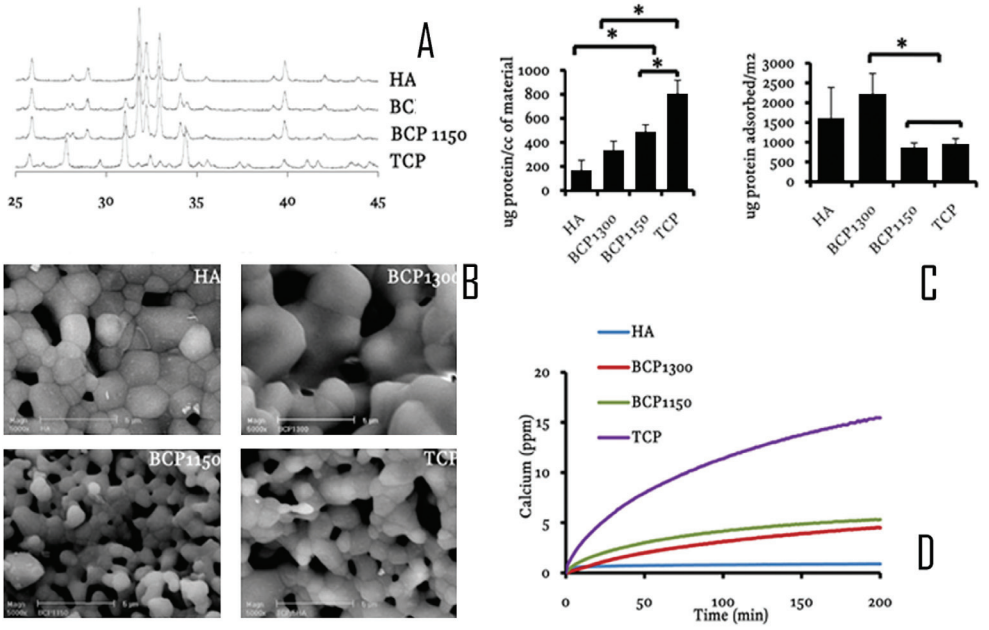

FIGURE 1: Characterisation of calcium phosphate ceramics. (A) XRD analysis showing the composition of the four different ceramics with their characteristic peaks indicated. (B) Environmental SEM photographs depicting their microstructure. (C and D) Protein adsorption and calcium release profile of the different ceramics, respectively. The error bars represent standard deviations. (*) denotes statistical difference (one-way ANOVA and Tukey's test, $P < 0.05$).

showed no differences in macrostructure between the different ceramics, although the micro pore size and volume varied (Fig. 1A and 1 B). The average grain size of BCP1150 and TCP was smaller and the number of micro pores higher on comparison with HA and BCP1300 as shown by SEM (Fig. 1B). As a consequence, the specific surface area of the four ceramics varied from 0.1 m2/g for HA to 1.2 m2/g for TCP, resulting in differences in adsorption of serum proteins, with TCP adsorbing more proteins per volume of material than BCP1150 and BCP1300 (Fig. 1C). In contrast, when adsorption of protein was expressed per surface area, BCP1150 and TCP bound less proteins than BCP1300 and HA. Despite variations in the quantity, no qualitative differences were observed in the type of proteins adsorbed on different ceramics (Fig. 1C). We assessed the rate of calcium release from the four ceramics and found that it was significantly faster for TCP compared to the other three ceramics (Fig. 1D). An overview of the materials characteristics is given in Table 1.

	HA	TCP/5HA	BCP1150	BCP1300
Chemistry	HA	5HA+95TCP	20TCP/80HA	20TCP/80HA
Particle size (mm)	1-2	1-2	1-2	1-2
Specific surface (m2/g)	0.1	1.2	1	0.2
Percentage of materials (%)	46.4±2.4	49.9±1.8	45.6±2.2	44.6±1.9
Microporosity (%)*	3.1	8.7	41.1	48.7
Ca release (ppm)	0.9±0.1	15.3±0.2	5.4±0.1	4.2±0.4

* Volume percentage of micropores smaller than 10 µm within the ceramic.

Materials Support Osteogenic Differentiation of Human Bone Marrow-Derived Multipotent MSCs

The effect of material properties on tissue development is mediated via cell-material interaction and therefore we decided to analyze the effect of the four different materials on osteogenic differentiation of human multipotent marrow stromal cells (hMSCs) *in vitro* and *in vivo*[18,19]. First, we seeded hMSCs [previously tested for their multipotency and phenotypically characterized following protocols described elsewhere[20]; see Figs. 2 and 3] on the four different ceramics and cultured them for 7 days in osteogenic differentiation medium, after which quantitative PCR was performed on a panel of genes indicative of osteogenic differentiation.

As a control, we grew hMSCs on tissue culture flasks in control and osteogenic medium and observed the well-documented increase in alkaline phosphatase (ALP) expression in osteogenic medium (Fig. 4)[21]. With the exception of ALP and collagen type I, gene expression of all genes was higher on the four ceramics than on tissue culture flasks, suggesting that the ceramics favour osteogenic differentiation (Fig. 2A and Fig. 4) Furthermore, marked differences in expression levels of genes encoding osteocalcin, bone sialoprotein, and osteopontin were found in hMSCs cultured on the different

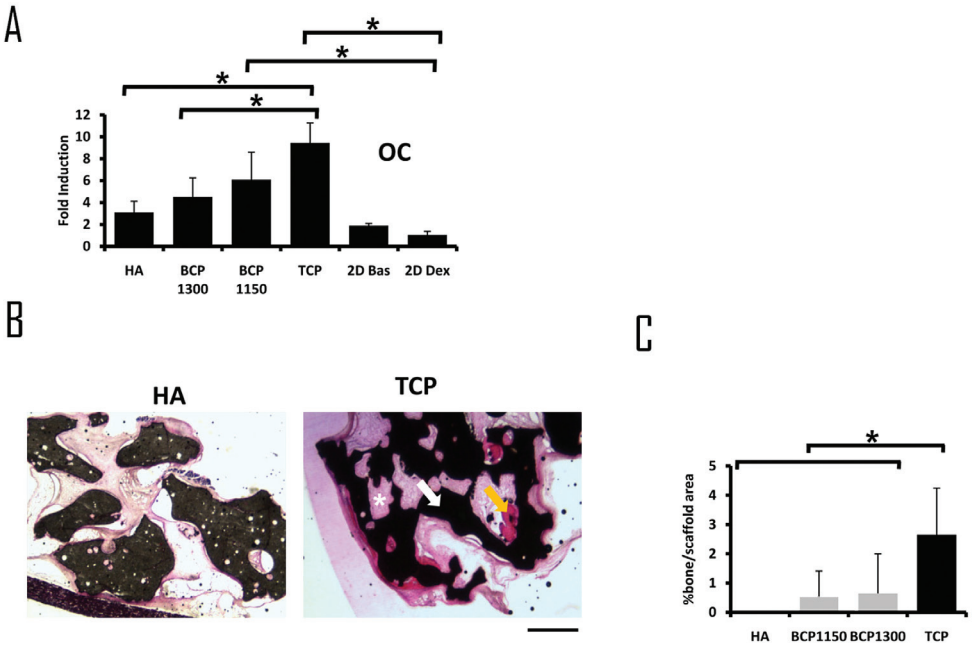

FIGURE 2: Osteogenic differentiation of hMSCs on ceramics of different composition. (A) Expression of the bone-related protein osteocalcin by hMSCs seeded in the different ceramics. Expression levels were normalized with 18S. Fold induction was calculated using the ΔCT method relative to dex-treated hMSCs in tissue culture plates. The error bars represent standard deviations. B and C indicate the bone forming potential of hMSCs seeded in different ceramics. Histological sections (B) and quantification of bone area per scaffold area (C) are shown. Basic Fuchsin stains bone red (orange arrow), methylene blue stains fibrous tissue blue (*), and the ceramic is shown in black (white arrow). The error bars represent standard deviations. (*) denotes statistical difference (one-way ANOVA and Tukey's test, $P < 0.05$). (Scale bar: 200 μm.)

ceramics. A similar but less profound effect for S100A4 andRunx2 was also observed. Interestingly, hMSCs on TCP consistently displayed the most osteogenic profile, and on HA the least (Fig. S4).

Next, we assessed bone apposition by hMSCs on the different ceramics. Porous calcium phosphate ceramics are frequently used in bone tissue-engineered constructs, in which culture expanded MSCs are seeded onto the ceramic *in vitro* and then implanted. Upon implantation, MSCs will differentiate into osteoblasts and deposit bone tissue onto the ceramic surface[22]. We cultured hMSCs *in vitro* for 7 days on the four calcium phosphate ceramics (HA, BCP1300, BCP1150, and TCP) in osteogenic medium. The constructs were implanted subcutaneously into immunodeficient mice for 6 weeks after which formation of new bone tissue was assayed using histomorphometry. No bone formation was observed either on scaffolds without cells or on HA scaffolds seeded with hMSCs (Fig. 2B and Fig. C). In contrast, we did observe apposition of bone tissue on grafts of BCP1300, BCP1150, and TCP ceramics seeded with hMSCs. On TCP, we observed an amount of bone five times higher than on BCP1300 and BCP1150 (2.7 _ 1.6% in TCP, 0.7

_ 1.3% in BCP1300, and 0.6 _ 0.9% in BCP1150), demonstrating that ceramics stimulated osteogenic differentiation *in vitro* and bone formation *in vivo* depending on their physicochemical and structural characteristics.

In Vivo Osteoinduction by Different Calcium Phosphate Ceramics

As previously mentioned, osteoinduction is a critical parameter of any bone graft in large bone defects. To analyse the osteoinductive potential of our ceramics, we used both ectopic implantation in the muscle tissue of eight dogs and a clinically-relevant posterolateral spinal fusion model in which two materials can be compared in a paired manner. For this purpose, we selected the two extremes from the previous experiments, HA and TCP. After 12 weeks of implantation histomorphometric analysis showed that the area percentage of bone in available pore space was five times higher in TCP than in HA, both in muscle and in the spine, demonstrating that calcium phosphate ceramics

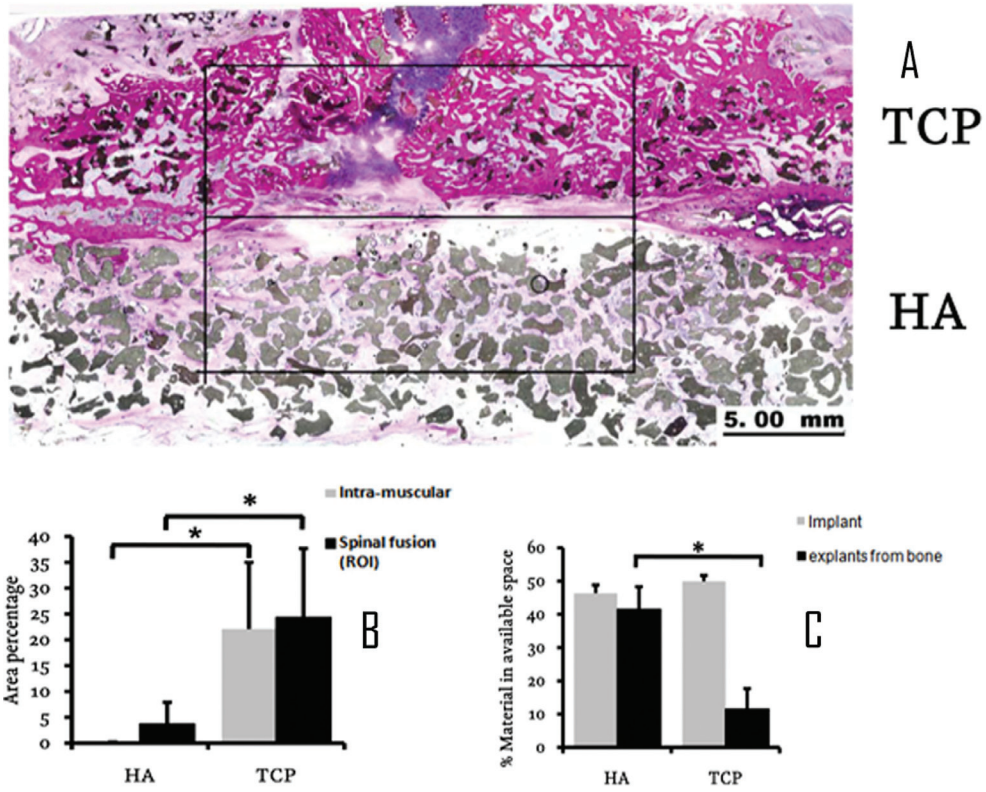

FIGURE 3: Posterolateral spinal fusion in dogs. (A) Histological overviews showing newly-formed bone in TCP and HA implants (square, region of interest). (B) The area percentage of bone for HA and TCP ceramics in the case of intramuscular implantation (grey bars) and in the spinal fusion (black bars). (C) The percentage of material available before implantation (grey bars) and upon explantation (black bars). Note that HA ceramic was not resorbed during the 12 weeks implantation in contrast with TCP. The error bars represent standard deviations. (*) denotes statistical difference (Student's paired t test, $P < 0.05$).

with differing chemical composition have different osteoinductive potential, which was in accordance with *in vitro* results (Fig. 3 A and Fig. B).
We observed a resorption of 77% of the implanted TCP after 12 weeks of implantation, whereas no detectable resorption of HA was found (Fig. 3C).
To demonstrate that not only chemistry but also structural characteristics can influence the osteoinductive potency of ceramics, we implanted BCP1150, BCP1300, and TCP in muscle of ten sheep. Unfortunately, HA was omitted from this study due to technical difficulties. Twelve weeks after implantation in paraspinal muscles, we observed that bone induction had occurred in all three calcium phosphate ceramics; however, the amount of bone that was formed varied between the different ceramics (Fig. 4A). Again bone apposition was highest in TCP (28.7 _ 4.8% of available pore space) followed by BCP1150 (17.7 _ 5%). Significantly less bone was observed in BCP1300 (11 _ 7.5%) indicating that both chemistry and structural properties can influence the *in vivo* osteoinductive potential of the ceramics (Fig. 4B).

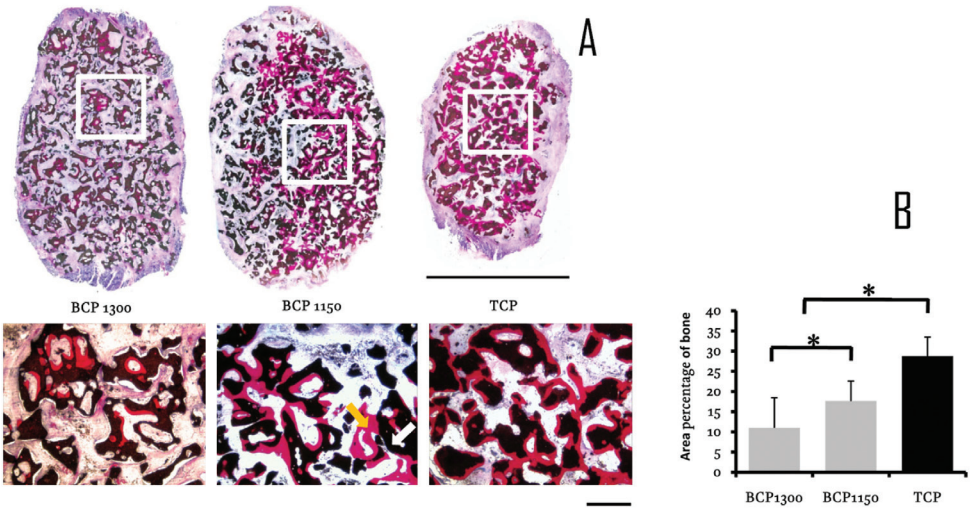

FIGURE 4: Osteoinductive potential of different calcium phosphate ceramics implanted intramuscularly in sheep. (A) Histological sections showing the newly-formed bone (orange arrow) and the calcium phosphate ceramic (white arrow) upon 12 weeks implantation. Basic Fuchsin stains the newly-formed bone red, methylene blue stains fibrous tissue blue, and the scaffold is shown in black. (B) Quantification of newly formed bone. The error bars represent standard deviations. (*) denotes statistical difference (one-way ANOVA and Tukey's test, $P < 0.05$). (Top, Scale bar: 10 mm.) (Bottom scale bar: 200 µm.)

Calcium Phosphate Ceramics as a Bone Graft Substitute in a Critical-Size Defect

The studies above indicate that TCP is a superior ceramic with respect to stem cell differentiation and osteoinduction *in vivo*. We next tested the ability of TCP to heal a critical-sized orthotopic defect in comparison with standard treatments. To this end, we

implanted TCP in a bilateral iliac wing defect in sheep with a critical-size diameter of 17 mm. As a negative control, we included a group in which the defect was left empty, and as a positive control, we used two groups of sheep in which the defect was either treated with autologous bone or with a preparation of recombinant human BMP-2 (rhBMP-2) delivered in a collagen sponge (Infuse® Bone Graft, Medtronic). The latter treatment is a commercially available product used for spinal fusion surgery[23,24].

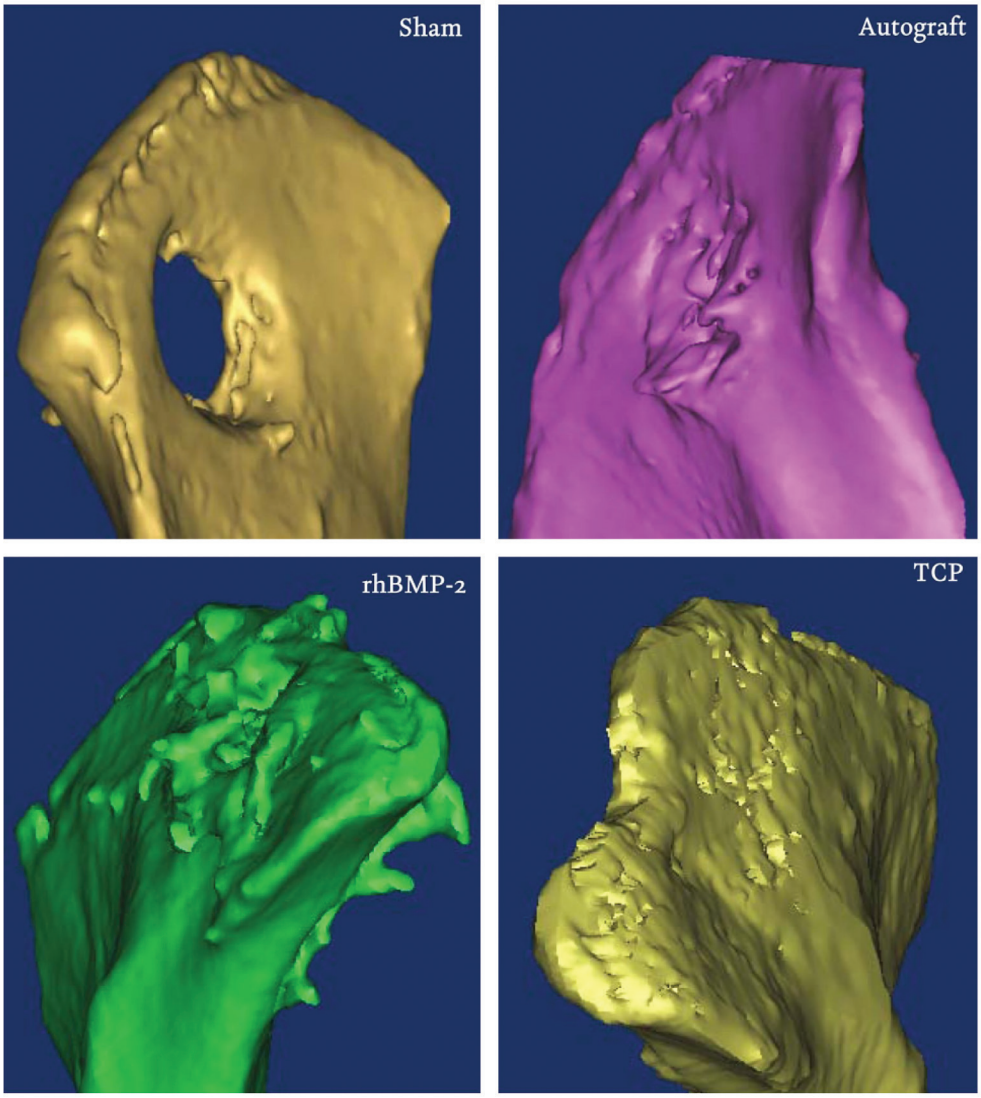

FIGURE 5: Ilium defect. Three-dimensional models of the os ileum 12 weeks after implantation. Bone formation outside the margins of the defect was found in the rhBMP-2 group, whereas in the TCP group, the material remained within the defect with new bone formation and implant resorption observed at 12 weeks. (scale bare: 1cm

Twelve weeks after implantation, we observed that the iliac wing defect was not able to heal spontaneously, although some newly-formed bone could be seen along the host bone bed (3.1 _ 1.8% of the defect was covered with bone; Fig. 5 and Fig. 6), confirming that the defect was critically sized[25]. In contrast, when autologous bone was used as an implant, bone was found throughout the defect (32.2 _ 6.5%). As it is difficult to distinguish between implanted bone auto graft and newly-formed bone, the percentage of bone measured represents both residual bone auto graft and newly formed bone. Histological analysis revealed tight bonding between new bone and the host bone bed, without an interspersed fibrous tissue layer (Fig. 6). However, in all animals tested less

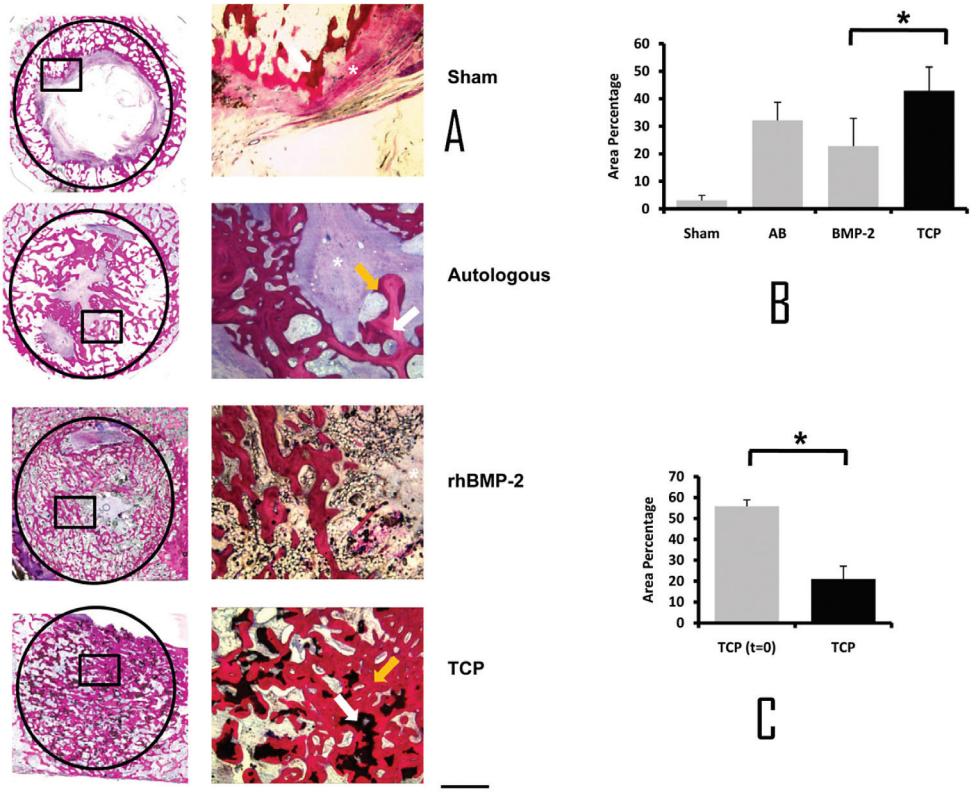

FIGURE 6: Performance of calcium phosphate ceramics, autologous bone, and rhBMP-2 in a critical-size defect in the ilium of sheep. (A) Histological sections depicting the newly-formed bone within the defect created in the ilium of sheep. The defect margins are indicated by the black circle (17 mm in diameter) on the left panel and on the right panel the demarked region can be seen in detail. Basic fuchsin stains bone red, methylene blue stains fibrous tissue blue, and the scaffold is shown in black. Newly-formed bone is indicated by an orange arrow, autologous bone or the ceramic material are indicated by a white arrow, and fibrous tissue is indicated by an asterisk (*). B and C represent the area percentage of bone per available area between the different conditions (B) and the resorption of the ceramic 12 weeks after implantation (C). The error bars represent standard deviations. (*) denotes statistical difference (one-way ANOVA and Tukey's test, $P < 0.05$ (B) and Student's paired t test, $p < 0.05$ (c). (Scale bar: 200 μm.)

bone was observed in the centre of the defect than in the periphery of the defect, and large areas were filled with fibrous tissue.
Results obtained upon implantation of rhBMP-2 were similar to those of autologous bone. In the rhBMP-2 treated defects, 22.8 _ 10.1% of the defect was filled with new bone. Fibrous tissue was observed in the centre of the defect of all animals tested. In addition, ectopic bone formation outside the defect area was found in 8 out of 10 animals, probably due to diffusion of rhBMP-2 from the graft into the adjacent soft tissue (Fig. 5).
Interestingly, implants made of TCP ceramic showed similar performance to autologous bone regarding bone formation. With an area of new bone covering 33.9 _ 6.8% of the defects, they outperformed the rhBMP-2 group, an overview of the histology and quantification of bone formation can be seen in Fig. 6A and B.
Significant resorption of the ceramic material was observed: the percentage of ceramic in the defect area had decreased from 56% to 21% after 12 weeks of implantation (Fig. 6C). Similar to auto graft, new bone formed in the TCP treated defect formed a tight bond with the host bone, and no fibrous tissue was observed in the periphery of the defect. In the central area of the defect, only 2 out of 10 animals showed the presence of fibrous connective tissue.

DISCUSSION

The fully synthetic implant based on calcium phosphate ceramic reported in this study was equally as successful as autograft and rhBMP-2 in the treatment of a critical-sized bone defect.
Unlike many other synthetic bone graft substitutes which are considered solely osteoconductive, the ceramic presented here possesses intrinsic osteoinductivity, comparable to autograft, DBM, and BMPs. This intrinsic osteoinductivity could, in part, explain its performance orthotopically and represent a paradigm shift in the treatment of bone defects.
So far, porous ceramics have been used as bone fillers for small bone defects, where osteoconduction is sufficient. Due to its autologous nature and strong clinical performance autograft is still the preferred treatment of complex and large bone defects. Although BMPs are potent molecules and represent an alternative to autograft, limitations remain in terms of dose, release kinetics, and mechanical properties.
In the United States alone, annually around 300,000 patients undergo spinal fusion procedures involving mainly autologous bone grafting and BMPs At an estimated cost of $11.25 billion. In our study, TCP proved equally efficient as autologous bone in inducing bone apposition in the preclinical iliac wing defect model in sheep, with even less fibrous tissue in the central regions of the implant than autologous bone. Fibrous tissue formation is one of the main reasons for unsuccessful healing of large bone defects

and non-union as well as for implant failure. Bone formation induced by TCP remained within regions of the defect, whereas we observed BMP-induced bone formation in soft tissue surrounding the defect as well. Based on the findings reported in this paper, we have embarked on clinical trials to evaluate the possibility of using TCP only as a bone graft in large bone defects.

Our data show that the ability of ceramics to instruct cell and tissue development can be controlled merely by changing either the chemical composition or structural properties. The calcium phosphate ceramic formulations investigated in this study represent a process of optimization in the search for an optimal alternative to autograft. First, chemistry was varied from pure HA, to a mixture of HA and TCP (BCP), and finally to TCP with traces of HA. Second, the macrostructure of the four ceramics was kept equal in each of the four ceramics. The presence of macro structural features, such as macro-pores[26-28], concavities/channels[29,30], and voids between particles[31] has previously been shown to be a prerequisite for osteoinduction by synthetic biomaterials. By keeping the macro porosity of the four ceramics similar, we attempted to avoid the effect of this parameter on their osteoinductive potential. Finally, micro structural properties were controlled by the processing parameters: micro structural surface properties of the two BCPs, with equal chemistry and macrostructure, were varied by controlling their sintering temperature. Significant differences in osteoinductive potential were observed between the BCPs sintered at 1150 and 1300 °C: An increase in micro porosity and a decrease in grain size, resulting in an increase of the specific surface area of the ceramic, was shown to render a ceramic osteoinductive[32]. Similarly, a comparison between HA and BCP, sintered at the same temperature, thus with similar macro- and micro structural features but different chemistry, showed a more pronounced bone formation in BCP, which contains highly resorbable TCP[33]. The suggestion that both an increase in specific surface area and an increase in resorbability of a ceramic may be beneficial for its osteoinductive potential eventually led to the development of a calcium phosphate ceramic with higher TCP content and higher surface area—the TCP formulation presented herein. Many other formulations could be developed; however, the processing parameters and the fact that osteoinduction is mainly observed in large animals limit the number of formulations to be tested. The high osteoinductive potential of TCP begs questions about the cellular and molecular mechanism behind it. Although we have not pinpointed a particular signal transduction pathway yet, the fact that TCP shows higher expression of osteogenic markers by hMSCs *in vitro* and more *de novo* bone formation *in vivo* on comparison with the other investigated ceramics suggests a mechanism in which TCP triggers osteogenic differentiation. Our working hypothesis is that pericytes, the smooth muscle cells aligning with the invading capillary blood vessels, encounter a milieu in which the cells differentiate into osteoblasts[34].

We are testing the hypothesis by focusing on the interaction between TCP and hMSCs *in vitro* and in the ectopic bone formation model using microarray analysis and genetic

interference studies. Another question concerns the other side of the molecular interface: Which biomaterial properties play a role in osteoinductivity and how do they influence the osteogenic process?

Differences in dissolution behaviour of the ceramic, which can be obtained either by changes in chemical composition (calcium phosphate phase) or by changes in structural properties (crystallinity, grain size, porosity, specific surface area) seem to be associated with osteoinductive potential *in vivo*[17]. We observed that the ceramic with the strongest osteoinductive potential displays the most pronounced dissolution *in vitro* and degradation *in vivo*.

It is plausible to think that the calcium release plays a role in the process but further studies are needed to identify the responsive cell types and the pathways activated by calcium. Similarly, we observed differences in *in vitro* protein adsorption with TCP as the ceramic with the highest protein adsorption per volume of all ceramics. Nevertheless, despite these quantitative differences, we did not observe differences regarding the nature of the proteins adsorbed by the four ceramics. It is important to investigate whether the higher protein content of TCP reflects changes in the pro/anti osteogenic protein ratio and, if so, which proteins are involved in the process. However, it should be emphasized that differences in protein adsorption do not necessarily have a causal relationship with osteoinductive potential *in vitro* and *in vivo*.

Resolving the molecular mechanism of osteoinduction will offer tools to develop new osteoinductive materials, e.g., based on polymeric materials, in order to meet other requirements for successful bone repair, such as mechanical and handling properties. Furthermore, by understanding biological processes involved in osteoinduction by biomaterials, we will obtain more fundamental insight into biomaterial–tissue interactions. Data presented in this manuscript on ceramic biomaterials in the area of bone graft substitution provide preclinical proof of concept for a generation of smart materials, displaying superior biological performance through modulation of cell behaviour.

Bio instructive materials have found their way in a plethora of applications, from biodegradable sutures to contact lenses and stents, and in all instances, the challenge lies in finding the optimal parameters for the particular biomedical application. To do so, *in vitro* bioassays that predict the performance of a material for intended application in the human body are essential. In this manuscript, the prospective knowledge of the osteoinductive capacity of different ceramics helped us in identifying a suitable *in vitro* cell system. For other biomaterials and other clinical applications, cell biologists and material scientists need to team up to streamline the process of identifying tissue instructive biomaterial properties.

MATERIALS AND METHODS

Synthesis and Characterization of Calcium Phosphate Ceramics. HA ceramics were prepared from HA powder (Merck) using the dual-phase mixing method and sintered at 1250 °C for 8 h according to a previously described method[35].

BCP ceramics were fabricated using the H2O2 method using in-house made calcium-deficient apatite powder and sintered at 1150 °C (BCP1150) and 1300 °C (BCP1300), respectively[36]. The method used to synthesize the BCP ceramics was also used for preparation of TCP. TCP ceramics were prepared from TCP powder (Plasma Biotal) and sintered at 1100 °C. Ceramic particles (1–2 or 2–3 mm) were prepared, cleaned ultrasonically with acetone, 70% ethanol and demineralized water, dried at 80 °C, and sterilized by gamma irradiation prior to use.

The macro- and microstructure of the different ceramics was evaluated using an SEM (XL30, Environmental SEM-Field Emission Gun, Philips). Composition of the ceramics was determined by XRD (Miniflex). Specific surface area of the different ceramics was analysed with mercury intrusion (Micromeritics Instrument, Inc.).

The calcium release profile of the ceramics was determined by immersing 0.5 mL of 1–2 mm ceramic particles in 100 mL of simulated physiological saline (0.8% NaCl, 50 mM Hepes, 37 °C, pH 7.3) and monitoring the calcium concentration using a calcium electrode for 200 min.

To calculate the concentration of protein adsorbed, 1 mL of ceramic particles was incubated for 3 d in 1% FBS in PBS at 37 °C. Protein adsorption was measured using a protein assay kit (micro-bicinchoninic acid™, Perbio) in accordance with the manufacturer's protocol. Analysis of the proteins adsorbed to the different ceramics was performed using gel separation by electrophoresis after incubation of the ceramics for 1 d in serum. Equal amounts of proteins were loaded onto the gel and, upon separation, the gel was stained with Coomassie blue.

RNA Isolation and Quantitative PCR

To analyse the effect of the various ceramics in the gene expression profile of hMSCs, 2 × 105 cells were seeded per three particles and cultured for 7 d in osteogenic medium. As a control, we seeded 5.000 cells⁄cm2 on tissue culture flasks either in basic or osteogenic medium for 7 d. Total RNA was isolated using Trizol and the Nucleospin RNA isolation kit (Macherey-Nagel) in accordance with the manufacturer's protocol. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Six hundred and fifty nanograms of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (BioRad) in accordance with the manufacturer's protocol. PCR was performed on a Light Cycler real-time PCR machine (Roche) using SYBR® green I master mix (Invitrogen). Data was analysed using Light Cycler software version 3.5.3, using fit point method by setting the noise band to the exponential phase of the reaction to

exclude background fluorescence. Fold induction relative to cells grown on tissue culture flasks in osteogenic medium was calculated using the ΔCT method after normalization with 18S as a housekeeping gene.

Ectopic Bone Formation by hMSCs

hMSCs were isolated from adult bone marrow and cultured as previously described[37]. To evaluate the effect of different calcium phosphate ceramics on ectopic bone formation by hMSCs, we seeded 2 × 105 cells per three particles of approximately 2–3 mm. Cells were cultured *in vitro* for 7 d in the presence of osteogenic medium. Prior to implantation, the tissue-engineered constructs were washed with PBS. Six immunodeficient mice (HsdCpb:NRI-nu, Harlan) were anesthetized using isoflurane, surgical sites were cleaned with ethanol, and four subcutaneous pockets created. Three particles of each of the calcium phosphate ceramics were implanted in these pockets for 6 weeks.

Posterolateral Spinal Fusion Model in Dog

The experiments were performed following approval of local Animal Care and Ethics Committee (Animal Center, Sichuan University, Chengdu, China). The surgical operation was performed under general anaesthesia (30 mg pentobarbital sodium per kilogram body weight) and sterile conditions. Firstly, the spinous processes of L3 and L4 were identified, and small bilateral incisions were made. Secondly, the spinous processes and the vertebral body between L3 and L4 were exposed on both sides by blunt separation. After injuring the exposed bone using a scraper, materials (HA or TCP, 1–2 mm, 5 mL) were placed in both sides. Finally, the muscles from both sides were tightly closed with sutures.

After surgery, penicillin was intramuscularly injected for three consecutive days to prevent infection. In addition, 1 mL of ceramic particles of HA and TCP were implanted in the paraspinal muscle to evaluate the osteoinductive potential of the ceramics in this model. Twelve weeks after surgery, the animals were killed and samples were harvested with surrounding tissues.

The samples were then fixed, dehydrated, embedded in methyl methacrylate (MMA), and undecalcified sections were made parallel to the posterolateral processes and stained with methylene blue and basic fuchsin for histological and histomorphometrical analysis. Sections obtained 5 mm away from the end of spinous processes were used for histomorphometry and the area between the two processes and 5 mm from the middle was selected as region of interest. The area percentage of bone in the region of interest and in available space, and area percentage of materials in the region of interest, were calculated and data obtained from eight animals were pooled for quantitative analyses.

Sheep model (Intramuscular Implantation and Iliac Implantation)

All the experiments were performed following approval of the University Animal Care and Ethics Committee from the University of New South Wales, Randwick, Australia. Ten adult female sheep (3 years old) were used for intramuscular (ectopic) implantation. Variuos calcium phosphate ceramic (BCP1300, BCP1150, and TCP) particles with a size of 1–2 mm were implanted intramuscularly in separate pockets for 12 weeks. For the iliac implantation (orthotopic implantation), 22 adult female sheep (3 years old) were sedated using an intramuscular injection of Zoletil and anaesthesized using a mixture of O2 (4 L /min) and isoflurane (1.5–2.5%). Pain relief was administered prior to commencement of the surgical procedure. An incision was made over the exposed length of both iliac crests, through the periosteum. Upon exposure of the os ilium, a 17-mm defect was created and the implants (autograft, rhBMP-2, or TCP) were placed into the defects. The periosteum, muscles, fat tissue, and skin were closed over the iliac crest by suturing layers using resorbable sutures (3-0 Dexon, Davis and Geck). In the case of autograft, the bone was harvested at the same time as the creation of the defect and reduced to 1–2-mm particles using a rongeur. Recombinant human BMP-2 (Medtronic) at a concentration of 0.4 mg/mL (0.72 mg/defect) was soaked on 17-mmdiameter Helistat absorbable collagen haemostatic sponges (Integra Life-Sciences Corp.) for 15 min prior to stacking in the defect. During the implantation period, animals were fed with a standard diet and had continuous access to water.

Histology and Histomorphometry

Implants were retrieved and fixed in 0.14 M cacodylic acid buffer pH 7.3 containing 1.5% glutaraldehyde. Fixed samples were dehydrated in ethanol series and embedded in MMA. Sections were processed on a histological diamond saw (Leica SP1600) and stained with 1% methylene blue (Sigma) and 0.3% basic fuchsin solution (Sigma). Sections (one section per sample across the middle, or otherwise specified) were scanned using a digital scanner (Dimage Scan Elite 5400II, Konica Minolta Photo Imaging, Inc.) to obtain an overview, and representative images were used for histomorphometrical analysis using Photoshop software (Adobe). Either the area percentage of bone in the samples or the area percentages of bone in the available space were obtained for quantitative analyses.

Statistical analysis

Statistical analysis was performed using a one-way ANOVA followed by a Tukey's multiple comparison test or paired t test ($P < 0.05$). All animals mentioned in the experimental setup were used for statistical analysis. No incidents were registered during the course of animal experiments.

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Chapter 4

ß-TCP vs. Autologous bone for repair of alveolar clefts in a goat model

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INTRODUCTION

Bone grafting for repair of the alveolar process in cleft patients comprises more than just the treatment of a local bony defect[1]. Repair of the alveolar defect helps not only to restore dental arch continuity, but also stabilizes the maxilla, supports the nasal alar base and restores the volume to the upper lip[2,3].

In order to repair the alveolar cleft, autologous bone grafts are harvested from elsewhere in the body. After filling the alveolar cleft with bone, functional loading needs to be established, either by resumption of eruption of the cuspid or by orthodontic guidance of viable teeth adjacent to the cleft into the new bone[4].

Until recently, the traditional sites for harvesting were amongst others mainly the autologous iliac crest or mandibular symphysis[5-7]. Obviously, harvesting autologous bone has several disadvantages, such as prolonged operation time and the occurrence of co-morbidity. The risk of complications at the donor site ranges between 10 to 30 percent, comprising postoperative pain, hypersensitivity, pelvic instability, infection, meralgia paresthetica (injury to the lateral femoral cutaneous nerve also called Bernhardt-Roth's syndrome)[8], apical root damage and visible cutaneous scarring[9,10]. Therefore, to bypass the drawbacks related to the grafting procedure, the search for alternatives, such as synthetic bone substitutes, would seem to be the next logical step. A prerequisite for this alveolar cleft repair with bone substitute is a resorbable scaffold and should be replaced by vital bone, thereby allowing teeth to move or to be moved into vital, thus living bone.

In this study we introduce and describe a novel animal model in order to test a bone substitute as an alternative to autologous bone for repair of alveolar clefts. The aim of this study in goats (N=10) was to test the hypothesis that ß-TCP works as well as autologous bone as grafting material for alveolar cleft repair.

In addition, this goat model also allowed the investigation of the feasibility of subsequent orthodontic tooth movement of adjacent teeth into the repaired cleft.

MATERIALS AND METHODS

The goat as novel animal model for creation and repair of alveolar clefts, the study and its protocol were approved by the Dutch Animal Care and Use Committee (DEC-UMC 05.01.003). In a split mouth study in 10 adult Dutch milk goats (Capra Hircus - Wetering, Kerkdijk, Rosmalen, The Netherlands), bilaterally created alveolar clefts (Figure 1) were repaired using ß-TCP (CuriOs™, X-Pand Biotechnology, Bilthoven, The Netherlands) on one side and autologous iliac crest bone crest on the other.

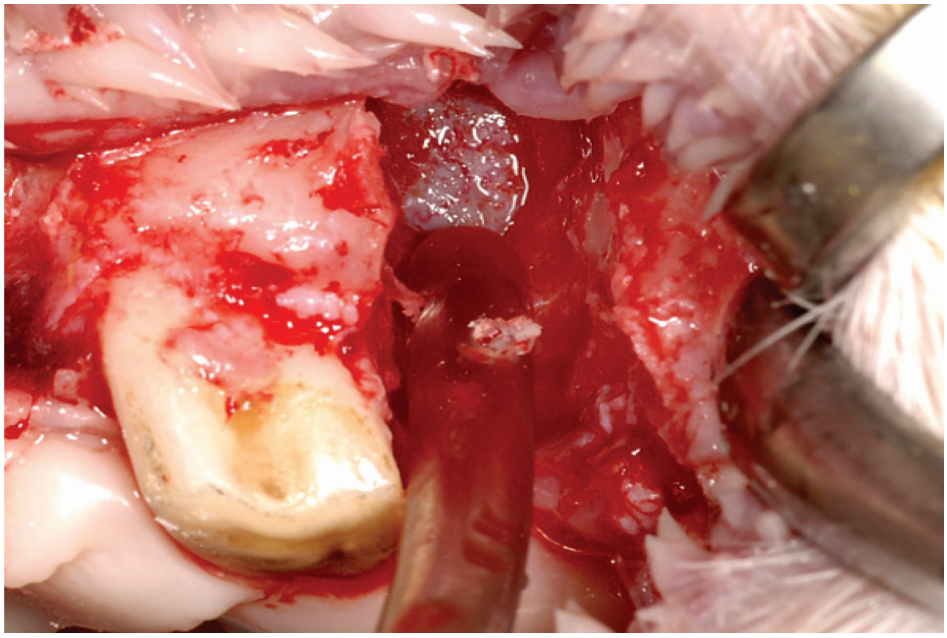

FIGURE 1: Created alveolar cleft after extraction of the second premolar and removal of buccal, palatal and cranial bone walls; the oro-nasal periosteum is visible (black arrows).

Surgical procedure

Each surgical procedure was performed under general inhalation anesthesia. Following extraction of the second premolars, bilaterally, two-wall bony alveolar defects of 3 cm^3 were created in the maxilla by elevating the buccal and palatal mucopereosteum and simultaneously removing all buccal, palatal and nasal cortical bone and vitalizing the mesial and distal bone sides of the extraction wound by removing the periodontal layer. Only the nasal mucosal layer was left intact (Figure1).

FIGURE 2a: ß-TCP granules, moistened with physiological saline solution and to be suctioned into a syringe for grafting; red line is one centimeter.

Figure 2b: Iliac crest 100% cancellous bone, prepared in chips for grafting; red line is one centimeter.

The alveolar cleft-like defects on one side were filled with approximately 3 cc β-TCP granules, with a particle size of 1-2 mm, and moistened with physiological saline (Figure 2a). The contra lateral defect was repaired using 3 cc autologous iliac crest bone graft chips. The autologous iliac crest bone was 100% cancellous bone (Figure 2b).
Finally, the restored defects were closed by suturing prepared mucoperiosteal flaps (Vicryl 3-0, Ethicon, Brussels, Belgium). In total 10 goats were operated on. Subsequently, the goats were sacrificed by means of an overdose of pentobarbital (Euthesaat, Organon, Oss, The Netherlands).

Bone substitute

CuriOs™ (X-pand Biotechnology BV, Bilthoven, The Netherlands) is a 65±15% porous and >90% phase-pure beta tricalcium phosphate (ß TCP) graft material that is synthetic, osteoconductive, osteoinductive and resorbable[11].

Orthodontic devices and procedure

Orthodontic devices, custom-made (Figure 3a) from plaster models, were manufactured by a dental laboratory (Oosterwijk, Utrecht, The Netherlands).

FIGURE 3a: Inside view of the orthodontic device. It consists individually cast parts: molar crowns connected by a transpalatal bar. A fixed round sliding bar attached to these crowns and traction hooks situated on the buccal side. A separate crown for the first premolar to be moved. Attached to this crown a tube with a traction hook. The inside of the tube was coated with a layer of Teflon in order to prevent traction-friction.

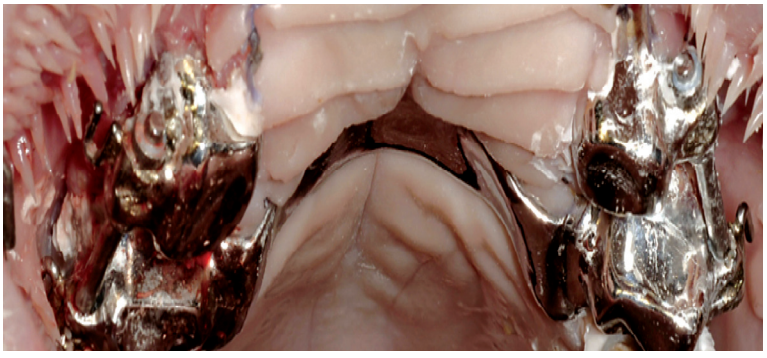

FIGURE 3b: Intra-oral: Orthodontic device *in situ.* Three months later, again under short-acting general anaesthesia, the orthodontic device was activated by means of closed, eyeleted coils using a force of 50cN. In this way, a traction force was transmitted to the first premolar, bodily distalizing it. This orthodontic procedure took six months.

Hooks for fixation of the traction coils were soldered onto the crowns (Gac Sentinol-Lomberg, Soest, The Netherlands). One week after the first surgical procedure, again under general anaesthesia, the orthodontic device was placed in its entirety (Figure 3b).

Animal care

The goats were fed with pre-moistened, ground chunks of hay (PULP 6mm, foodstuff beet pulp with raw protein 8.5%, raw fat 0.5% with additional linseed, raw protein 28.6%, raw fat 11.1%, De Heus Diervoeders, Rijsbosch, Beusichem, The Netherlands) to prevent dehiscence of the flap and damage to the orthodontic device. Throughout the experiment the dentition and orthodontic device were cleaned daily.

Objectives

After the surgical procedure, neo-angiogenesis and bone formation were allowed to take place for a period of three months. Then followed the orthodontic treatment. Nine months after the initial surgery, all animals were sacrificed.

Radiographic assessments

Measurements of orthodontic movement were taken from two-dimensional, semi-standardized radiography[12] was carried out intraorally immediately after surgery, during the12 week follow-up, and at the end of the experiment.
The specimens retrieved from the sacrificed animals were analyzed as blinded samples by means of quantitative 3D radiographic analysis. To calculate the bone volume and bone volume percentage, a standard interdental region was selected in the repaired defect site. The 3-D images were manufactured by placing them vertically onto the sample holder of a micro-CT imaging system, Skyscan 1072 desktop X-ray Micro-tomography System (SkyScan, Kontich, Belgium), with the long axis of the implant perpendicular to the scanning beam. Subsequently, a high-resolution scan was recorded at a 30-µm-voxel resolution. Then, using Nrecon V1.4 (SkyScan, Kontich, Belgium), a cone beam reconstruction was performed on the projected files. Finally, by using 3D creator software, a 3D reconstruction of the reconstructed cleft area was obtained.

Histological procedure and assessment

The samples retrieved from the sacrificed animals were blinded, dehydrated in a graded series of ethanol (70–100%), washed with acetone, embedded in methylmethacrylate, stained and sectioned. Subsequently, the histological sections were scanned using a Konica Minolta Dimage slide scanner. Using Photoshop software, a constant region of interest (ROI) was selected in the defect site which was the same size in all sections. Hereafter, the bone and bone substitute material in the ROI were pseudo-coloured and the number of pixels in both bone and former ß-TCP material and the total number of

pixels per image, were determined. Pixel numbers were used to calculate the volume of bone per available total volume using the following formulas: [pixels bone / total pixels x 100%] and [pixels former β-TCP / total pixels x 100%].
Subsequently, the samples were sectioned using a LeicaSP1600 saw microtome (Saint Gobain Diamantwerkzeuge GmbH, Norderstedt, Germany) for microscopic assessment.

Statistical analysis

The paired-samples t-test was used to determine differences in bone volume between the results obtained using the synthetic bone substitute β-TCP and those using autologous bone. The test was also used to determine differences in results between two investigators. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Clinical and surgical observations

All operation sites healed successfully. Clinically no wound dehiscence was observed, although some minimal loss of ß-TCP particles in the recipient area in one single animal was encountered.

Orthodontic tooth movement

When taking the thickness of the crowns into consideration, maximum orthodontic tooth movement of the first premolar was observed in all goats (Figure 4).
Orthodontic tooth movement was measured twice by the same researcher at an interval of one month, and expressed as a percentage of the extraction diastema (Table 1).
In the clefts reconstructed with iliac bone an average tooth movement of 43.2% was measured; in the clefts reconstructed using β-TCP, this average was 41%. No statistical difference was noted between these treatment modalities.

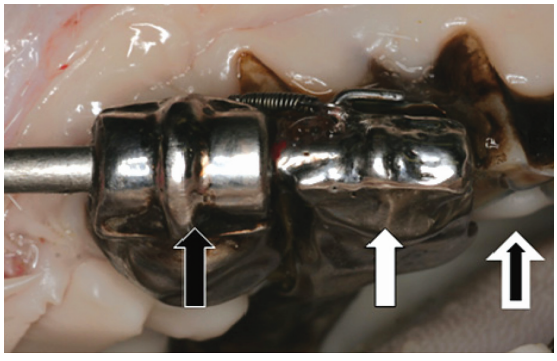

FIGURE 4: Occlusal view of the maxilla specimen showing the first premolar (black arrow), the third, anchor premolar (white arrow) and part of the first molar (black/white arrow). Considering the thickness of the crowns there is maximal orthodontic closure of the created and repaired alveolar cleft. Also note the de-active status of the closing coil after the six month activation period.

TABLE 1: Orthodontic displacement assessments 1 and 2 expressed as a percentage of the original width of the extracted second premolar.

Goat number	Iliac crest or β-TCP	Side of jaw	Displacement-1 %	Displacement-2 %	Displacement average %
3253	iliac crest	left	31.3	31.0	31.2
	β-TCP [a]	right	10.5	10.1	10.3
3254	iliac crest [b]	right	38.7	39.0	38.9
	β-TCP	left	63.3	63.8	63.6
3406	iliac crest	right	51.8	50.7	51.3
	β-TCP	left	50.5	51.2	50.9
9965	iliac crest	left	64.5	63.9	64.2
	β-TCP c	right	3.7	3.8	3.8
3397	iliac crest	right	31.5	31.8	31.7
	β-TCP	left	52.4	51.1	51.8
3369	iliac crest	left	41.6	41.2	41.4
	β-TCP	right	52.4	52.1	52.3
3209	iliac crest	right	51.3	52.1	51.7
	β-TCP	left	51.3	51.8	51.6
2136	iliac crest	right	51.4	50.9	51.2
	β-TCP	left	51.4	51.0	51.2
2178	iliac crest	left	37.8	38.0	37.9
	β-TCP	right	35.9	36.2	36.1
2097	iliac crest	left	33.2	32.9	33.1
	β-TCP	right	38.1	38.7	38.4
average ± sd	iliac crest		43.3 ± 11.0	43.2 ± 10.8	43.2 ± 10.9
	β-TCP		41.0 ± 19.5	41.0 ± 19.5	41.0 ± 19.5

[a] loose coil; [b] root remnant; [c] food remnant

Radiographic 3-D Coned Beam scans

The 2-D radiographs taken *in vivo* showed sufficient bone height during the progress of the orthodontic movement of the first premolar. A clear difference in the height of the bone and the width of the periodontal ligament was observed between the pressure and tension sides of the premolar (Figure 5a).

The 3-D assessments are depicted in Figure 6.

They show the mean and the standard error of the mean of the bone volume for both the ß-TCP and the autologous bone reconstructed sides, expressed in number of pixels counted[3]. Sufficient bone height was shown (Figure 5b), but no significant differences were observed between the two treatment modalities (paired-samples t-test).

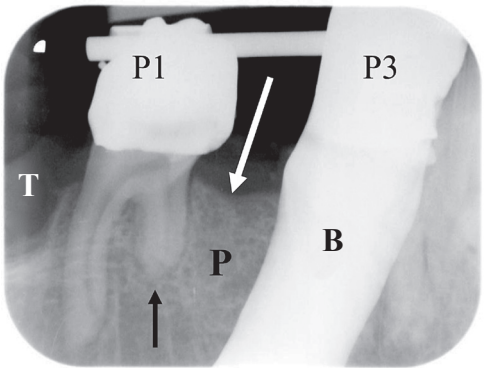

FIGURE 5a: Two-dimensional radiograph, taken during orthodontic displacement of the first premolar. Showing the premolar (P1), the anchor premolar (P3), hidden behind the bar of the orthodontic device (B). The premolar (P1) is moved to the anchor premolar (P3). Note the difference in width of the periodontal ligament at the tension and pressure sides (**T** and **P**). The white arrow points to the height of the reconstructed area. As a result of the orthodontic displacement, the distal root of the premolar P1, although originally shorter than the mesial root, is shortened (black arrow).

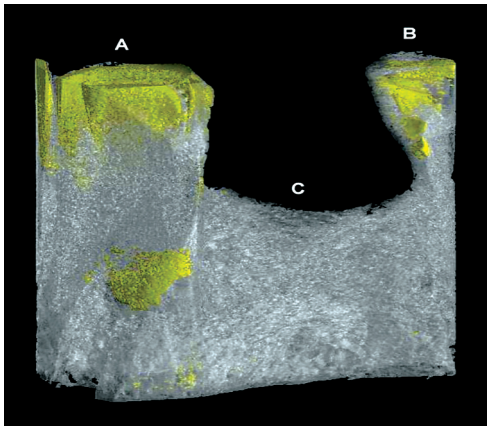

FIGURE 5b: 3-D microscan image of a pilot MMA specimen (TCP graft after 3 months and no orthodontic displacement) showing the interdental bone structure. A=molar, B=premolar, C=new interdental bone.

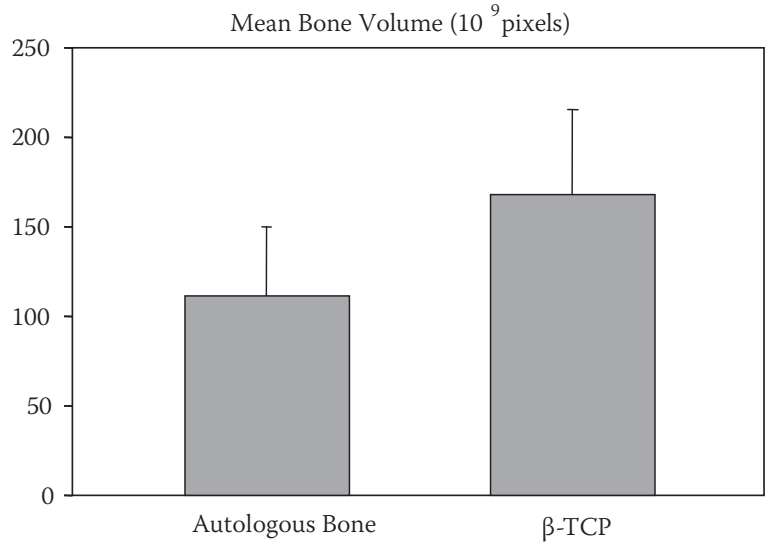

FIGURE 6: Mean and S.E.M. of the bone volumes as measured in the alveolar defect repaired by either iliac crest bone or ß-TCP.

Histological sections

Neither inflammatory cells such as mast cells nor macrophages could be distinguished in the sections, whereas bone marrow regions with a border of osteocytes and also the

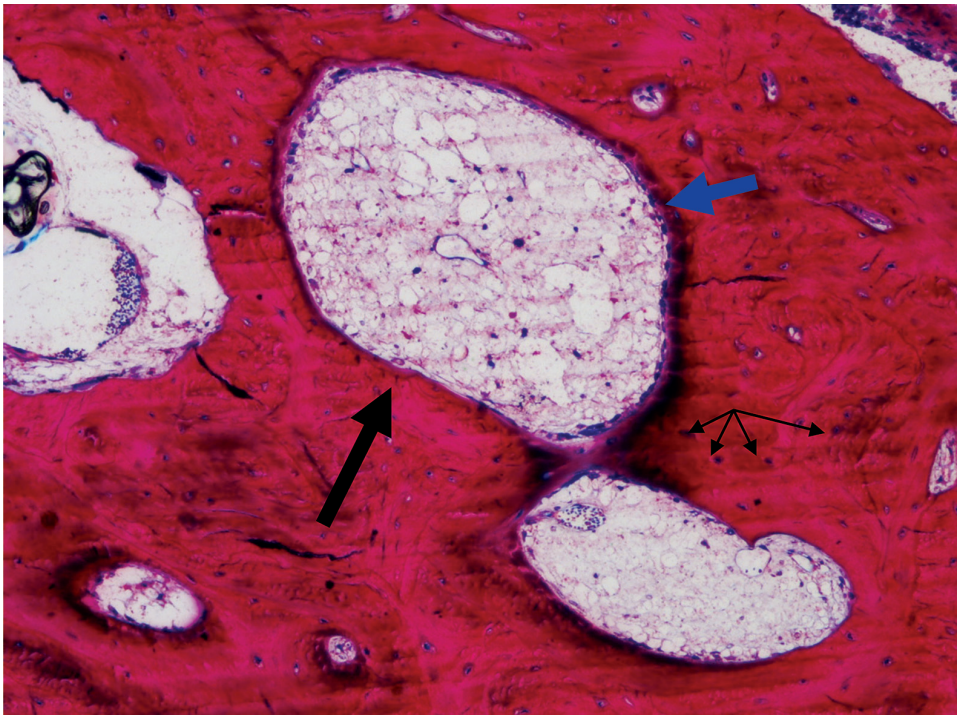

FIGURE 7: Bone marrow (black arrow) with a border of osteocytes (blue arrow) and haversian canules (small arrows).

haversian systems were clearly visible (Figure 7).

This depicts a normal bone structure. Figure 8 shows formation of bone after implantation of bone harvested from the iliac crest and Figure 9 shows bone formation after implantation of ß-TCP. These figures illustrate the bridging repair of the original defects as well as the resorption of the ß-TCP particles.

Two investigators counted the percentages of bone in both the autologous bone and the ß-TCP bone samples [pixels bone / total pixels x 100%] and [pixels β-TCP / total pixels x 100%]. No significant differences were found between the results from Investigators 1 and 2 (paired-samples t-test). Their combined results are shown in Figure 10.

It depicts the area percentage of total bone volume found for grafting with autologous iliac bone (20.87%; st dev 5.40) and ß-TCP substitute (22.80%; st dev. 5.62).The differences per goat are illustrated in Figure 11.

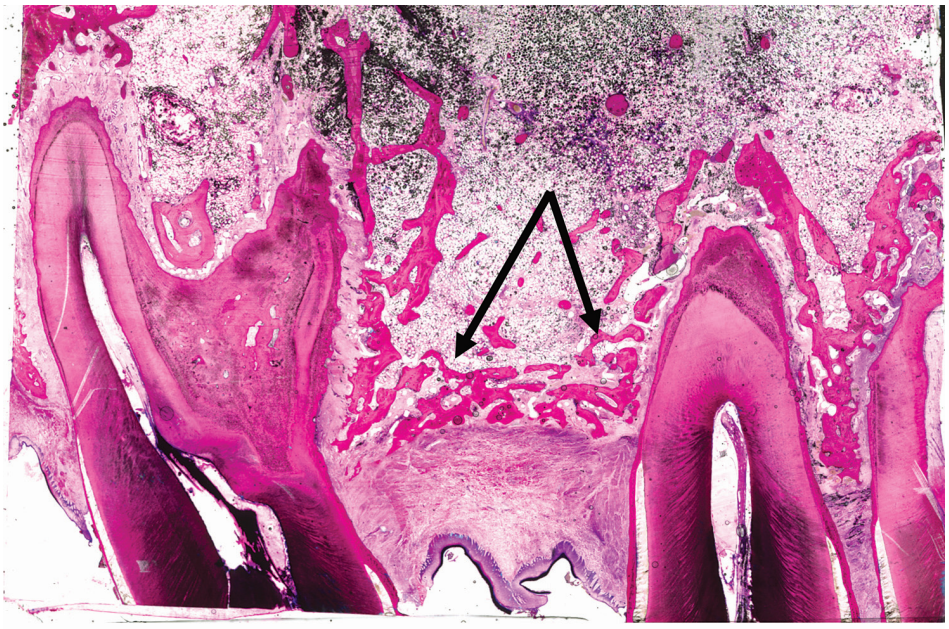

FIGURE 8: Bone formation in an alveolar cleft grafted with iliac crest bone chips (black arrows).

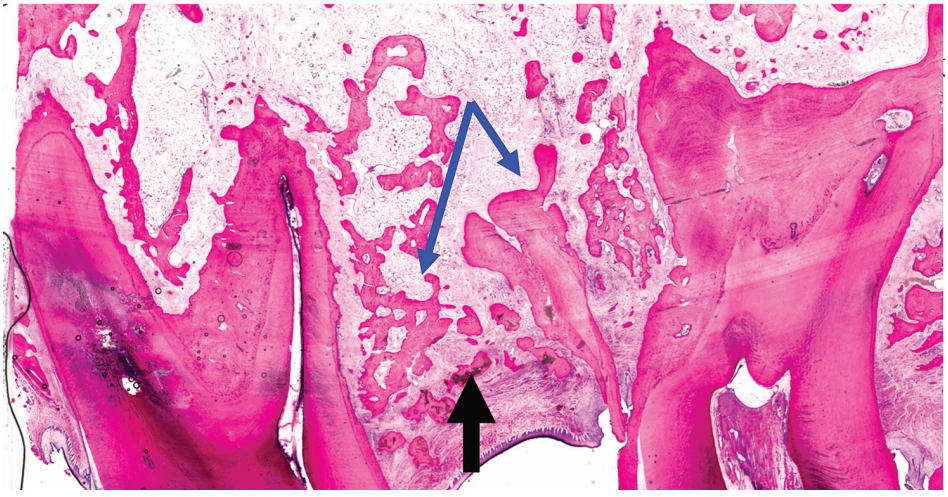

FIGURE 9: Bone formation (blue arrows) in an alveolar cleft grafted with ß-TCP with scarcely any rests of ß-TCP (black arrow).

DISCUSSION

This study emphasizes that the goat model can be used for studying alveolar cleft repair, for orthodontic tooth movement studies and for a combination of both. The goat as an

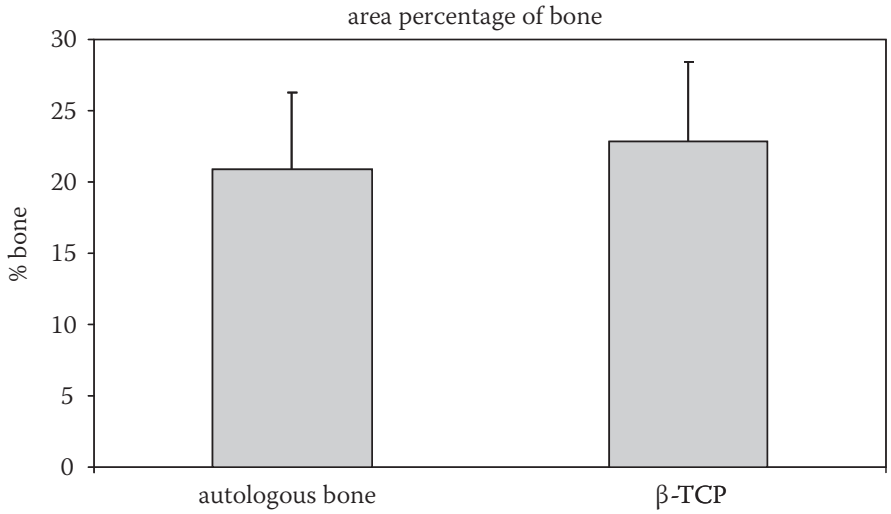

FIGURE 10: Amount of bone found in 10 goats.

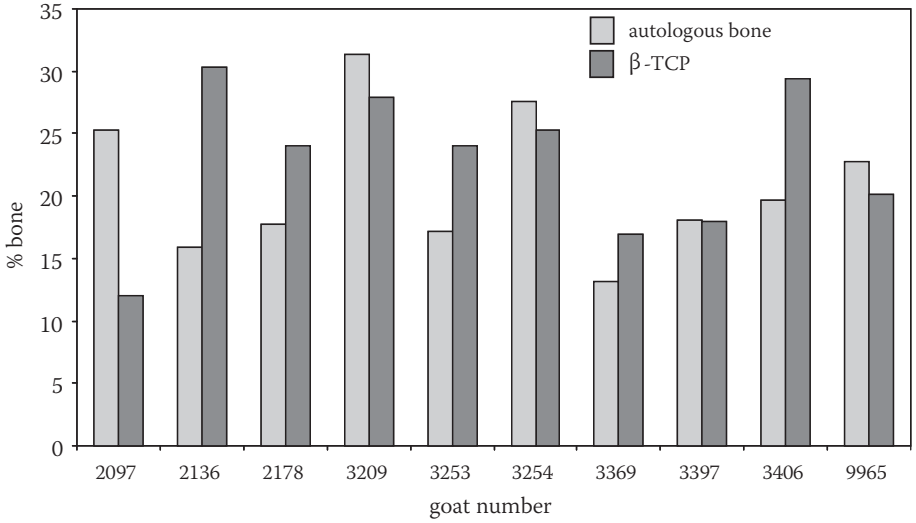

FIGURE 11: Percentages of autologous bone and β-TCP bone in 10 goats.

experimental model was chosen for several reasons. Alveolar defects similar in size to human alveolar cleft defects can be created. They can be defined as an alveolar defect of critical size with two bone walls and four mucosal walls and are in fact similar to the surgical acceptor site we create when repairing alveolar clefts in humans. Would we repair these with mucosal layers only, without bone grafting, we would not expect bone development inside the repaired site or a bony connection between the two sides of the alveolar cleft. Therefore we do not have a control to prove the critical size of the defect.

Further, the metabolic rate of the goat is more or less equivalent to that of a child of 10-11 years old[13], the age at which most alveolar clefts are repaired in humans. There was a trend for the alveolar cleft defects repaired with ß-TCP to show more bone in growth than the sites repaired with iliac bone grafts, although this difference was not significant. No differences in orthodontic movement were observed between the sites grafted with iliac bone and the sites grafted with ß-TCP.

Surgical grafting considerations

The anatomy of the goat is such that it fulfils the requirements of this study, i.e. investigating if the use of the synthetic bone void filler ß-TCP is as good as an iliac bone graft in repairing an alveolar cleft defect, and if orthodontic movement is feasible. Although distraction osteogenesis procedures aimed at closing alveolar clefts at the age of cleft repair have been widely researched[14-16], secondary bone grafting is still the preferred method of treatment[7,17], also because there is some controversy about early primary bone grafting and midfacial growth[1]. Some authors suggest mixing autologous bone grafts with another material[18,19]. They describe cases in which the use of inorganic deproteinized bovine bone in combination with autogenous bone seemed to be preferable. They claim that the addition of bovine bone mineral to autogenous bone is beneficial to graft success because it acts as a slowly resorbing space maintainer. Others advise adding growth factors, such as Bone Morphogenic Proteins (BMPs) or Platelet Rich Plasma (PRP) to the autologous bone graft to improve healing[20,21]. Donor site surgery requires prolonged operating time and causes morbidity[22]. Therefore the introduction of a bone substitute would be ideal to bypass the drawbacks that are linked to the harvesting procedure of an autologous bone graft. The resorption process of such a bone void filler should be in balance with the process of the formation of new bone; the space left by the resorbing bone substitute needs to be replaced by new bone. This process of 'creeping substitution' has been reported extensively in the literature[22-24].
The sole use of ß-TCP as a bone substitute without mixing it with autologous material for repair of alveolar clefts is novel and has not yet been studied in alveolar cleft repair. Encouraging results on the use of a limited volume of ß-TCP for preimplantation sinus floor elevation procedures have been published[18,25-29]. Because of its versatility, low complication rate and long-term results, synthetic, pure-phase beta-TCP is a suitable material for the filling of bone defects in the alveolar region[19,25]. To date, ß-TCP was already reported to be used in cleft repair, but only to function as a barrier to cover the bone graft, hereby protecting the bone graft from resorption[17].

Orthodontic considerations

From a clinical perspective, the orthodontic movement of the first premolars was problem-free, although the average displacement of the first premolars into the alveolar clefts was over 40 % (Figure 12).

This can be partially ascribed to a root remnant or a loose coil as seen on the radiographs of goats 3253 and 3254 (see a, b, Table 1). It should also be realized that because of the intrinsic qualities of the design of the orthodontic device, i.e. the thickness of the walls of the crowns and the location of the palatal bar (Figure 12), over 40%, the maximum possible closure of the diastema was achieved.

A threshold for a force magnitude that would switch on tooth movement cannot be defined[30]. For this reason, in an earlier pilot study in two goats orthodontic displacement was started at a force of 75 cN. Clinical inspection showed that at as early as three months, the entire extraction diastema had already been closed. However, minimal root resorptions from the cement surface of the roots of the displaced premolar, as well as some small signs of ankylosis were observed (Figure 13).

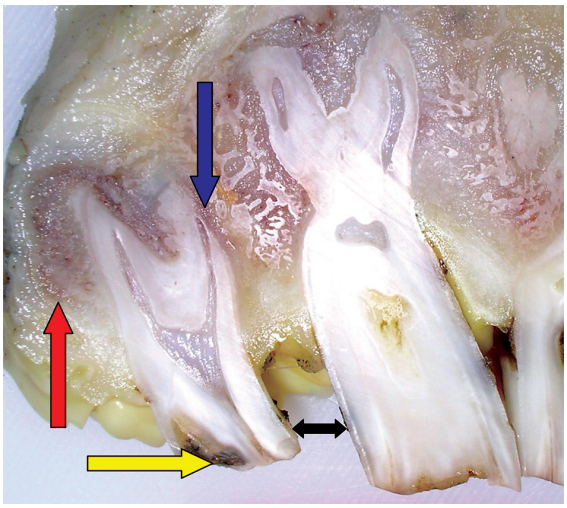

FIGURE 12: Specimen of the goat maxilla after grafting with ß-TCP and orthodontic movement of the first premolar into the newly formed bone (yellow arrow=direction of movement). Note the rest diastema due to the thickness of the crowns (black arrow) and the distinct bone formation at the tension side of the premolar (red arrow) and the small apical root resorption at the pressure side (blue arrow).

For this reason we reduced the orthodontic retraction force to 50 cN which certainly induced gradual and more limited orthodontic movement but also a minor degree of *apical* root resorption (Figure 13). This phenomenon is analogous with the human situation and therefore acceptable[31-33].

Radiographic considerations

Volumetric data measured by the 3-D Cone Beam scanner showed a fair amount of new bone formation. Other animal studies have reported on comparisons between implanted materials such as autologous and xenologous and synthetic graft materials, but they do not report any data on the complete healing of the created alveolar cleft defect[34-36]. Our findings did not allow us to significantly differentiate in favor of either autologous bone or ß-TCP as a grafting material, although ß-TCP showed slightly better result (see Table 1).

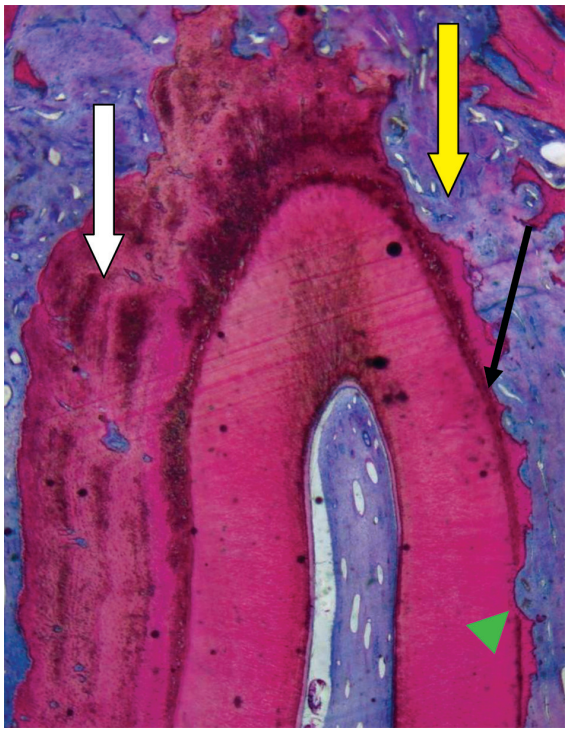

FIGURE 13: A goat premolar root after orthodontic movement into the newly formed bone after grafting the alveolar cleft. Note the possible sign of ankylosis (black arrow) and a small area of root resorption (green arrow head); also a distinct difference is visible between pressure (yellow arrow) and tension (white arrow) sides.

Another reason why the interdental alveolar clefts were not completely refilled with bone may be that orthodontic movement was limited and therefore total functional loading of the grafts did not take place - which always means that interdental bone height is lost[37]. Significantly more bone has been reported to regenerate in grafted load-bearing site defects in tibial fractures[38].

Histological considerations

The sections were stained with methylene blue and basic fuchsin in order to make bone formation visible in general overview. This method of staining does not allow the distinction between macrophages, inflammatory cells and mastocytes or between osteoblasts and osteoclasts.

Nevertheless this study shows that after grafting of either ß-TCP or autologous iliac crest bone comparable bone volumes are formed in the alveolar clefts. The total amount of bone found in all ten goats after grafting with ß-TCP was approximately 10% greater. However, this difference was not statistically significant and no relationship between percentage of bone volume and the extent of orthodontic movement was observed. Scarcely any discernible remnants of ß-TCP were seen (Figure 14).

The implanted ß-TCP did not block tooth movement yet it was resorbed. Histology showed cellular processes on both the pressure and tension sides of the periodontal ligament that are analogous with the human situation. One particularly interesting

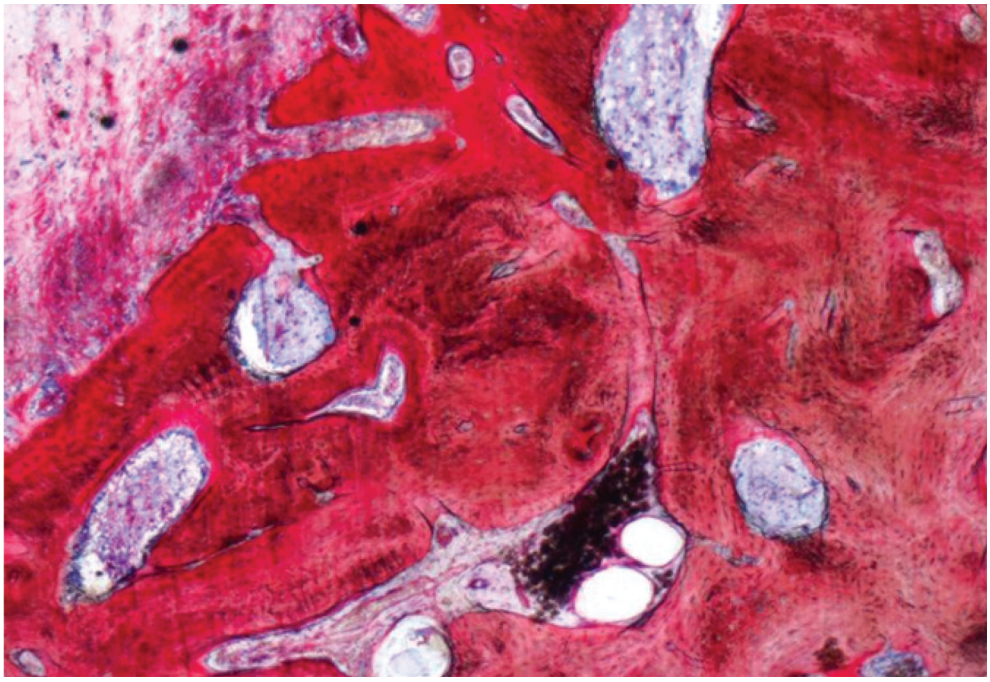

FIGURE 14: Bone (red) formation and almost complete inclusion and resorption of the ß-TCP (black particles).

observation is that once the premolar had moved into the new bone, the β-TCP had been totally resorbed (Figure 9). This implies that both the osteoclast and osteoblast activity that is induced by orthodontic tooth movement and the load-bearing characteristics of a bone-borne tooth favorably influence the resorption of β-TCP[39,40]. This has been confirmed by a qualitative histological study in beagle dogs where biodegradable TCP exhibited a greater ability for adaptive remodeling in response to orthodontic force than did particulate marrow and cancellous autologous bone, suggesting its suitability for clinical use during orthodontic tooth movement[38].

We allowed for neo-angiogenesis and bone formation to take place for a period of three months after the initial surgery, because immediate tooth movement into a non-bony environment will lead to root exposure rather than bone formation.

Namely, the characteristic properties of the fibrous periodontal ligamental joint (amphiarthrose) between a tooth and its socket - the gomphosis - allow for the attachment and load bearing characteristics of a tooth[41]. It is from this area too, that tooth movement is initiated throughout the activation time of the orthodontic forces. These forces initiate a remodeling process in the tension and pressure sides of the bone surrounding the tooth. However, in order for this process to take place, bone must be present.

Although we still do not completely understand the mechanism of bone generation stimulated by ß-TCP or the effects on the osteostimulation that are exercised by synthetic bone substitute materials when they are orthotopically implanted *in vivo*, these initial results show the potential of CuriOs β-TCP for use in clinical cleft surgery.

CONCLUSIONS

The goat model meets all the requirements of the experiments that we have described. Its additional benefits include lower associated costs and a higher degree of social acceptance. It has shown that orthodontic tooth movement into the newly formed tissue with only slight partial root resorption is feasible.

This study has shown from the surgical, orthodontic, histological and radiological standpoints, that in the repair of alveolar clefts created in goats, the bone substitute ß-TCP (CuriOs™) is at least as effective as autologous iliac crest bone. At those sites where bone regeneration from iliac crest bone and bone induction from ß-TCP took place, there is evidence that the synthetic bone substitute was slightly superior to autologous bone. The hypothesis that the synthetic bone substitute ß-TCP can be used equally as well as an autologous bone graft from the iliac crest for grafting and repair of alveolar clefts is not rejected. Its use in the clinical human alveolar cleft situation appears to be warranted and further investigations into its use will be reported on the near future.

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Chapter 5

Effect on the contour of bone and soft tissue one year after harvesting chin bone for alveolar cleft repair

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INTRODUCTION

Several donor sites have been described for harvesting autologous bone grafts for the treatment of an alveolar cleft in cleft lip and palate (CLP) patients[1-5]. Autologous bone harvested from the mandibular symphysis has favourable properties: its ectomesenchymal and membranous origin enables it to maintain its volume and viability due to early vascularization[6-8], it responds well to orthodontic loading of the alveolar process[6], and as a donor site it has low objective and subjective morbidity[1,8,9]. This makes the mandibular symphysis the first-choice donor site in many cleft centres[4,10].

After the harvesting procedure, the bony defect can be filled with a biologically resorbable gelatine sponge (Spongostan®) in order to restrict bleeding, to provide platelet rupture and to support fibrin threads[13]. Although studies show limited and acceptable postoperative morbidity, a number of patients experience sensory loss, pulp obliteration and/or necrosis due to apical damage after the harvesting of chin bone[1,9,11,12]. In addition, lateral cephalogram analysis from one year postoperatively onwards, frequently shows a residual defect in the mandibular symphysis. Unfortunately, donor site morbidity is an inherent side effect of harvesting chin bone[1,11,12], thus challenging investigators to look for suitable allograft alternatives for the repair of alveolar clefts.

The aim of this study was therefore to evaluate and quantify this residual bony defect in the mandibular symphysis, and the effect of harvesting chin bone on the soft tissue contour of the chin at a minimum of one year postoperatively.

Patients

Ninety-two consecutive CLP patients operated on between 2001 and 2006 at the Department of Oral and Maxillofacial surgery at the Wilhelmina Children's Hospital Utrecht, the Netherlands, were examined. Thirty-three patients were excluded because of incomplete data, earlier operations in the chin region, severe co-morbidity such as diabetes or a congenital syndrome. Ultimately, 59 patients with unilateral or bilateral CLP who underwent a surgical procedure to harvest chin bone for repair of the alveolar cleft were included. The study group comprised 39 males and 20 females: 24 right-sided, 17 left-sided and 18 bilateral CLP patients. The mean age of the patients was 12 years (range 8.4 to 19 years). All patients underwent a standardized surgical procedure to harvest the chin bone graft. Surgery was performed by four surgeons who all used the same surgical technique.

Surgical procedure

The buccal cortical surface of the mandibular symphysis was approached through a horizontal incision in the vestibule of the lower lip within the intercanine region. After dissecting a submucosal flap, an incision was made through the mental muscles on each side and down to the bone. After elevating the muco-periosteal flap the mental nerve

was localized on both sides. Using the position of the apices of the incisors and the germs or roots of the permanent canines as seen on preoperative orthopantogram, the outline of the bone graft was marked with a fissure burr. The monocortical bone graft was sawn out with a reciprocal saw, irrigated with physiological saline solution 0.9%, and harvested using a chisel. To collect the maximum amount of bone, the remaining cancellous bone within the symphysis was harvested using excavators. A biologically resorbable gelatine sponge (Spongostan®) was used to fill the bony defect[13]. Closure was achieved by approximating the periostium and both mental muscles to their origins with a Vicryl® 2-0 suture (Ethicon, Johnson & Johnson Intl.); the mucosa was closed with a Vicryl® 4-0 running mattress suture. Directly postoperatively the chin was taped with elastic tape to minimize swelling from oedema and restrict haematoma formation. The tape was removed after 72 hours.

To prevent wound infection, a standard prophylactic antibiotic regime (Clindamicyn, Hameln Pharmaceuticals, 31789 Hameln, Germany, 10 mg/kg per 24 hours) was prescribed for 48 hours postoperatively. A soft diet was prescribed to prevent wound dehiscence.

METHODS

Standardized digital lateral cephalograms were used to evaluate and quantify the bony defect. Adobe Photoshop 9.0 was used to adjust the radiographs for proper analysis. The mandibular and maxillary regions of each lateral cephalogram were selected, resulting in a detailed image of the mandibular symphysis with a standard image size of 6.5 x 8.3 cm at a resolution of 300 DPI. To measure the bony defect directly postoperatively and at a minimum of one year postoperatively, digital tracings were made. To achieve maximal standardization and restrict variability to a minimum, each tracing included a number of cephalometric hard-tissue landmarks (Figure 1).

Menton (Me), the most inferior point of the mandibular symphysis; Gnathion (Gn), the most anterior inferior point of the bony chin in the midsagittal plane; Pogonion (Pog), the most anterior point of the bony chin in the midsagittal plane; B-Point (B), the deepest (most posterior) midline point on the bony curvature of the anterior mandible; Infradentale (Id), the most superior anterior point on the mandibular alveolar process between the central incisors[14].

Each tracing included the mandibular symphysis including the defect if present. The ventral outline of the defect was standardized by tracing a straight line (Figure 2) from the caudal labial border to the cranial labial border of the defect.

The size of the defect area was calculated by counting the number of pixels making up the defect as well as the number of pixels depicting the whole symphysis in the same radiograph. In this way the postoperative defect (Figure 2) could be expressed as a

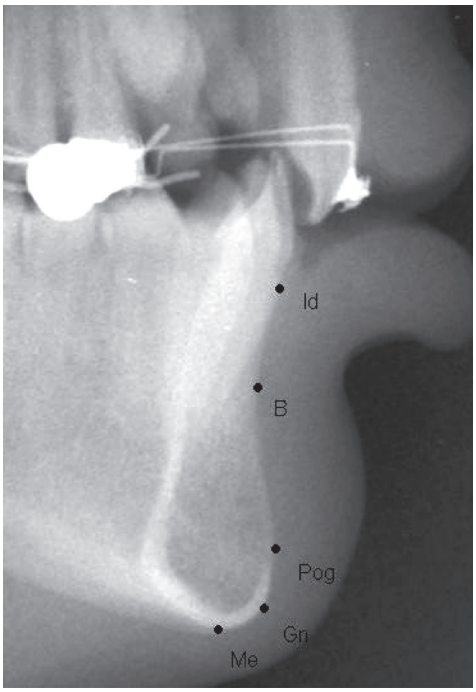

FIGURE 1: Detail of the mandibular symphysis showing hard tissue landmarks: Menton (Me), Gnathion (Gn), Pogonion (Pog), B-Point (B), Infradentale (Id).

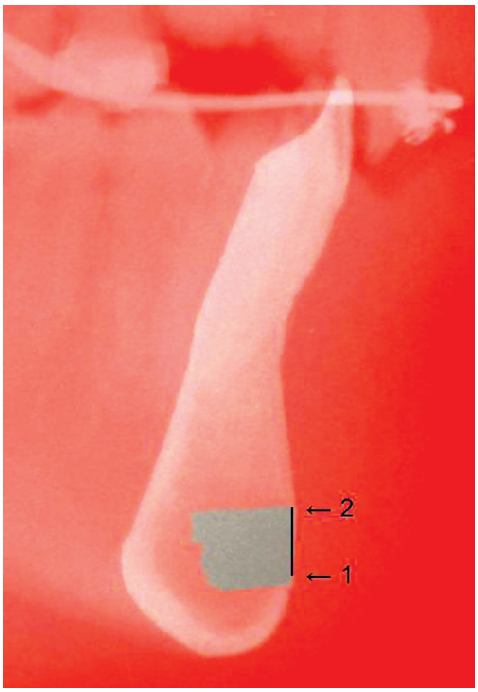

FIGURE 2: Digital tracing of the mandibular symphysis showing the bony defect directly postoperatively. Note the straight line from the caudal labial border (1) to the cranial labial border (2).

percentage of the symphysis. Using this method it was also possible to calculate the size of the residual defect one year or more after surgery (Figure 3) and to compare it with the defect directly postoperatively.

To analyse the effect of harvesting chin bone on the soft tissue at a minimum of one year after surgery, we used the same detailed images of the mandibular symphysis. Digital tracings of the soft tissue contour were made preoperatively, and at a minimum of one year postoperatively. The thickness of the soft tissue was measured by tracing a perpendicular line onto every tangent line of four standardized hard-tissue landmarks:

1. Menton (Me);
2. Pogonion (Pog);
3. The centre of the defect;
4. B-point (B);

Each perpendicular line (soft tissue thickness) was measured digitally (Figure 4).

Our study group consisted of 59 patients. Because complete data were not available for all patients we analysed the data in three groups A, B and C (Table 1).

FIGURE 3: Digital tracing of the mandibular symphysis including the bony defect one year postoperatively.

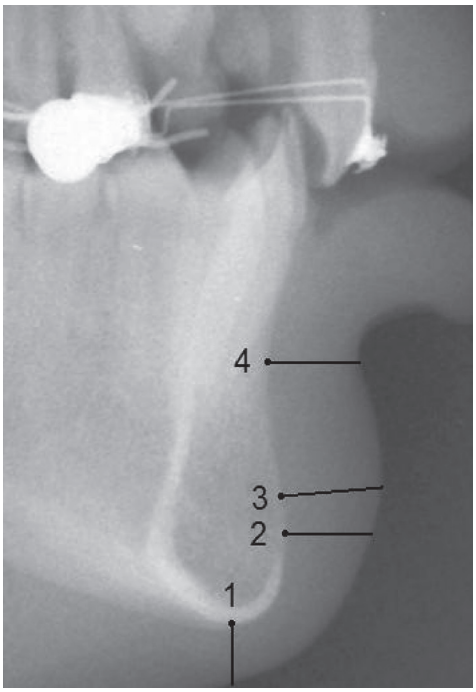

FIGURE 4:. Detail of the mandibular symphysis used to measure the soft tissue thickness showing hard-tissue landmarks 1: Menton, 2: Pogonion, 3: Centre of the defect, 4: B-Point.

TABLE 1: Study group classified by group (A, B and C) and comparison of cephalograms.

	Pre-op and 1 year post-op cephalograms	Post-op and 1 year post-op cephalograms	1 year post-op cephalograms
GROUP A (n=59)			Comparison of the residual defect with the mandibular symphysis
GROUP B (n=23)		Comparison of the post operative defect with the residual defect after 1 year	
GROUP C (n=31)	Analysis of the effect of chin bone harvesting on the soft tissue thickness after 1 year		

Group A

Lateral cephalograms of 59 patients taken at a minimum of one year postoperatively were available. Digital tracings were made to determine the size of the residual bony defect and to compare it with the mandibular symphysis one year postoperatively (Figure 3). The tracings were made by one investigator.

Group B

Lateral cephalograms taken directly postoperatively and at one year postoperatively were available for 23 patients. In these 23 patients we compared the direct postoperative bony defect to the residual bony defect after one year (Figures 2 and 3).
In this group we also determined inter- and intra-observer variability. Digital tracings were made by two independent investigators. Each investigator traced two radiographs per patient times on three different occasions.

Group C

Lateral cephalograms taken preoperatively and one year postoperatively were available for 31 patients. One investigator made digital tracings to analyse the effect of harvesting chin bone on the soft tissue thickness one year postoperatively (Figure 4).

Statistical methods

Analysis of variance (ANOVA, SPSS 11.5; SPSS, Chicago, Il, USA), was applied to test the nil hypothesis that there would be no statistical difference between the results obtained from the radiographs (taken directly or one year postoperatively), the two independent investigators, and three individual tracings. Pearson's correlation coefficients were calculated to determine possible relationships between the age of a patient and the residual defect after one year, and a relationship between the defect directly postoperatively and one year later. In addition, we studied a possible relationship between gender and the defect after one year. In our soft tissue analysis we investigated the change in soft tissue thickness one year after harvesting chin bone (paired t-test). We also studied the relationship between the change in soft tissue thickness and gender as well as the size of the residual defect. A p-value of less than 5% was considered significant.

RESULTS

Group A

The mean size of the defect after one year was 4.8 mm^2 ± 5.5 mm^2, ranging from 0 to 22 mm^2. The defect was on average 1.4% ± 1.6% of the mandibular symphysis. In addition, we observed a significant correlation between the age of the patient and the size of the defect after one year ($p<0.01$ $r=0.34$). In older patients a larger defect remained at a minimum of one year after bone harvesting (Fig. 5). We found no significant correlation between gender and the size of the defect after one year ($p>0.05$).

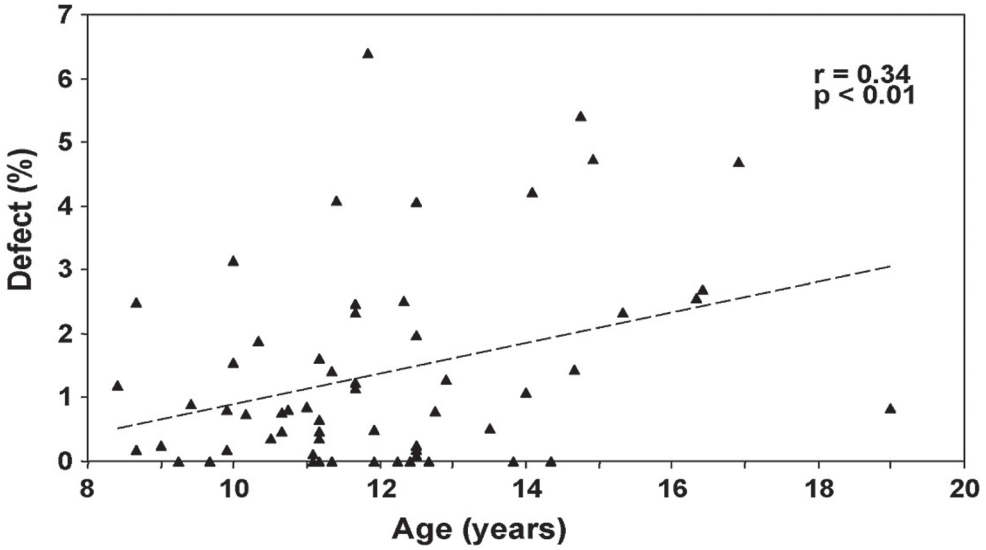

FIGURE 5: Individual bony defects of 59 patients (group C) 1 year post-operatively (expressed as a percentage of the mandibular symphysis) as a function of the age of the patients. The dashed line indicates linear regression. A significant correlation between defect and age was observed ($p < 0.01$;$r=0.34$).

Group B

The mean size of the donor site defect immediately postoperatively was 35 mm^2 ± 28 mm^2, ranging from 5 to 129 mm^2. The defect was on average 10% ± 7% of the mandibular symphysis. After one year we measured a significant residual mean defect of 1.4% ± 1.8% of the mandibular symphysis which is 14% of the peroperative defect. ($p<0.001$) (Fig. 6). A mean repair of 86% of the original defect had taken place. The repair of the defect ranged from full repair (100%) N=10, to only 16% repair N=1. A nearly significant correlation (trend) was found between the size of the defect directly postoperatively and the defect one year postoperatively($p=0.06$ $r=0.39$). Thus, there was a tendency for a larger direct postoperative defect to result in a larger residual defect after one year.
The results showed no significant differences between the tracings carried out by the two independent investigators ($p>0.05$). No significant differences were found between the three individual tracings carried out by the same investigator on three different occasions ($p>0.05$).

Group C

A significant increase in soft tissue thickness one year after surgery was measured at points 2, 3 and 4 ($p<0.003$). No significant increase in thickness was measured at point 1 ($p=0.39$). No correlation was found between gender and the increase of soft tissue thickness one year after harvesting chin bone. A negative correlation was found between

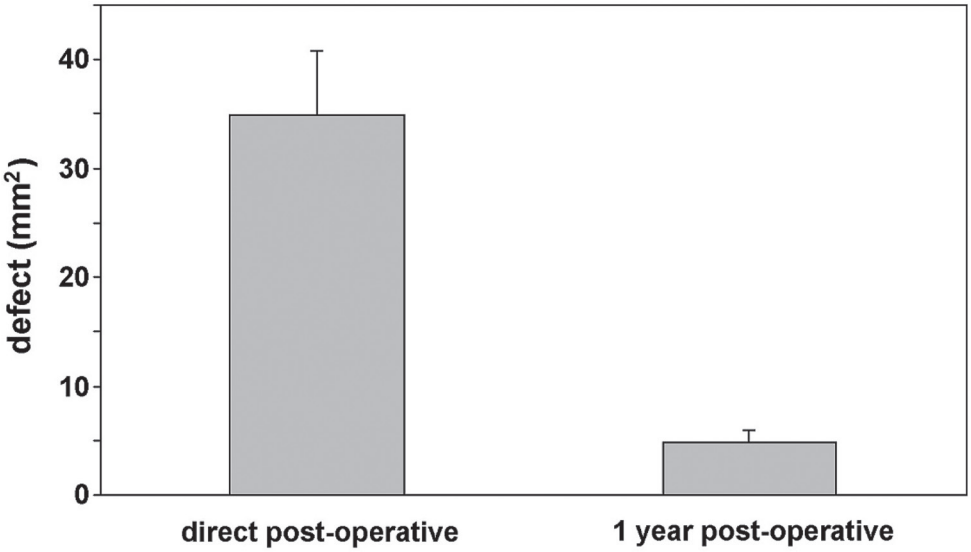

FIGURE 6: Average bony defects and standard error of the mean (expressed in mm2) directly postoperatively and 1 year postoperatively for a group of 23 patients (group B). A significant residual defect persisted ($p < 0.001$).

the size of the bony defect one year postoperatively and the soft tissue thickness at point 3 ($p=0.011$, $r=-0.45$).

DISCUSSION

The results of this study show an evident residual bony defect in the mandibular symphysis at a minimum of one year after harvesting chin bone. Evidently full repair of the donor site does not occur. Several studies investigating bone healing have found a negative correlation between age and the healing of bone defects[15,16]. In our study, we also found a significant correlation. One year after harvesting chin bone, the residual defect was significantly larger in older patients than in younger patients. As in other studies it seems that increased age has a negative effect on bone healing and results in a larger residual defect later on. No correlation was found between gender and residual defect.

Soft tissue analysis on our patient group with a mean age of 12 years (range 8.4 to 19 years) showed a significant increase in the thickness of the soft tissue contour at points 2, 3 and 4 of the mandibular symphysis one year after harvesting chin bone. These measurements suggest a correlation between harvesting chin bone and an increase in soft tissue thickness at the donor site. Studies investigating growth changes of the facial skeleton and soft tissue in non-CLP children and in CLP children, in the same range of

age, who were treated with an iliac crest bone graft describe a similar increase in soft tissue thickness as a result of growth[17-22]. We can therefore state that the increase in soft tissue thickness in our study is a result of growth rather than a result of surgery. Several growth studies show a correlation between gender and the amount of soft tissue growth. Boys show significantly more enlargement than girls[17,20]. In our study we could not endorse this correlation.

To perform the measurements we used Adobe Photoshop 9.0. (Adobe Systems Inc. California, USA). Dedicated software such as DICOM (Digital Image and Communication in Medicine) was available but was not shown to be significantly better than non-dedicated software[23]. We performed our analysis on two-dimensional radiographs. As measurements from conventional or digital radiographs do not accurately reflect the clinical situation they usually result in underestimation[24]. From this perspective it is plausible that the residual defect we measured was actually an underestimation of clinical reality.

Although a residual defect one year after harvesting a bone graft from the mandibular symphysis is of minor clinical significance, the donor site does not repair itself fully, especially in older patients. The use of allograft bone void fillers for repair of donor site defects is suggested with the aim of restoring the contour of the mandibular symphysis more completely. Consequently, current research in our department will reveal the effects on the residual defect after filling the mandibular symphysis donor site with a bone substitute. Our study model with its standardized radiographic measurements is eminently suitable for this purpose.

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Chapter 6

Micro-structured Calcium Phosphate ceramic for donor site repair after harvesting chin bone for grafting alveolar clefts in children

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submitted

INTRODUCTION

The frequency of occurrence of various forms of congenital facial clefts varies throughout the world and between different ethnic groups, local areas and time span[1]. In Europe, the occurrence of clefts of lip, alveolus and palate (CLP) among Caucasians has been reported to be somewhere between 0.69 and 2.35 per 1,000 births[2]. One of the major interventions in the treatment of patients with unilateral (UCLP) or bilateral (BCLP) cleft of lip, alveolus and palate is closure of the alveolar cleft. The importance of this procedure does not only lie in the closure of the alveolar cleft in order to allow the eruption of the surrounding teeth and in enabling orthodontic transfer in the grafted bone. The soft-tissue profile of lip and ala nasi is also improved by the bone correction and even so the dental treatment which follows orthodontic treatment. This is of great social importance in every culture in the world. Transplantation of autologous bone is still the gold standard in alveolar cleft repair strategy[3]. Using the chin as standard donor site is routine practice in most cleft centres or cleft surgery repair in the Netherlands and well accepted with low objective and subjective morbidity[4-11]. We have observed almost no adverse effects of the chin graft method. Since it began to be used routinely use in 1995 surgeons have built up a thorough experience in harvesting chin bone for repair of unilateral and even bilateral alveolar clefts[12,13].

However, one problem continues to be inherent to harvesting autologous bone: wherever in the body the bone is harvested, it is accompanied to a greater or lesser extent by co-morbidity[14]. The question is: can this be avoided?

A resorbable bone substitute is a potential answer to this question. The bone substitute that we used is a micro-structured resorbable calcium phosphate that has been tested in animal experiments and has been shown to be equally as effective as autologous bone[15]. The application of bone substitute only, i.e. with no autologous bone and/or cell material or bone morphogenetic proteins (BMP's), for the repair of alveolar clefts is new in children. We wanted to exclude the risk of complications accompanying the use of artificial grafting material for filling clefts in CLP patients. To this end we chose to first investigate Micro-structured resorbable calcium phosphate as a filling material in the chin, the Utrecht donor site of autologous bone for repair of the alveolar cleft[16] and the only clinically available defect in our patients.

The critical size defect (CSD) in a child's mandible was never determined and probably never will be. CSD's were originally defined as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal[17]. However Cooper in 2010[18] stated *'After a review of the existing literature and a critique of the clinical applicability of the models studied, it is suggested that the use of the term "critical-sized-defect" be discontinued'*. But the defects following chinbone harvest for alveolar bone closure obviously are not beyond this size. In a

previous study[12] we have shown that these defects heal spontaneously '*leaving a residual defect* (compared with the original) *of about 14%'.*

Aim

The aim of this study was to evaluate the safe clinical use of a newly developed calcium phosphate based micro-structured and resorbable bone substitute in children. We used it as a filling material in the autologous chin bone donor site in young patients with a cleft of lip, alveolus and palate (UCLP and BCLP). It was also important to determine the degree of dimensional stability of the used micro-structured resorbable calcium phosphate granules in bone formation and the process of bone graft resorption and remodelling.

PATIENTS, MATERIAL, METHODS

Patients

This study was carried out in accordance with the principles expressed in the Declaration of Helsinki. Permission for this study was obtained from the Medical Ethical Committee of the University Medical Centre, Utrecht, The Netherlands (Protocol nr. 06-210).
Before the operation, parents and children were given extensive information about this experimental study. In order to avoid being influenced by the researchers, the parents and children were given the opportunity to speak to an independent but informed physician and to ask any questions they may have had. Patient information forms were developed especially for this study. Patients were included in the study after written informed consent had been obtained. The blinded patient group comprised 20 UCLP and BCLP patients, all with an indication for alveolar cleft repair. The group was sorted by gender and type of cleft (Table 1) and average age at the time of repair surgery (Table 2).

TABLE 1: number and gender

	MALE	FEMALE	Total
UCLP	7	5	12
BCLP	6	2	8
Total	13	7	20

TABLE 2: average age at time of cleft repair surgery

	MALE	FEMALE
UCLP	11y 1m	11y 2m
BCLP	12y 2m	10y 9m

Material (Bone Substitute)

The synthetic bone graft material is comprised of 1-2mm sized Micro-structured calcium phosphate particles that contain >90% tricalcium phosphate and <10% hydroxyapatite (RevisiOs BV, The Netherlands) (Figure 1).
It is a resorbable material for clinical application in bone regenerative surgery. The Micro-structured surface of the material renders the material osteoinductive as

FIGURE 1: 1-2 mm sized micro structured calcium phosphate particles. (red scale bar: 1 cm)

demonstrated in various preclinical models[20-22], without the addition of bone growth factors or other bone inducing agents or cells. These materials have superior ability to accelerate bone healing, compared to non-osteoinductive ceramics.

Methods

Surgical method

Alveolar cleft repair surgery comprised repair by means of a bone transplant using autologous bone taken from the mandibular symphysis. The bone-harvesting site was then filled with the Micro-structured calcium phosphate. The buccal cortical surface of the mandibular symphysis was approached by a horizontal incision in the vestibule of the lower lip in the intercanine region. After making a submucosal flap, an incision was made through the mental muscles on each side and down to the bone. After elevating the muco-periosteal flap the mental nerve was localized bilaterally. Using the position of the apices of the incisors and the germs or roots of the permanent canines as seen on preoperative radiograph, the outline of the bone graft was marked with a fissure burr. The monocortical bone graft was cut out with a reciprocal saw, irrigated with physiological saline solution 0.9%, and harvested using a chisel. To collect the maximum amount of bone, the remaining cancellous bone in the symphysis was harvested using excavators. To refill the harvesting site, a five-wall bony defect, Micro-structured calcium phosphate granules, 1-2 mm in size, were mixed with fibrin glue (= human material) and placed in the defect (Figure 2).

Closure was achieved by approximating the periosteum and both mental muscles to their origins with a Vicryl® 3-0 suture (Ethicon, Johnson & Johnson Intl.). The mucosa was closed with a Vicryl® 4-0 running mattress suture. Directly postoperatively the chin was taped with elastic tape to minimize swelling from oedema and restrict additional

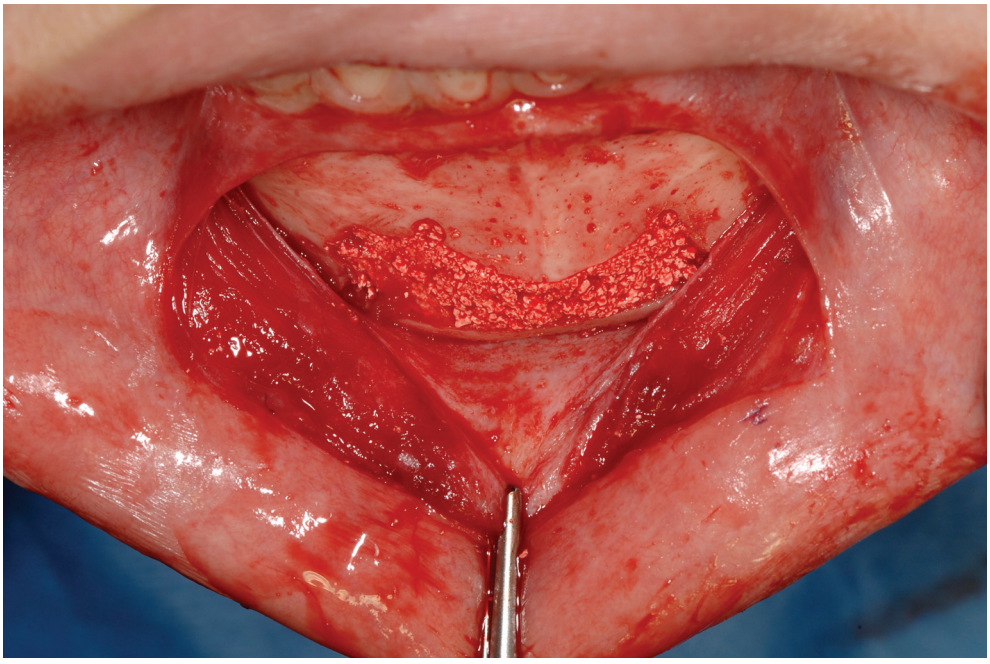

FIGURE 2: Micro structured calcium phosphate granules, 1-2 mm in size, mixed with fibrin glue (= human material) and placed in the symphyseal defect.

haematoma formation. This also served to stabilize the soft tissue profile of the lower lip with the mandible. The tape was removed after 72 hours.

To prevent wound infection, a standard prophylactic antibiotic regime (Clindamicine, Hameln Pharmaceuticals, 31789 Hameln, Germany, 10 mg/kg per 24 hours) was prescribed for 48 hours postoperatively. A soft diet was prescribed to prevent wound dehiscence. Oral hygiene was delivered by a dental professional.

Study method

The study took the form of a prospective randomized clinical trial of 20 patients. We followed an established method of using lateral cephalograms[11]. These had been taken both peroperatively and at twelve months postoperatively. The area of interest: the mandibular symphysis, was imaged according to the method described by Dik et al.[19] (Figure 3).

The data obtained were digitalized and the treatment outcomes expressed in numbers. Comparisons with a previous study[19] were made. Patients from both studies together are a cohort from all newly incoming patients, operated upon by the same surgeons in similar settings. Mean age and gender ratio are also similar in both studies.

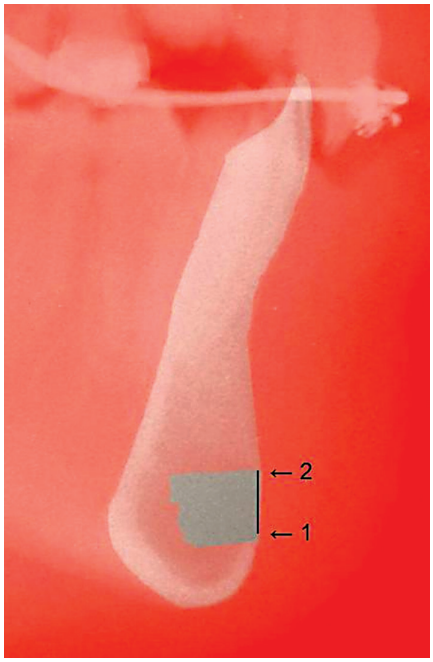

FIGURE 3: Digital tracing of the mandibular symphysis showing the bony defect directly postoperatively. Note the straight line from the caudal labial border (1) to the cranial labial border (2).

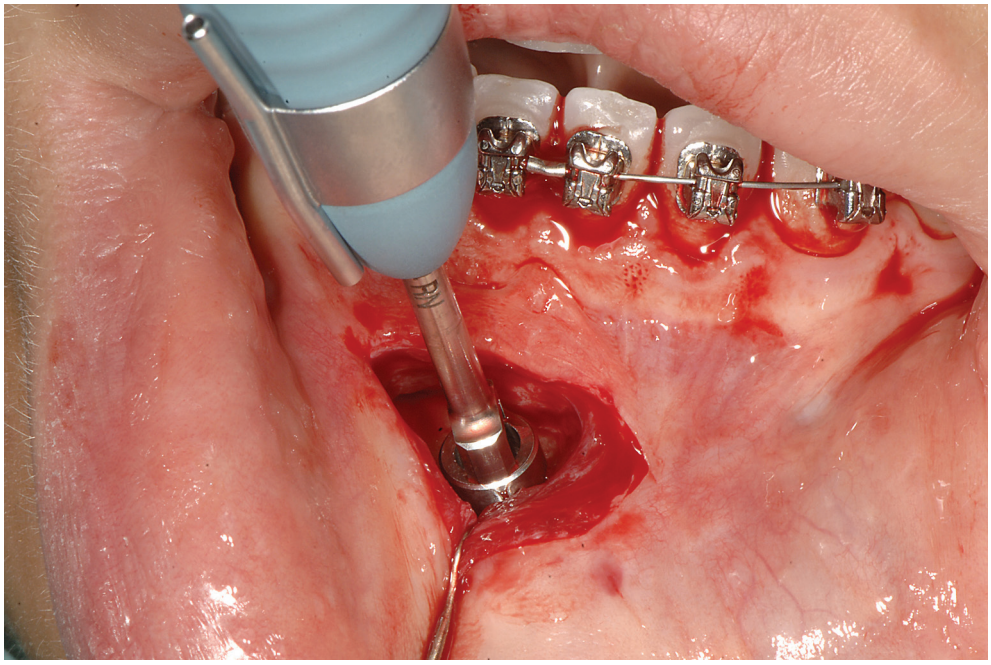

FIGURE 4: Taking the biopsy with a hollow drill.

Histological methods

In two patients we were able to take a biopsy from the area of the chin from which bone had been harvested and then filled with the Micro-structured calcium phosphate more than 18 months previously (Figure 4).

The two biopsies were taken during re-entry and with strict permission from patients and parents. This mandibular symphysis bone biopsy material was then prepared for histology (Figure 5).

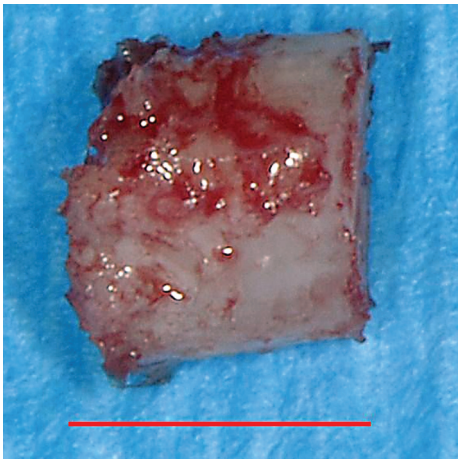

FIGURE 5: Specimen of the biopsy material. (red scale bar: 1 cm)

The biopsies were not decalcified and after dehydration through a graded series of ethanol (Merck) were embedded in cold curing MMA (K-Plast, L.T.I. Bilthoven, The Netherlands). Sections approximately 20 micrometre thick were made using the Leica SP1600 saw microtome. They were stained with 1% methylene blue and 0.3% basic fuchsin (both Sigma-Aldrich) and mounted on glass slides using UV curing glue (Permacol, Ede, The Netherlands).

CT Scans

On medical indication the CT scans of one patient taken pre-operatively, and at three months and one year postoperatively were available. They were both studied and interpreted visually.

RESULTS

Defect measurements outcome

Table 3 shows the digital measurements in pixels and in mm^2 van of the contour of the symphysis (sym) and of the defect (def). Measurements were taken peroperatively

(per) and one year postoperatively (pos). The total group was divided into unilateral cleft patients (uni) and bilateral cleft patients (duplex). The percentage (perc) of the defect per- and postoperatively was measured and compared with that of the symphyseal contour.

TABLE 3: Group Statistics

	duplex_uni	N	Mean	Std. Deviation	Std. Error Mean
sym_per	uni	12	43715.00	4.180.556	1.206.823
	duplex	8	44165.25	7.883.710	2.787.313
sym_pos	uni	12	44994.25	4.648.109	1.341.794
	duplex	8	49674.88	6.346.719	2.243.904
sym_per_mm^2	uni	12	3.133.667	2.996.975	865.152
	duplex	8	3.165.875	5.652.169	1.998.343
sym_pos_mm^2	uni	12	3.225.417	3.329.249	961.071
	duplex	8	3.560.875	4.550.273	1.608.764
def_per	uni	12	12970.00	2.253.532	650.539
	duplex	8	12133.83	4.705.651	1.663.699
def_pos	uni	12	171.88	321.361	92.769
	duplex	8	249.83	374.688	132.472
def_per_mm^2	uni	12	929.750	1.615.471	466.346
	duplex	8	869.750	3.375.355	1.193.368
def_pos_mm^2	uni	12	12.417	229.722	.66315
	duplex	8	18.000	267.689	.94642
perc_per	uni	12	301.483	510.202	147.283
	duplex	8	276.827	910.290	321.836
perc_pos	uni	12	.4109	.79544	.22962
	duplex	8	.5417	.87797	.31041
perc_original_def	uni	12	12.308	215.902	.62325
	duplex	8	27.163	447.436	158.192

TABLE 4: Inter-Group Statistics

	study	N	Mean	Std. Deviation	Std. Error Mean
sym_per_mm^2	Dik et al.	23	3.366.087	4.525.156	943.560
	present	20	3.146.550	4.122.643	921.851
sym_pos_mm^2	Dik et al.	23	3.437.174	4.547.175	948.151
	present	20	3.359.600	4.109.509	918.914
def_per_mm^2	Dik et al.	23	349.791	2.780.065	579.684
	present	20	905.750	2.408.170	538.483
def_pos_mm^2	Dik et al.	23	48.139	567.292	118.289
	present	20	14.650	240.291	.53731
perc_per	Dik et al.	23	101.704	713.637	148.804
	present	20	291.621	686.546	153.516
perc_pos	Dik et al.	23	14.152	164.482	.34297
	present	20	.4632	.80909	.18092
perc_original_def	Dik et al.	23	162.978	1.834.148	382.446
	present	20	18.250	326.065	.72910

The percentage of the residual defect after one year was also compared with the original defect (per). The mean defect peroperatively for the unilateral cleft patients was *93* ± *16* $mm^{2,}$ for the bilaterals *87* ± *34* mm^2. The mean defect peroperatively (def_per_mm2) for the total group was 91 ± 24 mm^2. The mean defect postoperatively for the unilateral cleft patients was *1.2* ± *2.3* mm^2, for the bilaterals *1.8* ± *2.7* mm^2. The mean defect 1 year postoperatively (def_pos_mm2) for the total group was 1.5 ± 2.4 mm^2. Table 4 shows the outcomes of the comparison of the current study and a previous study[12]. In this study the average peroperative defect was *35* mm^2 and *4.8* mm^2 one year postoperatively. The percentage of the residual defect compared with the original defect was *16*%. The average defect was *91* mm^2 peroperatively and *1.5* mm^2 one year postoperatively. The percentage of the residual defect on comparison with the original defect was *1.8*%.

Statistics

Analysis of variance (ANOVA, SPSS 15.0; SPSS, Chicago, Il, USA) was applied to test the null hypothesis that there would be no statistical difference between the results obtained from the radiographs (taken directly (=peroperatively) or one year postoperatively), and between the two independent investigators and three individual tracings. Independent sample t tests were performed to test possible differences in the defects between unilateral and bilateral cleft patients (peroperatively and 1 year postoperatively). Furthermore, the independent sample t test was used to compare the present results with those of our previous study[12]. One-sample t tests were performed to test whether after one year the defect differed from zero (Tables 5 and 6). Pearson's correlation coefficients were calculated to determine possible relationships between the age of a patient and the residual defect after one year, and a relationship between the defect peroperatively and

TABLE 5: One-Sample Statistics

study		N	Mean	Std. Deviation	Std. Error Mean
.	def_pos_mm^2	0(a,b)	.	.	.
Dik et al.	def_pos_mm^2	23	48.139	567.292	118.289
present	def_pos_mm^2	20	14.650	240.291	.53731

a t cannot be computed because the sum of case weights is less than or equal 1.
b t cannot be computed. There are no valid cases for this analysis because not all case weights were positive.

TABLE 6: One-Sample Test (a)

		Test Value = 0					
						95% Confidence Interval of the Difference	
study		t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Dik et al.	def_pos_mm^2	4.070	22	.001	481.391	23.608	72.671
present	def_pos_mm^2	2.727	19	.013	146.500	.3404	25.896

a No statistics have been computed for one or more split files

one year later. In addition, we studied a possible relationship between gender and the defect after one year. A p-value of less than 5% was considered significant.

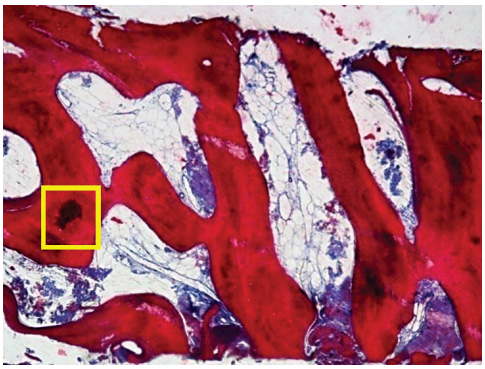

FIGURE 6a: 40xs magnification. Bone is stained red/pink in this image and the yellow square indicates the presence of a remnant of the implanted tricalcium phosphate material.

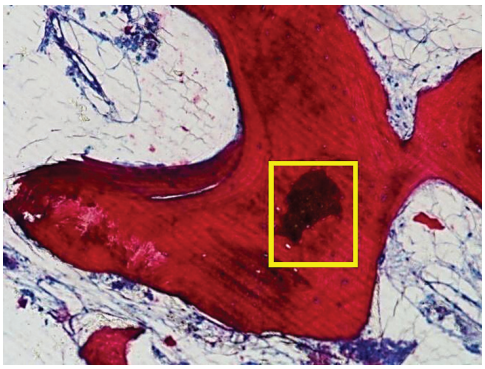

FIGURE 6b: 100xs magnification of a CaP remnant (yellow square) fully embedded within the bone. The open structure in between the bone is filled with bone marrow (white).

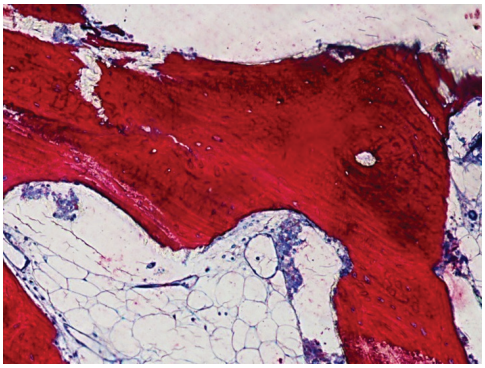

FIGURE 6c: 100xs magnification of a typical bone structure; an osteon with Haversian channel in the top right.

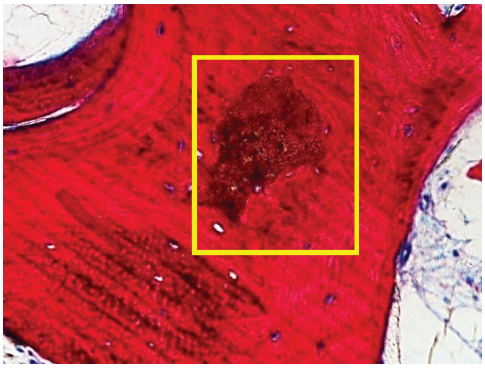

FIGURE 6d: 200xs magnification of the tri-calcium phosphate remnant, (yellow square) which is in direct contact and fully embedded within the newly formed bone.

Histological evaluation of human mandibular symphysis bone biopsies

Figure 6A shows an overview at 40 x magnification of the biopsy taken from patient 2.000.401. Bone is stained red/pink in this image and the yellow square indicates the presence of a remnant of the implanted tricalcium phosphate material.
Figure 6B was taken at a magnification of 100 x magnification and shows (yellow square) a remnant of the implanted tricalcium phosphate material fully embedded within the bone. The open structure in between the bone is filled with bone marrow (white).
Figure 6C shows a higher magnification (100 x) of a typical bone structure; an osteon with Haversian channel in the top right.

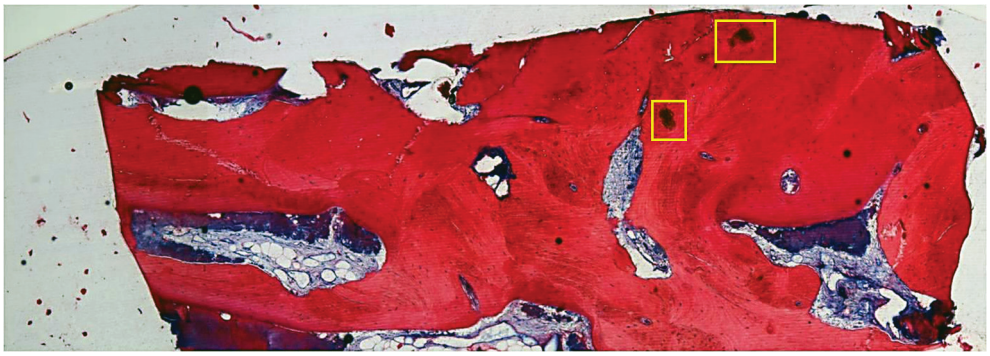

FIGURE 7a: An overview of the entire biopsy taken from patient 3.058.885 at 40xs magnification. The yellow squares indicate remnants of the implanted micro structured calcium phosphate material. The open structure in between the bone is filled with bone marrow.

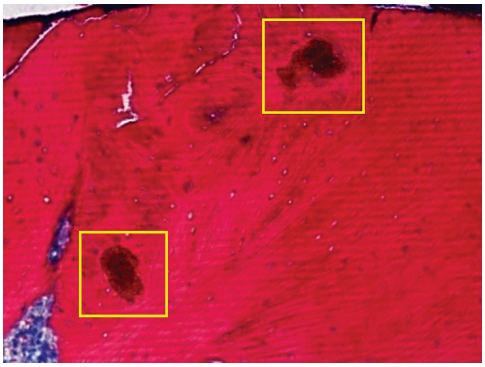

FIGURE 7b: Magnification of 100 xs. It shows the presence of remnants of the implanted tricalcium phosphate material fully embedded within the bone (yellow squares).

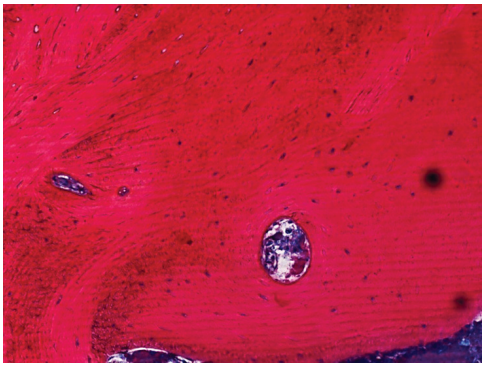

FIGURE 7c: Higher magnification of 100 xs of a blood vessel inside the bone.

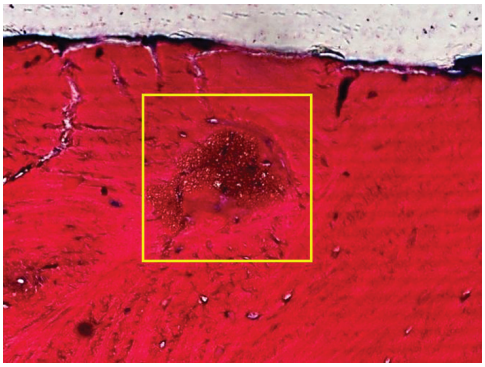

FIGURE 7d: 200xs magnification of a tricalcium phosphate remnant (yellow square) in direct contact with the newly formed bone.

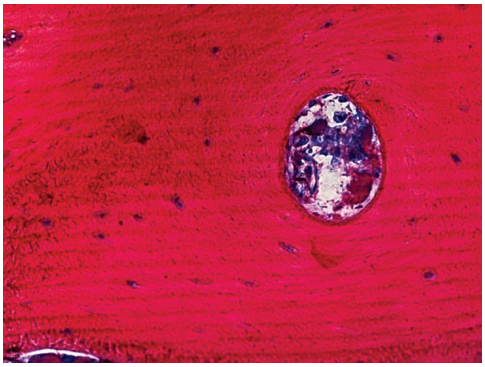

FIGURE 7e: 200xs magnification of the blood vessel inside the bone.

Figure 6D shows a 200 x magnification of the tri-calcium phosphate remnant, (yellow square) which is in direct contact and fully embedded within the newly formed bone.
Figure 7A shows an overview of the entire biopsy taken from patient 3.058.885 at 40x magnification. The yellow square indicates remnants of the implanted Micro-structured calcium phosphate material. The open structure in between the bone is filled with bone marrow.
Figure 7B is taken at a magnification of 100 xs and shows the presence of remnants of the implanted tricalcium phosphate material fully embedded within the bone (yellow squares).
Figure 7C shows a higher magnification (100 xs) of a blood vessel in the bone.
Figure 7D shows a 200x magnification of a tricalcium phosphate remnant, (yellow square) which is in direct contact with the newly formed bone.
Figure 7E shows a 200x magnification of the blood vessel. In both patients the bone structure resembles the normal bone structure, with only very small remnants of the implanted calcium phosphate ceramic.

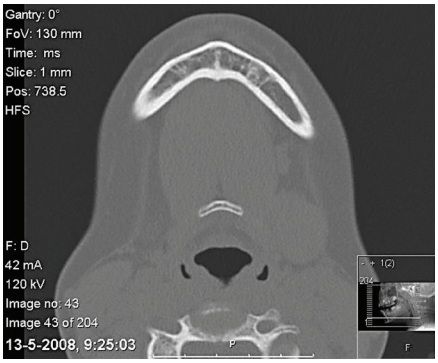

FIGURE 8: Axial image of the harvesting site preoperatively

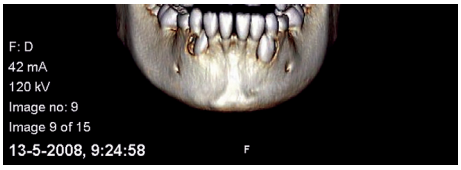

FIGURE 9: Frontal image of the harvesting site preoperatively

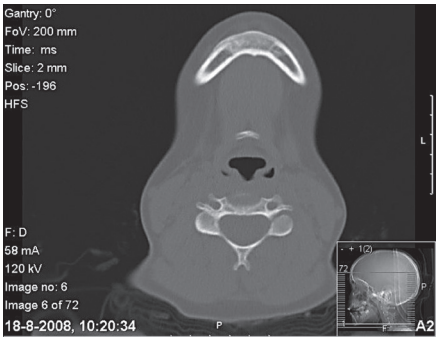

FIGURE 10: Axial image of the repaired symphysis 3 months postoperatively with grafting material (micro structured calcium phosphate) still visible

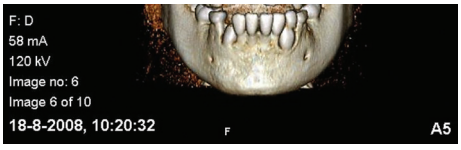

FIGURE 11: Frontal image of the repaired symphysis 3 months postoperatively and no grafting material (micro structured calcium phosphate) visible

3D CT Scans

3D CT scans of one patient were available. Figures 8 and 9 show the preoperative mandibular symphyseal harvesting site. Figures 10 and 11 show the symphysis three months postoperatively. Figures 12 and 13 show it one year postoperatively.

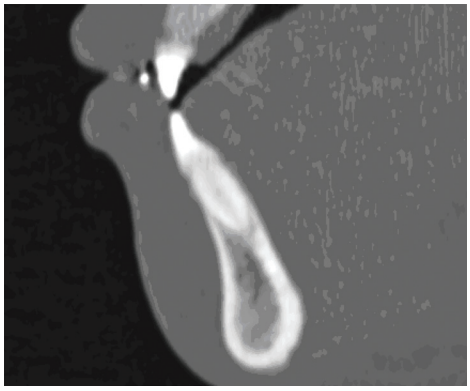

FIGURE 12: Sagittal image of the repaired symphysis one year postoperatively

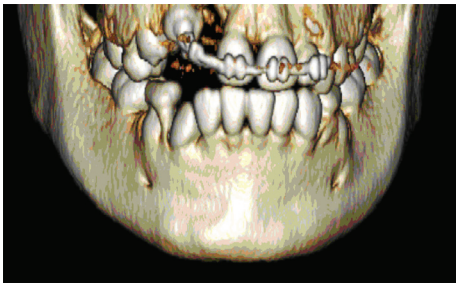

FIGURE 13: Frontal image of the repaired symphysis one year postoperatively

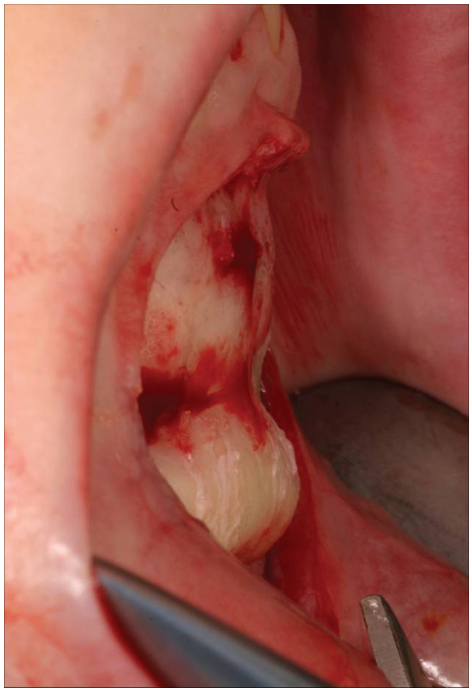

FIGURE 14: The hollow-curved mandibular symphysis shows a non-regenerated bone defect at the time of secondly harvesting chin bone for a redo of alveolar cleft repair

DISCUSSION

This study demonstrates that the Micro-structured, resorbable calcium phosphate ceramic provides excellent regeneration properties for the repair of the chin bone harvesting defect in children. Although with a different β-TCP, Horch, in five bony wall defects as well, showed this after the surgical removal of large pathological jaw defects[23]. One year postoperatively, the measurements taken from lateral cephalograms show that there is scarcely any visible residual defect. This is in contrast with the results from our previous study[19] and the Weinbull study[11] that states: *'at radiographic* (lateral cephalograms) *examination bone healing after chin graft harvesting did not regenerate to the preoperative level'.* This is depicted in Figure 14, showing a non-regenerated bone defect along the hollow-curved mandibular symphysis at the time of secondly harvesting of chin bone for a redo of the alveolar cleft repair at later age.

The main reason for the use of the accepted method of lateral cephalogram assessment in this study was the non-existence of 3-D radiographic imaging in our retrospective previous study[19]. Because we wanted to be able to compare the results of both studies we used this similar method and assessed the defect two-dimensionally as it is depicted in Figure 14 three-dimensionally.

The histological investigations of the two bone biopsies show proof of solid, induced bone formation and almost complete resorption of the Micro-structured calcium phosphate.

The data from the results of both per and post-operative visual-imaging show no difference between patients with a unilateral and bilateral alveolar cleft. We had expected this as repair of a bilateral alveolar cleft generally requires the harvesting of more autologous bone than repair of a unilateral alveolar cleft[24].

Indeed, statistical tests showed that there were no significant differences between the groups (p-values>5%). It was striking that the peroperative defects measured in this study appeared to be larger than those in the previous[19]. This difference proved significant (p=0.000). The explanation for this can be found in the visibility of the defect on lateral skull radiograph. The presence of the calcium phosphate material peroperatively marks the defect better.

The size of the defect after one year is also different in each study. Both show a significant residual defect. However, in the previous study (t=4.1; p=0.001) the residual defect was significantly larger than in the current study (t=2.7; p=0.013). This means that the residual defect is nearer to nil despite the much larger original defect. The final defect is significantly smaller than found by the previous study (p=0.019). At one year the percentage of the final defect compared with the original defect is also different in each study: 14 % previously and now 1.8%. This means that the percentage of the original defect is now much smaller than in the last study (p=0.001). For this reason we can conclude that Micro-structured resorbable calcium phosphate fills the defect better than Spongostan which is normally used.

The one year symphysis bone biopsies from these two patients show that the implanted Micro-structured calcium phosphate granules have almost been fully resorbed and replaced by viable bone normal histological appearance. These results indicate the biocompatibility and efficacy of this novel synthetic bone graft. This shows that the revascularization that was responsible for the changing process of remodeling of the bone substitute had taken place. If we were to transpose these data to the alveolar cleft itself, then it is only in a vital bone setting that tooth movement can be initiated throughout the activation time of the orthodontic forces. These forces initiate a remodelling process in the tension and pressure sides of the bone surrounding the tooth. It is the characteristic properties of the fibrous periodontal ligamental joint between a tooth and its socket - the gomphosis – that allow for the attachment and load bearing characteristics of a tooth[25,26]. Functional loading is of the utmost importance for the preservation of the newly-acquired of bone. However, in order for this process to take place, bone must be present.

A question asked frequently by the parents of patients was: Why don't you put the bone substitute directly into the alveolar cleft itself? This question should be seen in its social context. Dutch patients like to be actively involved in their treatment and their doctors are prepared to a shared transparent decision treatment. Our decision not to do this was a considered one. As the implantation of bone substitute in general and this Micro-structured, resorbable calcium phosphate ceramic in particular is new in children, we wanted to first safely test it at a less critical site than the alveolar cleft.

The Dent-Scan CT views taken preoperatively, three-months postoperatively and one-year postoperatively show a real restoration of integrity of the mandibular symphysis following peroperative filling with the Micro-structured calcium phosphate bone graft. The compacta contour of the defect that was caused by harvesting autologous chin bone to repair the alveolar cleft in the upper jaw was completely healed after three months. After three months some remains of ceramic granules are still visible in the cancellous bone. The one-year postoperative scan demonstrates complete healing and progressive resorption of the Micro-structured calcium phosphate granules.

On the grounds of these results and of the results from an earlier animal study[15], the Medical Ethics Committee has given permission for these Micro-structured and resorbable calcium phosphate granules to be grafted directly into the alveolar cleft. A new coned beam computer tomogram (CBCT scan) radiography protocol is currently investigating bone quality and bone quantity[27].

CONCLUSIONS

The findings of this study have illustrated the biocompatibility and efficacy of a new class of Micro-structured, resorbable calcium phosphate granules, as well as its dimensional

stability throughout remodelling. Histological examination of biopsies indicates the overall presence of newly-formed vital bone and the resorption of the bone substitute, a *conditio sine qua non* for maintaining shape and volume of bone for the orthodontic moving of teeth.

The use of Micro-structured calcium phosphate bone grafts in alveolar cleft repair is now warranted. At such time harvesting autologous bone elsewhere in the body, and by extension its inherent concomitant morbidities is avoided. And that will be winning progress.

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Chapter 7

Micro-structured β-TCP for repair of the alveolar cleft in CLP patients: a pilot study

Ad de Ruiter, Nard Janssen, Robert van Es, Andries van der Bilt, Gert Meijer and Ron Koole

submitted

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 (CLP) among Caucasians has been reported to be between 0.69 and 2.35 per 1,000 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 the autologous bone graft harvested either from the iliac crest or from the mandibular symphysis[6-9]. Both grafting procedures carry potential risks for grafting 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]. In order to prevent donor site morbidity, as well as shorten the operating 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=6) was to generally evaluate the repair of the alveolar cleft after grafting with micro-structured 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 (UCLP) were randomly included for alveolar cleft repair with β-TCP in 2009/10. Male-female ratio was 5:2 and mean age was 11.16 years (SD +/- 1.83). 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.

Reference group

In 2001 Van der Meij[19] evaluated the outcome of iliac crest bone grafts in cleft palate patients (N=42 UCLP) in order to assess the amount of bone necessary for eruption of

adjacent teeth. All his assessments were distilled from computed tomography scans, so a volumetric outcome could be realised. Accordingly, we interpreted the results of our pilot-study group in the same way.

Surgical procedure

Alveolar cleft repair was performed under general anaesthesia with naso-endotracheal intubation. Antibiotic prophylaxis was given intravenously for three days (Clindamicine, Hameln Pharmaceuticals, 31789 Hameln, Germany, 10 mg/kg per 24 hours). 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. (Figure 1). Then the palatal mucosa was sutured and a vestibular mucoperiosteal layer was constructed with transpositional mucoperiosteal flaps. All sutures used were resorbable (Vicryl 4.0, Ethicon, Brussels, Belgium).

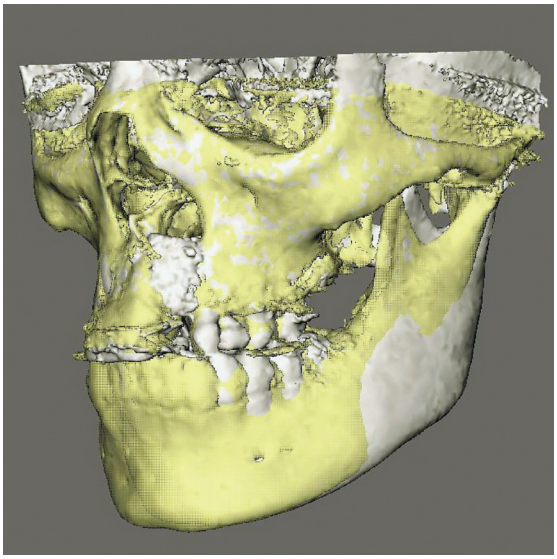

FIGURE 1: micro-structured β-TCP granules inserted for repair of the alveolar cleft

Orthodontic procedure

Three months postoperatively orthodontic therapy was started without actively engaging erupting teeth adjacent to the cleft.

Bone substitute

The micro-structured β-TCP used in this study is a 65±15% porous and >90% pure phase beta tricalcium phosphate graft material (X-Pand Biotechnology BV, Bilthoven, The Netherlands). It is synthetic, osteoconductive, osteoinductive and resorbable[20].

Radiographic assessment

In the study group a Coned Beam CT-scan (CBCT) was carried out directly preoperatively, one week postoperatively and after six months. Radiation hazards refrained 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. CBCT scan analysis was performed by two independent investigators using a Osirix Dicom Viewer (Apple Inc.)[21]. 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-existent bone defect was compared in percentages with the total graft volume and with the residual bone volume six 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 ANOVA (SPSS 15.0, SPSS, Chicago, IL., USA) 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. $P < 0.05$ was considered significant.

RESULTS

Bone healing

The six-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. Eruption of the adjacent canine or lateral incisor into the repaired cleft occurred spontaneously in all patients.

The outcome of the volume of the original clefts prior to surgery is depicted in Tables 1 and 2.

Statistical analysis

Examiners did not have a significant influence on the determination of the bone volume ($p=0.58$). Also repetition of the determination did not significantly influence the outcomes ($p=0.21$). The moment of the measurement, however, significantly influenced the bone volume outcomes ($p < 0.001$). A significant increase in bone volume was observed directly after operation ($p <0.005$). The volume increased from on average 0.72

TABLE 1: Cleft volumes pre-operatively, post-operatively and 6 months after operation. (nc: not calculated)

	Cleft volume preop	Graft volume postop	Bone volume 6 months postop
	Outcome cm^3	Outcome cm^3	Outcome cm^3
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

TABLE 2: Percentages of cleft volumes pre-operatively, post-operatively and 6 months after operation. (pre-operatively was set at 100%; nc: not calculated)

	Cleft volume preop	Graft volume postop	Bone volume 6 months postop
	Outcome %	Outcome %	Outcome %
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

cm^3 to 0.93 cm^3, an increase of 32%. A significant decrease in volume occurred in the following 6 months period, from 0.93 cm^3 to 0.53 cm^3 ($p < 0.001$). The bone volume after 6 months was significantly lower than the initial value, 73% ($p < 0.001$).

DISCUSSION

Six months after the operative grafting of micro-structured β-TCP into the alveolar cleft the bone volume thus acquired was satisfactory. We performed an independent samples t-test comparing our 6-months results and the results of 18 unilateral left-sided cleft lip and palate patients assessed one year after grafting by van der Meij[19] (Table 3). We found no significant differences between these groups ($p=0.09$): 73 ± 6 % (our group) vs. 60 ± 28 % (Table II of van der Meij[19]).

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

TABLE 3: (=Table I of Van der Meij19) Median percentage of residual bone per group after one year and the significance of differences between the groups.

Group	Clefts	Median (%)	P value
Group 1 UL	18	71.5	P > .1
Group 1 UR	8	68.9	-
Group 2 UL	10	65.0	P > .1
Group 2 UR	6	78.4	-
Group 1 B	7	53.9	Not tested
Group 2 B	6	34.0	-

Group 1, early; group 2, late secondary bone grafting. UL, Unilateral left-sided; UR, unilateral right-sided; B, bilateral

outcome as volumetric percentages bearing in mind that the number of patients and the scanning techniques were different in each group.

ALARA (As Low As Reasonably Achievable) is an accepted principle in medicine and dentistry that dictates that healthcare professionals should use the smallest amount of radiation required to produce the information needed to diagnose and treat the patient[22]. Conventional medical CT scanning of a maxilla subjects the patient to 200-300 times the amount of radiation required for a panoramic radiography. Cone beam scanners utilize a narrow, collimated cone beam of radiation that can scan the maxilla only. This requires only 2-8 times the amount of radiation used in a panoramic radiograph. Compared to the 2-D data delivered by panoramic radiographs, the 3-D data generated by an CBCT give 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 bone volume with regard to the original cleft showed a mean percentage of 73%. This means a considerable resorption has occurred. Repairing an alveolar cleft does obviously not mean that the alveolar process needs to be completely restored. Restoration of only seventy-three percent 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 watertight closure of the mucosa of the defect virtually impossible and may have caused salivary contamination of the granules. We therefore excluded this case from further analysis.
The authors heavily stress the importance of watertight 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.
Histological data could not be provided since in the Netherlands taking biopsies in humans for research purposes is strictly forbidden. The spontaneous eruption that was scored for all 6 patients, was considered as proof of the presence of bone.

CONCLUSIONS

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 towards the clinical use of micro-structured β-TCP bone substitute for repair of the alveolar cleft. Because of the considerable amount of bone resorption after overfilling the alveolar cleft only re-contouring 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 8

General Discussion

GENERAL DISCUSSION

Where do we stand and how did we arrive here?

After Bosker and van Dijk[1] published their results on grafting bone from the chin to the alveolar cleft in 1980 the Wilhelmina Children's Hospital (Utrecht, The Netherlands) made mandibular symphysis bone the first choice for cleft grafting in 1994. Besides its intraoral location, also the fact that the mandibular symphysis is of ectomesenchymal origin is beneficial[2-4].

The particular suitability of using the mandibular symphysis bone for grafting to the alveolar cleft was also underlined by several other authors[5-10]. They all preferred working at one operation site only and they also described the advantage of the associated low incidence of morbidity. The graft operation using autologous ectomesenchymal mandibular symphysis bone resulted in an alveolar process of sufficient volume[10] enabling teeth on either side of the grafted cleft to erupt either normally (66.70%) or with surgical assistance (33.3%).

When a grafting procedure has resulted in the availability of sufficient bone, orthodontic treatment is capable of moving the teeth into the new bone and of uprighting cleft-adjacent teeth in the repaired cleft in an overcorrected upright position so that functional loading can occur.

Although the use of autologous mandibular bone as graft material for repair of the alveolar cleft is an approved method, still some drawbacks are noted; for harvesting an autologous bone graft an extra operation is needed, leading to co-morbidity and probable extra cost. Moreover postoperative numbness of the chin region and avital teeth were reported after harvesting chin bone[11], so..

Can we do better?

The literature suggests that in order to avoid *any* side effect from harvesting autologous bone, resorbable bone substitutes are likely to be a better treatment option in non-cleft deformities[12-16].

Many synthetic bone graft substitutes are considered solely osteoconductive and are used as bone fillers for small bone defects only, where osteoconduction is sufficient. The fully synthetic bone substitute based on calcium phosphate ceramic, as used in our study, already showed to be at least as successful as an autograft or rhBMP-2 in the treatment of a critical-sized bone defect[17].

Which experimental animal model?

According to several authors[18-21] experimental animal research is the first step prior to a novel application in the human situation. However, the focus of public opinion makes it obligatory for the investigator to have valid, objective and scientifically evidence-based

reasons for choosing a particular animal model. Clearly each animal model has its own specific disadvantages and the decision to choose the goat as an experimental model was made for number of reasons. In contrast to goats, sheep have to be kept in pens in the open air which is more costly and also makes surveillance difficult. Smaller animals such as guinea pigs[20], mice or rats are unsuitable due to their micro-anatomy, unlike goats in which alveolar defects similar to human alveolar defects can be created more easily. In addition, the metabolic rate of the goat is more or less equivalent to that of a child of 10-11 years old[22-26], the age at which most alveolar clefts are reconstructed in children.

Obviously differences exist between goat anatomy and human anatomy in the eruption mechanism of the teeth and in the surrounding periodontal ligament[27]. Initially these differences caused doubt about the possibility of moving teeth horizontally in a goat model. Also the use of standard orthodontic devices is not possible. For this reason dental models were cast individually for each goat and orthodontic devices were manufactured.

Grafting considerations

Donor site surgery for harvesting autologous bone requires prolonged operating time and causes morbidity[28]. Therefore the introduction of a bone substitute is ideal for avoiding these drawbacks. Results on the use of a limited volume of ß-TCP for preimplantation sinus floor elevation procedures are encouraging[29-34]. Because of its versatility, low complication rate and long-term results, synthetic, pure-phase ß-TCP is a suitable material for the filling of bone defects in the alveolar region[35,36]. The use of ß-TCP in cleft repair has already been reported but its only function was as a barrier to cover the bone graft thus protecting the graft from resorption[37]. The sole use of ß-TCP as a bone substitute without mixing it with autologous material for repair of alveolar clefts was novel and had not yet been studied in alveolar cleft repair.

Another problem had to be investigated: the resorption process of such a bone-void filler should be in balance with the process of the formation of new bone; the space left by the resorbing bone substitute needs to be replaced by new bone. This process of 'creeping substitution' has been reported extensively in the literature[38-40].

In the complex combined situation of orthodontic movement of teeth and β-TCP remodeling into bone several postoperative intervals would have been necessary. However cost and Animal Care Committee regulations limited us to histology studies at the end of treatment.

Histological considerations

The sections were stained with methylene blue and basic fuchsin in order to make bone formation visible in general overview. This method of staining does not allow the distinction between macrophages, inflammatory cells and mastocytes or between osteoblasts and osteoclasts.

Nevertheless our study shows that after grafting of either ß-TCP or autologous iliac crest bone, comparable bone volumes are formed in the alveolar clefts. The total amount of bone found in all ten goats after grafting with ß-TCP was approximately 10% greater than with autologous bone. However, this difference was not statistically significant and no relationship between percentage of bone volume and the extent of orthodontic movement was observed. Scarcely any discernible remnants of ß-TCP were seen. The implanted ß-TCP did not block tooth movement yet it was resorbed. Histology showed cellular processes on both the pressure and tension sides of the periodontal ligament that are analogous with the human situation. One particularly interesting observation is that once the premolar had moved into the new bone, the β-TCP had been totally resorbed. This implies that both the osteoclast and osteoblast activity that is induced by orthodontic tooth movement and the load-bearing characteristics of a bone-borne tooth favorably influence the resorption of β-TCP[41,42]. This has been confirmed by a qualitative histological study in beagle dogs where biodegradable TCP exhibited a greater ability for adaptive remodeling in response to orthodontic force than did particulate marrow and cancellous autologous bone, suggesting its suitability for clinical use during orthodontic tooth movement[43].

Orthodontic considerations

From a clinical perspective, the orthodontic movement of the first premolars was problem-free, although the average displacement of the first premolars into the alveolar clefts was over 40 %. This can be partially ascribed to a root remnant or a loose coil as seen on the radiographs. It should also be realized that because of the intrinsic qualities of the design of the orthodontic device, i.e. the thickness of the walls of the crowns and the location of the palatal bar, the maximum possible closure of the diastema was achieved.

A threshold for a force magnitude that would switch on tooth movement cannot be defined[44]. For this reason, in an earlier pilot study in two goats orthodontic displacement was started at a force of 75 cN. Clinical inspection showed that at as early as three months, the entire extraction diastema had already been closed. However, minimal root resorptions from the cement surface of the roots of the displaced premolar, as well as some small signs of ankylosis were observed. For this reason we reduced the orthodontic retraction force to 50 cN which certainly induced gradual and more limited orthodontic movement but also a minor degree of apical root resorption. This phenomenon is analogous with the human situation and therefore acceptable[45-47].

Does the donor site repair '*ad integrum*'?

The results of the study in Chapter 5 show an evident residual bony defect in the mandibular symphysis at a minimum of one year after harvesting bone. Apparently full repair of the donor site does not occur if after harvesting, the site is filled with

Spongostan® only. In our patient group soft tissue analysis showed a significant increase in the thickness of the soft tissue contour at some points of the mandibular symphysis one year after harvesting chin bone. These measurements suggest a correlation between harvesting chin bone and an increase in soft tissue thickness at the donor site. Studies investigating growth changes of the facial skeleton and soft tissue in non-CLP children and in CLP children, in the same age range who were not treated with a symphysis bone graft describe a similar increase in soft tissue thickness as a result of growth[48-53].

We performed our analysis on two-dimensional radiographs. As measurements from conventional or digital radiographs do not accurately reflect the clinical situation they usually result in an underestimation[54]. From this perspective it is plausible that the residual defect we measured was actually an underestimation of clinical reality.

Although a residual defect one year after harvesting a bone graft from the mandibular symphysis is of minor clinical significance, the donor site does not repair itself fully, especially in older children. The use of allograft bone void fillers for repair of donor site defects is suggested with the aim of restoring the contour of the mandibular symphysis more completely. Our study model with its standardized radiographic measurements is eminently suitable for studying this issue as described in Chapter 6.

Application of the bone substitute directly into the alveolar cleft?

During the research the parents of patients asked frequently: Why don't you put the bone substitute directly into the alveolar cleft itself? This question should be seen in its social context. Dutch patients like to be actively involved in their treatment and their doctors are prepared to make a shared and transparent decision on treatment. Our decision not to do this was a considered one. As the implantation of bone substitute was new in children, we wanted to first test it at a less critical site than the alveolar cleft. The choice to restore the chin defect that is left after harvesting the autologous bone graft has the advantage that it involves the repair of a five-bony-wall defect. The results as described in Chapters 2, 3 and 6, motivated us in our decision to repair the alveolar cleft with this bone substitute only even though an alveolar cleft is a two-bony-wall defect and for this reason only has two walls with viable bone.

The study reported in Chapter 6 demonstrated that the micro- structured, resorbable calcium phosphate ceramic afforded excellent regeneration properties for the repair of a considerable defect in children. Although with a different β-TCP, Horch confirmed this after the surgical removal of large pathological jaw defects[55]. One year postoperatively, the measurements taken from lateral cephalograms showed that there was scarcely any visible residual defect. Histological investigations of the bone biopsies showed solid, induced bone formation and almost complete resorption of the micro-structured calcium phosphate.

In studies reported in Chapters 5 and 6 the size of the defect after one year is different although both show a significant residual defect. However, in the study reported in

Chapter 5 (t=4.1; p=0.001) the residual defect was significantly larger than that found in the Chapter 6 study (t=2.7; p=0.013). The final defect in the Chapter 6 study is significantly smaller than that found in Chapter 5 (p=0.019). For this reason we can conclude that micro-structured resorbable calcium phosphate fills the defect better than Spongostan® which is normally used.

Have we reached our goal? Bone substitute for repair of the unilateral alveolar cleft?

On the grounds of aforementioned results and of the results from the Chapter 4 animal study[56] and from Chapter 6, the Medical Ethics Committee gave permission for micro-structured and resorbable calcium phosphate granules to be grafted directly into the alveolar cleft in children. Using coned beam computer tomography (CBCT) and subsequent analysis protocol we investigated bone quality and bone quantity[57] ***and*** tooth eruption after grafting the alveolar cleft with micro- structured β-TCP in a pilot study (Chapter 7).

The experimental studies (Chapters 3 and 4) and the clinical study (Chapter 6) and the initial successful findings of the pilot study motivate to more research into the use of micro-structured β-TCP bone substitute for alveolar cleft repair, and also point to a clear path towards its clinical use.

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Chapter 9

Summary
en
Nederlandse samenvatting

SUMMARY

Over six chapters this doctoral thesis straightforwardly describes the story of the analysis of the plaster model material of those children with cleft of lip, alveolus and palate (CLP) who were treated at the Wilhelmina Children's Hospital at the UMC Utrecht between 1990 and 2008 in accordance with the current protocols. On comparison with a multicentre international study on the results of closure of the hard palate, the WKZ epiperiostal closure group scores high. The statistical analyses showed high significance ($p<0.001$) in both the allocations of the outcome of the results before/after operation and following orthodontic treatment. (Chapter 2).

It then goes on to examine the question - how can we do it better?

A bone substitute micro-structured β-TCP is compared with and considered as an alternative to autologous bone transplant (Chapter 3).

To test the selected bone substitute and to test the use of the goat as an experimental animal model a split mouth study in goats is described. In this study iliac crest bone grafts are compared with the bone substitute micro-structured β-TCP. It has been shown from the surgical, orthodontic, histological and radiological standpoints, that in the repair of alveolar clefts created in goats, the bone substitute ß-TCP (CuriOs™) is at least as effective as autologous iliac crest bone. The hypothesis that the synthetic bone substitute ß-TCP can be used equally as well as an autologous bone graft from the iliac crest for grafting and repair of alveolar clefts is not rejected. Its use in the clinical human alveolar cleft situation appeared to be warranted. (Chapter 4).

The step to the human situation is taken after retrospective examination of the filling of the chin bone harvesting site with Spongostan®. The results of this study show an evident residual bony defect (14%) in the mandibular symphysis at a minimum of one year after harvesting chin bone. (Chapter 5).

A comparable prospective study of the results of filling the harvesting site with β-TCP which was new in children. The final defect is significantly smaller than found by the previous study (1,8 %, $p=0.019$). (Chapter 6).

The final chapter concerns a pilot study of seven children which describes the results after the implantation of micro-structured β-TCP in the alveolar cleft itself. Comparing the outcome of this group of only six patients (73%) with the outcome of the N=42 group of Van der Meij[19] (approximately 71% mean for unilateral cleft cases), assessed one year after grafting leads to the assumption that the bone formation in the alveolar cleft after grafting it with micro-structured β-TCP was successful. (Chapter 7).

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft in zes hoofdstukken een rond verhaal vanaf de analyse van het beeld- en modellenmateriaal van tussen 1990 en 2008 in het Wilhelmina Kinder Ziekenhuis (WKZ) van het UMC UTRECHT behandelde schisispatiënten (CLP) volgens tot nu toe geldend protocol. In een vergelijking met een internationale multicenterstudie naar de resultaten van de sluiting van het harde gehemelte scoort het WKC-CLP team hoog. Daarenboven laat statistische analyse grote significantie zien in de twee groepen van onderzoek: voor operatie en na operatie é*n* orthodontische behandeling. (Chapter 2)
Toen naar de vraagstelling: hoe kan het beter?
Een botsubstituut, microstructuur-β-TCP, wordt vergeleken met andere keramieken als alternatief voor het 'eigen' bottransplantaat, omdat oogsten van dit bot wel eens leidt tot bijverschijnselen. (Chapter 3)
Vervolgens naar het beschrijven van de geit als experimenteel diermodel en een split mouth studie in geiten om heupbottransplantaten te vergelijken met het microstruur-β-TCP als botsubstituut. Vanuit chirurgisch, histologisch en röntgenologisch standpunt werd aangetoond dat het synthetische microstructuur-β-TCP het net zo goed 'doet' als het autologe heupbottransplantaat. De hypothese dat voor de Bot-in-Gnatho (BIG)-operatie microstructuur-β-TCP met hetzelfde resultaat gebruikt kan worden als bot uit de heup werd niet verworpen. Het gebruik van dit β-TCP voor het repareren van de kaakspleet bij kinderen blijkt gerechtvaardigd. (Chapter 4)
De stap naar de humane situatie is genomen via retrospectie naar de opvulling van de kinbot-oogstplek met Spongostan. De resultaten van deze studie laten een significant restdefect zien (14%) in de symphyse van de onderkaak gemeten minimaal een jaar na operatief oogsten van kinbot (Chapter 5),
en een daarmee vergelijkend prospectief onderzoek naar de resultaten van opvulling van die oogstplek met het onderzochte β-TCP (nieuw voor kinderen). Het restdefect is nu significant kleiner dan in de studie uitgevoerd in Chapter 5 (1,8 %, p=0.019) (Chapter 6).
Afgerond wordt met een pilotstudie waarin bij zeven kinderen gekeken wordt naar het resultaat van het inbrengen van microstruur-β-TCP in de eenzijdige kaakspleet zelf. Wij vergeleken de resultaten van deze groep met de resultaten van de groep (N=42) van van der Meij, die gemiddeld ongeveer 71% botvolume kon meten na repareren van de kaakspleet met heupbot. Onze 73% gemeten botvolume doet ons veronderstellen dat de botvorming in de kaakspleet na vullen met het microstruur-β-TCP succesvol was. (Chapter 7).

Dankwoord

Dank wil ik uiten aan iedereen die het mij mogelijk gemaakt heeft dit proefschrift tot stand te brengen. Dat betreft niet alleen diegenen die er wetenschappelijk bij betrokken waren. Een goede werksfeer en vriendschap in het maatschappelijk leven is minstens zo belangrijk; dus allen die daaraan bijdroegen verdienen vermeld te worden.
Een klassiek dankwoord is afgezaagd; dus….
Daar gaan we, met opzet niet in alfabetische volgorde: namen en trefwoorden, een zoekplaatje

BEOORDELINGSCOMMISSIEDANKVOORUWINZETENGOEDKEURINGKIDSINOPLEI
DINGVEELMEEGEMAAKTENALTIJDGEZELLIGERICKAMERGENOOTMEDEAUTEUREN
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GEZETDOENSASPCPASSELHEUREDEPARISELLENONCOLOGIEENCURSUSGENOTEHARM
IENAANSTELLINGENSALARISSCHAALNETOPTIJDDAPHNEHAMMEREDOUTMYDUNGLIS
HANDTOUGHTMEENGLISHLANGUAGEEDITINGCARLAMIJNLIEFSTEDANSVRIENDINCEES
JAARGENOOTENAUTOSTHOMASAAKTEDEKUNSTFOTOSENCHRISHADPLEZIERMETDELAY
OUTTHILLYVRAAGBAAKJEANNETTEHULPELSGEDULDBERTINAORTHOMANAGERWAN
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METFLORENCENENOELSPELENWIJREBORNERONWARMEVRIENDENSTUWEDVERTROU
WENLINDAGEITENOPERERENENEINDELOZEHISTOLOGIEVANGEITENCOUPESPIETTOTH
EPOINTHISTOLOOGENMETHODISCHPREPARATENMAKERYVONNEBALIERUDIHARRYJU
DITHVAKANTIEENKAMERROOSTERGERTISOTISTCPROCKERENKRITISCHREVIEWERLYDIAU
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NADVISEURENESPRESSOLIEFHEBBERMONIQUEESCABLAPASCALMAAKTINLAYSENINCIS
ALEOPBOUWENALSGEENANDERMARIANDRAVERSJOSEWFEPDOFFICERGDLMETALINETA
MARAWENDYHESTERNICOHANSELLYJANNIEANJACEESDEDENWE10GEITENENERKOMEN
ERWEER10AANPETRAGEZELLIGENHARIENSASTITIAANCOGEITENBREIERENLONDENSGAS
THEERNICOLETTEWIJNSCHILDERIJMARIJNSEVEREHYPODONTIAHANNEKEENMARCEL
FRIENDSFORLIFETUSSENDEZUSTERSENDEADMINISTRATIERENIKHEENENWEEROPD5LAU
RENSDRANKENAUTOVRIENDSILSAUCEAUXTRUFFESSASSAGEITENFOTOSENSALADE
MARVICKAMMERMOSERENMANVANHETALTERNATIEFEMILIESIMONESANNEMONDHY
GIENEVERONIQUEOSSENWORSTVANDEHERGONARDMAATJEENDARTELEOPVOLGER
INSCHISISLANDNETTIEELEKTROFIETSTERBOUKENWIBONOGSTEEDSBUURVROUWEN
BUURMANENVRIENDENWILLEMBCROWDINGBEHANDELINGAATKOMENMONIQUEMA
RIANNICOLEBEUGELMEISJESPIETBENTLEYENSETUPSTOINESNELLELEERMEESTEROSTEOTO
MIEENSNURKMRAJANTHESISRUNNINGMATEENMETCMEDESTRIJDERHANSGOLFTECH
NIEKRUUDLEERMEESTERENVRIENDMATHIEUJARONMAARTJE3ZINTUIGENLAURABEUGEL
KIDSMICHAELNIEUWEKAMERGENOOTENGEZELLIGESPRAAKWATERVALHENDRIKCHABLIS
VERGIETENENNIEUWEJONGEVADERNUVANKPNJACQTROTSEOMADIETENDIETENENTAG
BARBARACLAUDIABTWAFAAARGANOLIEWILLEMVAUSTRALIEENBEDAARDPROFESSIO
NEELNIENKEMARIETTEETSGELENCAPSULESMARIJKEVANWELYMAAIKEMIJNTOEVER
LAATATTJEENHARMENJANTINAMIJNDIERBAREBURENANTOINETTEENSIPCOMIJNBEHULP
ZAMETUINMEESTERSSTONECREEKSILOVEYOUFOREVERAHDMAATJEENDEGROTEMAN
JKLEINEMANLUUKENMEISJENOAENMIJNHELEFAMILIEENVRIENDENDIEMIJDELAATSTER
UIMANDERHALFJAARZOWARMHEBBENBEGELEIDDEANGSTSTEPHANIERICKBRILCHRIS
TIESWATCHENBRITARALPHWILLEMWOUTERENJACQUELINEUCRIJAMMERGENOEGOPAF
STANDMETKEESGNATHOANDRIESSTATISTIEKINBIJNAALLEARTIKELENHUGOβTCPBE
SCHRIJVINGSIEGRITANJAMARTHAMIJNALTIJDKLAARSTAANDEHOUVASTINHARLINGEN

ENMIJNALLERLIEFSTEDIEUWKEDEKLEURINMIJNLEVEN