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# SEMI- INVASIVE BONE EVALUATION TECHNIQUES AFTER GUIDED TISSUE REGENERATION IN PATIENTS WITH PERIODONTITIS. THE INFLUENCE OF ORTHODONTICS DURING NEW TISSUE DEVELOPMENT

**PhD thesis**

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## 1. ABBREVIATIONS

BC: bony crest

BD: bottom of the defect

BDX: bovine derived xenograft

C: control

Ca: calcium

CAF: coronally advanced flap

CAL: clinical attachment level

CEJ: cemento- enamel junction

CO: collagen

CR: a coefficient of repeatability

DBBM: deproteinized bovine bone mineral

EDS: Energy Dispersive X-ray Spectroscopy

EMD: enamel matrix derivative

FMBS: full mouth bleeding score

FMPS: full mouth plaque score

G: graft

GR: gingival recession

GTR: guided tissue regeneration

HAP: hydroxyapatite

IC: intrabony component

LoA: Limits of agreement

M-MIST: modified minimally invasive surgical technique

NB: new bone

OCP: octacalcium phosphate

OTM: orthodontic tooth movement

P: phosphates

PPD: pocket probing depth

(PPi): inorganic pyrophosphate

PTM: pathologic tooth migration

SEM: Scanning Electron Microscopy

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SD: standard deviation

T1: Test 1 (tension site)

T2: Test 2 (pressure site)

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## 2. PREAMBLE

I earned a residency status at the Department of Periodontology, Faculty of Dentistry, Semmelweis University after graduation. Once I received my specialization my interest turned to the direction of interdisciplinary treatments. My main focus at that time was the understanding of periodontal regeneration. The first thing which opened my eyes was the seemingly good improvement of the clinical parameters after periodontal surgical regenerative interventions, where a non resorbable graft material was utilized inside the defects. The favorable results of the early healing were not sustainable every time in the long term. The healing process can only be better understood if we thoroughly examine human histological findings. However, in such settings a contradiction was found between the clinical improvement and the histological results.

The further challenge and difficulty was the orthodontic treatment of teeth with periodontal attachment loss. A literature review in this multidisciplinary field led me to the conclusion that there are no clear recommendations regarding treatment strategies. This is supplemented with the fact that there have not been published human histological data on the results of the previously mentioned combined treatment approaches. Thus, I decided 5 years ago to initiate a randomized controlled clinical trial in collaboration with the Department of Pediatric Dentistry and Orthodontics and with the representatives of biomaterial producing companies. In order to gain ample and clear evidence, we should not neglect human histologic evaluation, though we needed to find a less invasive approach for biopsy harvesting to fulfill ethical expectations.

Due to the fact that the pretreatment of the patients before being eligible for selecting them to the study is quite long, together with the prolonged healing and observation period, the duration of the trial spanned several years. After a 3-4 years of data collection and an accepted manuscript in a peer review journal, I submitted my application to the School of PhD Studies Advanced Program. Therefore my PhD thesis is mainly based on the findings of the randomized controlled clinical trial about the multidisciplinary periodontal- orthodontic treatment. Some of the presented results has not been published yet, while the major data are based on the author’s published and cited article in this topic, which contains preliminary data compared to the current one.

These preliminary findings were also summarized in an Oral Presentation during the Research Session of the latest Europerio (2018, Amsterdam). Nevertheless, our published data is considered to be the first human histological evaluation in this comprehensive treatment approach using a novel minimal invasive bone biopsy technique. Our findings were only tested before in preclinical settings, which can ensure the novelty of our study design and prove its clinical importance.

During my long term clinical studies we realized the need for an even less invasive technique, therefore together with my colleagues we intended to find new methodologies to evaluate tissue quality.

A second case series study was designed in order to introduce Raman spectroscopy for a better understanding of true healing processes in a minor chemical compound level. The question was whether Raman spectroscopy could provide enough and reliable data on examined tissues, and is able to detect differences within bone samples obtained from different patients with different periodontal states. The primarily outcome question is if this technique could serve as a possible alternative or a supplementary method next to the gold standard histology.

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## 3. INTRODUCTION

Periodontitis is described as a host-mediated inflammatory process of the periodontal supporting tissues induced by dental plaque accumulation. The formation and maturation of this microbial biofilm initiates gingivitis as a primary causative factor, but the progression to periodontitis is determined by several other factors. Some important dysbiotic ecological changes in the microbiome of the plaque are indispensable for periodontal tissue breakdown. Since the capacity of the innate and adaptive anti-bacterial mechanisms within the gingival sulcus are limited and individually determined, the apical spread of the biofilm bacteria along the root surface is sometimes inevitable [Socransky et al 1998]. However the individuals' genetically determined susceptibility and polymorphisms in immune-inflammatory responses can modify the progression and the severity of the disease. Since it is considered a multifactorial disease, other systemic and local risk factors can aggravate the inflammatory processes (e.g. smoking, uncontrolled diabetes, osteoporosis, immunosuppressive conditions, malnutrition, obesity, age, stress, local plaque retentive factors etc.) [Genco and Borgnakke 2013]. All these above mentioned factors are responsible in the modification of the pathophysiology of the progress, where microbiome induced inflammatory response leads to activation of host-derived proteinases that enable loss of periodontal supportive tissues. Tissue breakdown is characterized by marginal alveolar bone and periodontal ligament fiber loss, apical migration of the epithelial cells. The latter is correlated to the pathological formation of a periodontal pocket, combined with a bleeding upon provocation under inflammatory circumstances [Tonetti et al 2018].

The objectives of periodontal therapy are to arrest the progression of the diseases by eliminating the microbial etiological factors and to control other modifiable risk factors. Cause related therapy can arrest disease progression and maintain a healthy attachment apparatus for a lifetime when subjected to adequate oral-hygiene standards [Axelsson et al 2004]. The ultimate goal of a successful treatment is not just to eliminate the inflammation of the periodontal tissues, but rather to reestablish a pocket free structurally healthy gingival architecture over a preserved or reduced underlying alveolar bone level. A so- called infrabony defect with vertical bone loss impose a

critical and challenging clinical situation as the deepest portion of the defect (pocket) is located below the surrounding crestal bone level. In those cases the infrabony defects are further classified into interdental craters, in which the buccal or lingual bony walls maintained with an approximately same level of interdental bone loss around the adjacent root surfaces; and also into intrabony defects, where the bone loss is more advanced around one of the roots of the adjacent interdental root surfaces. The latter one is always correlated with a hemiseptum formation, which gives a minimum of one surrounding bony wall around the defect [Papapanou and Tonetti 2000, Newman et al 2011]. These type of infrabony lesions can be a candidate for regeneration, whereby the architecture and function of the lost or injured tissues are intended to be completely restored.

The main goal of regenerative periodontal surgery is to improve the prognosis of the teeth by the means of reconstitution of the attachment apparatus, which requires a synchronous formation of all periodontal tissues via osteogenesis, cementogenesis and formation of Sharpey-fibres. Evidenced based dentistry shows, that with the help of a regenerative surgery we are able to predictably eliminate only the infrabony defect (the intraosseous component of a vertical bone loss), and furcation type (class I and II) defects [Reynolds et al 2015].

There are several regenerative surgical techniques described in the literature, which can result satisfactorily probing pocket depth reduction and clinical attachment gain. Regenerative periodontal therapy has approximately 40 years of historical development, where in the first half of these four decades the aims of continuous research was to develop new materials in order to ensure the biological requirements for tissue reconstitution. Meanwhile in the last two decades histological observations [Polimeni et al 2009] as well as clinical trials [Trombelli et al 2010; Cortellini and Tonetti 2011] proved the innate regenerative capacity of the periodontal tissues under optimal circumstances (see below) and after a thorough debridement of the denudated root surface. The formation of a blood clot in an infrabony defect after elimination of causative factors can initiate numerous healing cascades. Growth factors are released from the thrombocytes and leukocytes trapped in the coagulum, which are responsible for the early phases of angiogenesis and newly forming tissues. In order to promote the native healing process of an intraosseous periodontal defect four prerequisites are

indispensable: bold clot integrity, space maintenance, primary wound healing, and promotion of mesenchymal cell originating from the intact periodontal ligament (PDL) space. However, several preclinical [Caton and Zander 1979] and clinical [Listgarten and Rosenberg 1979] studies demonstrated that repair, in particular the formation a long junctional epithelium is more likely to occur after a surgical or non-surgical debridement. The reason why the innate regenerative capacity of the periodontal tissue does not eliminate former defects is, that there are several factors which may cause deterioration of the blood clot, causing a wound failure with a consequential reparative healing pattern. These factors are associated with either the clinical management of the wounds or with the site and patient specific characteristics: open system through the sulcus, poor blood supply especially of the cementum, immobilization of hypermobile tooth and numerous cell sources with different ingrowth ability and speed into former defect. The minimization of these limitations of regeneration and the creation of the above mentioned prerequisites led to the development of tissue engineering materials. However, in the past two decades developments have focused on flap techniques and minimally invasive interventions in order to promote the native healing capacity of the coagulum.

Current evidence from systematic reviews shows clearly that regenerative material shows an acceptable but not outstanding incremental effect over the flap design. Different materials have 0.5-1.7mm additional clinical attachment level (CAL) gain, than an open flap debridement would achieve alone [Trombelli et al 2002; Needleman et al 2005; Esposito et al 2009]. In comparison, if we check in the literature the CAL gain tendency during flap design development, we will see the increasing effectivity of the flaps starting from the modified Widman like access flap, through the papilla preservation techniques to the minimally invasive flap designs [Graziani et al 2012; Ribeiro et al 2011; Cortellini and Tonetti 2011]. These important findings established the tendency toward the flap design development. Thanks to this knowledge, the regenerative strategies and decision trees regarding flap design and material selection are well described in the literature [Cortellini and Tonetti 2015, Cortellini 2012]. Incisions and flap designs should be based on local anatomical factors and infrabony defect configuration. A minimally invasive surgical technique is favorable, if defect morphology allows [Cortellini and Tonetti 2009, Trombelli et al 2012]. Studies showed

that containing intrabony defects handled with a minimally invasive surgical approach can heal without any regenerative material [Cortellini and Tonetti 2009, Cortellini 2012]. Animal studies confirmed this concept, that regeneration is more dependent on biological factors (space provision, blood clot stability, growth factors) than on regenerative materials [Chiu et al 2013]. Nevertheless, in some circumstances minimally invasive surgical techniques do not allow adequate access to the defect, especially if it is a multiple type defect, affecting several teeth. In the presence of a severe (circumdental crater or involves ¾ of the root surface) and deep defect, associated with missing buccal/lingual bony walls, an extended flap has to be also chosen, where more than one interdental sites are to be opened with the help of the papilla preservation technique [Cortellini et al 1995, 1999].

In the literature we can find nice techniques which combine a mesio-distally extended flap with papilla preservation techniques. The coronally advanced flap (CAF) technique in the treatment of vertical bony defects has been already described [Zucchelli and De Sanctis 2008], whereas the buccal flap was designed in a similar manner to the CAF used for the treatment of multiple recession defects in mucogingival plastic surgery [Zucchelli and De Sanctis 2000]. Modern surgical approaches aim not only for the elimination-regeneration of the bony defects, but also tend to achieve better aesthetic outcomes and a more predictable mucogingival situation after surgery. In ideal conditions, gingival recession should not increase as a consequence of the treatment procedure, especially when we are treating the anterior region of the maxilla. The CAF flap design is a considerable option in daily clinical practice, when specialists deal with numerous advanced and extensive vertical bone loss cases, where minimizing gingival recession is one of the principal goals next to tissue regeneration from a biological and clinical perspective. A further advantage of this technique is the split-full-split design in the buccal aspect, which can reduce the risk of an early wound failure and gingiva dehiscence by reducing the tension after flap closure [Burkhardt and Lang 2015].

The protocol for the treatment of these wide and so called non-containing (minimum one, or at the coronal third of the defect rather two missing bony walls) infrabony defects is a previously mentioned extensive flap supplemented with the combination of two regenerative materials. Evidence prefers either the combination of enamel matrix derivatives (EMD) and a graft or the guided tissue regeneration (GTR)

and a graft material [Hägi et al 2014, Cortellini 2012]. Both compositions use a graft material to provide cell scaffolding, to promote space maintenance, to prevent membrane collapse or use as a carrier for biological agents (EMD or growth factors) [Miron et al 2014, Reynolds et al 2003]. There is a wide selection of bone substitute materials according to its origin: autografts, allografts, xenografts and alloplastic materials. Particulate autografts have the best biologic behavior, although the harvesting can increase surgical morbidity, necessitate flap enlargement and adverse events are also described in the literature. Allogenic material can be a good alternative to autologous bone, but the utilization raises some ethical concerns and the acquisition in Europe is still difficult. Alloplastic grafts did not live up to expectations, due to their negative interference with periodontal regeneration [Sculean et al 2015; Reynolds et al 2003]. The most well-documented and examined bone substitute agents are the xenogenic materials, most commonly known as deproteinized bovine bone mineral (DBBM). The behavior of xenogeneic grafting materials is sometimes questionable in intrabony defects. While on one hand, these biomaterials in clinical studies show enhanced outcomes (PPD reduction and CAL gain) [Stavropolous et al 2010; Needleman et al 2005], on the other hand the true healing pattern in these studies remains unknown. Histological studies indicate that a high amount of particles, mainly located at the coronal aspect of the former defect, are sequestered in connective tissue without evidence of major bone formation [Sculean et al 2004]. According to the authors, the evidence of vital bone surrounding biomaterial particles, especially in the proximity of the surrounding original bony wall, is mainly an indication of biocompatibility, rather than a measure of osteoconductivity. Therefore these grafts are not likely to improve the host's innate regenerative capacity, moreover there seems to be a negative correlation between remaining particles and tissue regeneration, which serves as an "osteo-obstructive" phenomenon [Trombelli et al 1999, Wikesjö et al 1992]. It is concluded, once the management of a challenging defects requires the use of graft agents, they should have a porous structure and a resorb property within a few weeks without negatively influencing tissue formation, whilst maintaining structural integrity [Susin and Wikesjö 2013]. Some types of DBBM have extremely good porosity structure to help new bone ingrowth. On the other hand DBBM either with or without collagen content is considered a non- or minimally resorbing material in GTR

and GBR settings [Norton et al 2003, Araújo et al 2008]. The problem arises, if in a GTR approach the coronally located particles present connective tissue encapsulation, it will make the area susceptible for a later periodontal relapse or breakdown in case of not adequate plaque control. This justifies the question, how long can the successful clinical results be maintained.

A further challenge in periodontal therapy is the treatment of teeth presenting pathologic tooth migration (PTM). It occurs when there is a disruption in the forces that maintain the teeth in a normal relation [Brunsvold 2005]. This can result in a tilted, migrated or elongated position of the teeth. Once the periodontal attachment loss is combined with PTM and there is still existing opposing contact, the tooth gets into a secondary traumatic occlusion. It can cause not only functional and aesthetic problem for the patient, but it might have a negative influence on the progression and severity of a periodontal inflammation [Fan and Caton 2018]. The reported prevalence of PTM in periodontally compromised patients was 55% which represents a remarkable need for a comprehensive treatment of these patients [Martinez-Canut et al 1997]. In order to successfully handle these cases, it is necessary to comprehensively eliminate not only the inflammation, but reestablish a posterior bite zone with a proper masticatory function, where the teeth are loaded with physiologic occlusal forces. Treatment of severe PTM, in the case of avoidance of alternative extraction, frequently involves orthodontic therapy supplemented by periodontal surgical, non-surgical therapy and prosthodontic treatment. Some animal and several clinical studies report successful orthodontic treatment under periodontally compromised situations [Ericsson et al 1977, Re et al 2000; Boyd et al 1989]. It can produce very favorable results as long as periodontal inflammation is controlled before, during and after orthodontic tooth movement (OTM) [Mathews and Kokich 1997, Wennström et al 1993]. Periodontal disease control including pocket elimination and an adequate oral hygiene is extremely important, since there is evidence that tooth movement can apically displace plaque, causing possible progression of the inflammation and attachment loss [Ericson et al 1977]. In order to express the same torque on a tooth with a reduced periodontium as on a healthy one, we need to reduce the orthodontic forces due to the compensation of the increased leverage (distance from the attack point of the force until the rotational center)

[Antoun et al 2017]. On average the suggested forces used in these cases range from 5 to 15 grams per tooth [Rabie et al 1998].

Periodontal regeneration of infrabony defects combined with orthodontic tooth movement is still challenging, and weakly documented in the literature. The combined therapy may boost periodontal regeneration through reducing occlusal trauma and bone apposition on tensile side [Ogihara and Wang 2010, Juzanx and Giovannoli 2007]. Bone resorption on the pressure side and bone apposition on the tensile side occurs with every orthodontic treatment. The process and potential benefit of bone remodeling induced by orthodontic forces is correlated with the existence of periodontal ligaments, which may fail to occur when there is no attachment. Animal studies demonstrated that tooth movement after a solely conservative periodontal therapy into surgically created defect can reduce its size by bone apposition on the tensile site, but no changes in the level of connective tissue attachment level with simultaneous apical migration of the epithelium [Cirelli et al 2009; Polson et al 1984].Therefore it is necessary to regenerate periodontal tissues before initiating tooth movement into a bony defect [Reichert et al 2009; Nemcovsky et al 2007].

The application of GTR or its combination with a graft material in association with orthodontic treatment seems to improve periodontal conditions during teeth movement into or from a bony defect [Reichert et al 2009; Diedrich 1996; Diedrich et al 2003, Ogihara and Marks 2006]. There is available data that suggest tooth movement into regenerated areas can be performed without jeopardizing neither the formation nor the level of the new periodontal attachment apparatus [Nemcovsky et al 2007]. We still lack the data and evidence about which bone substitute should be indicated, how these materials are degraded, and on the long-term any side effects might occur due to the material’s breakdown or encapsulation into connective tissue. We also lack histological evidence about whether PDLs are responsible for the morphologic changes that occur in a grafted area. So far, only animal histologic evidence is available and suggests that OTM into a previously grafted area with bovine derived xenograft (BDX) can be performed safely [Araújo et al 2001]. Their finding was, that DBBM particles resorb completely on tensile side and partially on the pressure side (biopsy is in close proximity to the root surface in the direction of the tooth movement). These observations were explained by an enhanced osteoclast activity in the tooth movement

area, which could end up in a remodeling of the graft particle. No sign of resorption was detected where no orthodontic forces were exerted, in addition, according to the author the xenogeneic material was rather detracting the space from the bone marrow.

Mainly human clinical trials investigated the clinical success of the non-autogenous graft materials used with GTR technique, when the regenerative procedure is combined with orthodontic tooth movement. DBBM as well as allograft materials were evaluated in infrabony defects mainly in case series studies [Reichert et al 2009 and 2010, Ghezzi et al 2008, Cardaropoli et al 2006, Maeda et al 2005]. The findings of these multidisciplinary approaches confirmed safe and predictable treatment modalities with improved clinical parameters. Two from these clinical trials proved that following up patients for 12 and 18 months after surgery the results remain stable. According to the literature, the most widely documented grafting agents were the DBBM, however, no human histological examination proved the behavior of it under such clinical settings.

The initiation of OTM after the regenerative surgery has also been investigated in some clinical and preclinical settings. Traditional concepts originally suggest a period of 6 to 9 months of healing after regenerative surgery [Mathews and Kokich 1997], while modern treatment approaches utilize orthodontics immediately or at an early phase following 2 weeks of healing [Cardaropoli et al 2006]. A randomized controlled clinical trial found a statistically significant difference with immediate application of OTM regarding clinical parameters, radiologic bone density, and bone fill [Attia et al 2012]. The same group also demonstrated in an animal histological study that the immediate application of orthodontics showed an increase in the trabecular count and in the total surface area of newly formed bone compared to other groups, with delayed OTM or no orthodontics at all [Attia et al 2012]. Because there are only a few number of trials indicating that immediate orthodontic tooth movement has better effect on periodontal regeneration than the delayed one, more adequately designed randomized controlled clinical trials are necessitated to prove its superiority.

The knowledge acquired from literature addresses an important problem to clinicians, whether BDX material, which under many clinical circumstances cannot be neglected in non-containing infrabony defects, can be used predictably and safely, when the tooth is exposed to orthodontic forces. The initiation of OTM can also interfere with the healing phases occur after a regenerative surgery. Not only the timing of appliance

activation, but the orthodontic forces used in these cases might jeopardize the newly forming tissue integrity and cellular activity. Clinicians are still waiting for evidence-based information to get scientifically proven indications in these multidisciplinary treatment approaches to support them during decision making.

It is mentioned previously in this chapter that the number of human histological studies about this topic is extremely rare. This is not surprising in light of the fact that samplings for histological purposes are quite traumatic for the patients, therefore they are questionable from an ethical point of view. Nevertheless, they are still considered as the highest level of evidence in order to evaluate the surgical success. Clinical medicine is still looking for alternative devices which might substitute histology and can serve similar data about the quality and composition of the tissues. Raman spectroscopy as a sensitive imaging and non-destructive method might be an alternative to histology when it comes to assessing bony quality. This optical method based on laser beam scattering is able to gather information regarding chemical compounds from different tissues and to characterize and differentiate between initial normal bone, initial augmentation material and final augmented bone (a composition of graft material and newly formed bone) [Sfeatcu et al 2015, Corpas Ldos et al 2011].

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## 4. OBJECTIVES AND HYPOTHESIS

The main focus of the present dissertation is based on a prospective, randomized controlled clinical trial, which name is: "*Comparison of the guided tissue regeneration procedure alone and in combination with early orthodontic tooth movement in the treatment of non-containing intrabony periodontal defects."* The aim of our study is to evaluate the healing of a regenerative periodontal surgery, which is supplemented with orthodontics or used alone. We intend to treat so called unfavorable wide and non-containing intrabony defects, where the application of graft materials are necessary for the stabilization of the blood clot. The main interest is to histologically evaluate the behavior of a xenogeneic bone substitute material after a GTR procedure, when the augmented area is exposed to orthodontic forces. This type of healing pattern is compared with a "non-disturbed" healing, when the regenerated area and tooth do not undergo OTM. Histology primarily provides a qualitative description of the type of newly forming tissues and the cellular activity, which can give explanation about the clinically advantageous or detrimental healing outcomes. In addition, we also supplement it with quantitative analysis, therefore a histomorphometry examination is carried out to assess the amount of the developed structures. This is also important in order to reveal if newly formed tissues are similar in proportion and in composition around the orthodontically moved teeth compared to those, which has a normal healing course. Biopsy harvesting is aimed to be performed without sacrificing the tooth due to a "semi- invasive" technique. Throughout the trial, clinical and radiological evaluations are also performed as a routine diagnostic method in order to measure the periodontal improvements and to be able to statistically compare the different groups. Due to the fact that there is a reentry (prerequisite for biopsy) second surgical intervention, we have the opportunity to directly measure bone level changes as well. The latter measures on the examined tooth can be also radiologically detected. This gives us the opportunity to compare the same values between two different methodological pathways. So, among our secondary objectives is also to do a regression analysis between the outcomes of two mentioned diagnostic method.

Our null hypothesis is, that there is no statistically significant difference in terms of clinical parameter changes between the two healing patterns. This means that according

to the standard periodontal parameters, the different groups would show comparable improvements at the end of the study. It is assumed that the histological section might reveal a difference between the two treatment options. If the same phenomenon, which has been proved in a preclinical setting [Araújo et al 2001], might also occur in humans, then orthodontic tooth movement will result a different amount of resorption on the pressure and on the tensile side. Early initiation of orthodontic forces supposed neither to cause any adverse event nor inflammatory processes within the newly forming tissues. Histomorphometry results might show fewer amount of graft particles rate and higher amount of de novo bone formation in those patient, who receive orthodontic appliance after the GTR procedure.

Moreover, it is also intended to further seek for alternative techniques, which can supplement or even substitute the results obtained from histology. The main focus of an alternative evaluation should be a less invasive intervention, which causes less trauma for the patient, but ensures a good reliability and repeatability. Therefore Raman spectroscopy is introduced in a different case series study as a non- invasive technique in bone quality evaluation in the field of dental medicine. It is hypothesized that this optical method can serve data not only about the chemical compounds of the bone, but also reveals the possible occurrence of a mature and crystalline phase of bone indicating a true regeneration process. The further objective of this case series is to detect any potential differences between the bone (both of an original and an augmented one) of patients with or without the history of periodontitis.

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## 5. MATERIALS AND METHODS

### *5.1. PATIENT SELECTION*

In the randomized, controlled clinical trial patients are selected among the patients referred for periodontal treatment to the Department of Periodontology at the Semmelweis University Budapest. The patients need to display at least one vertical bony defect with an intrabony component deeper than four millimeter (≥ 4 mm). The defect has to be a wide (radiological angulation > 25° measured on the standardized parallel periapical X-ray), non-containing defect (1 or maximum 2 bony walls with missing buccal or lingual bony walls) to be included in the study. Furcation class III [Hamp et al 1975] lesions are excluded, since there is no human histologic evidence for periodontal regeneration of these defects [Sanz et al 2015]. Examined study teeth must have suffered a pathologic tooth migration (migrated, elongated, tilted tooth, etc.) or must be located in the dental arch at an incorrect position, which can serve as an indication for orthodontic treatment. If the patient presents more than one eligible defect, only the one with the deepest intrabony component will be included in the study. The reason for one tooth per patient serving data to statistics is to improve the power of the trial by eliminating the risk of result torsion, which might happen if too many teeth per patient were included.

Every candidate has to meet all the inclusion and none of the exclusion criteria detailed below.

Inclusion criteria:

1) Patients' age has to be between 18 and 65.

2) Present periodontitis with well-defined non-containing intrabony defect: intrabony component ≥ 4mm, defect radiological angulation > 25°, 1 or maximum 2 bony walls where either buccal or lingual bony wall is missing. Furcation involved molars are accepted with maximum class I or II lesions [Hamp et al 1975].

3) Pathologic migration (migrated, elongated, tilted tooth, etc.) must be present on the issued tooth, or it must be a candidate for orthodontic treatment due to primary malocclusion.

4) Patients must not be heavy smokers (<5 cigarettes/day).

5) Adequately reduced periodontal inflammation and plaque control levels. Full mouth plaque and bleeding scores (FMPS and FMBS) have to be <20% [O'Leary et al. 1972].

6) The patient has to be able to comply with the study related procedures (i.e. good level of oral hygiene, follow-up procedures).

7) The patient must be able to fully understand the nature of the study and give signed informed consent.

Exclusion criteria:

1) Pregnant women.
2) Participation in another clinical study within 30 days prior to study start.
3) Alcoholism, drug dependency, heavy smoking (>5 cigarettes/day).
4) Known infection with HIV, HBV, or HCV.
5) Patients requiring chemo- or radiotherapy.
6) Previous or current radiotherapy of the head.
7) Uncontrolled or insulin-dependent diabetes mellitus.
8) Clinically relevant osteoporosis or systemic disease affecting bone metabolism.
9) Clinically relevant cardiovascular disease e.g., decompensated cardiac insufficiency, hemodynamically relevant heart valve defects, or myocardial infarction during the last three months.
10) Clinically relevant blood coagulation disorder.
11) Previous or current treatment with systemic corticosteroids (within 2 months prior to screening visit) of more than 5 mg/day prednisone equivalent.
12) Previous or current therapy with bisphosphonates for at least 30 days within the last 12 months before screening visit.

The study protocol was approved by the Regional and Institutional Committee of Scientific and Research Ethics of the University (Nº 90/2015). Signed informed consents are collected from every patient during inclusion assessment, who are willing to participate in the clinical trial.

All candidates go through initial, cause-related periodontal therapy before the starting point of the study. Supragingival scaling, polishing and oral hygiene instructions are given to each individual. Root surface debridement is performed on those teeth's surfaces, where pocket probing depth is higher or equal to 4mm. Six weeks

after the last debridement session the candidate is recalled for a screening visit. Meanwhile periodontal clinical and radiographic assessment is performed by an independent examiner in order to check the eligibility of the patient for inclusion. Full mouth plaque and bleeding scores, and the deepest pocket representing the examined tooth have to be in agreement with the inclusion criteria. During the same visit a preoperative standardized periapical radiograph using long cone paralleling technique on an analog film is made. This serves for the baseline radiological intrabony defect's values, where its morphology requirements can be checked for eligibility. The evaluation of radiological images are carried out by scanning the analog periapical films, than with the assistance of a digital software (ImageJ, Laboratory for Optical and Computation, UW-Madison, USA) and with the help of a calibrated ruler and goniometer the intrabony component depth and defect angulation was recorded. In order to calibrate digital measurement, a manual periodontal probe (UNC-15, Stoma, Tuttlingen, Germany) was placed parallel to an X-ray beam at an unconcerned place next to the tooth, and the millimeter notches located on the probe served as the basis for calibration.

In case of increased tooth mobility (Class II and III cases [Marya 2014]), the study tooth is splinted to adjacent teeth with the help of a temporary composite splinting. The advantage of the temporary splint is its removability, since it is changed at a later phase of the treatment according to the randomization of the patient (see later in chapter 3.4.). If the enrolled patient presents more than one periodontal defect indicated for any type of surgery, then these ones are handled before initiating the study-related interventions. Ergo the tooth selected to be evaluated in the trial remains the last one to be treated, which ensures that in case of OTM, no existing inflamed pocket remains in the mouth.

### *5.2. OUTCOME VARIABLES, MEASURES*

Primary outcome variable is the histological analyses with respect to the bone quality and the quantitative histomorphometrical evaluation of the developed tissues at the place of the previous defect site. Secondary outcome variables are as follows: clinical attachment gain (ΔCAL), percentage of the intrabony fill of the defect, pocket probing depth reduction (ΔPPD), gingival recession changes (ΔGR), and finally bone

level changes specified by bone gain of the defect and crestal bone loss (ΔCEJ-BD and ΔCEJ-BC respectively). In addition an arbitrary bone quality analysis is also performed at the reentry visit (see chapter 3.5.).

Description of clinical, radiological measurements, histological evaluation and other records utilized through the study:

All clinical parameters are recorded at six surfaces of the study and adjacent teeth using the UNC-15 manual periodontal probe, and will be performed by the same calibrated investigator (intra-examiner reliability is added to chapter 3.6.) masked to the main objective of the protocol. UNC-15 probe is calibrated by every millimeter, therefore every parameter is measured as an integer, whereas the recorded value is nearest to the marked millimeter. The baseline and endpoint measures are examined on the day of the regenerative surgery (pre- or intraoperatively) and at the time of the reentry (right before or during the procedure), respectively. Between the two surgical interventions 9 months must elapse.

The following clinical variables are recorded at baseline and at the 9th month's endpoint:

1. *Pocket probing depth* (PPD) measured as the distance from the gingival margin to the bottom of the pocket.

2. *Gingival recession* (GR) measured as the distance from the cemento-enamel junction (CEJ) to the gingival margin.

3. *Clinical attachment level* (CAL) calculated as: PPD + GR (the distance from the CEJ to the bottom of the pocket). (*Figure 1*.)

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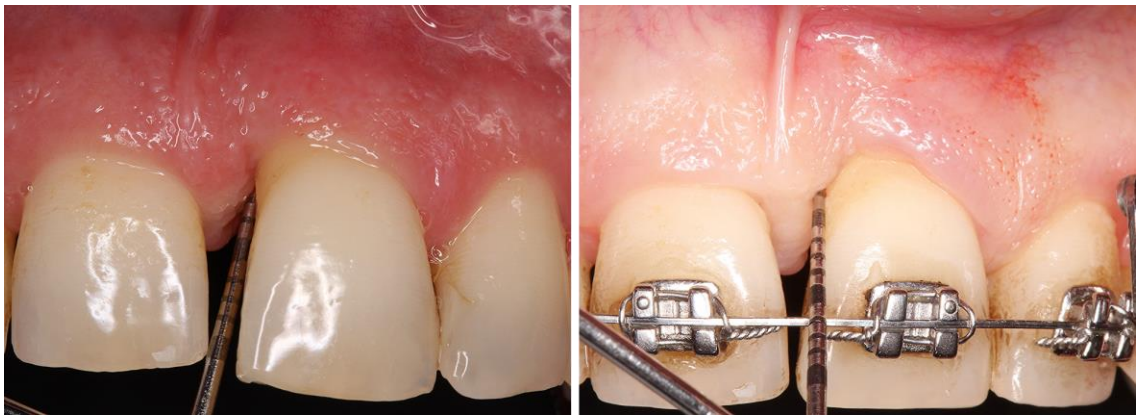

***Figure 1.*** *Baseline (left) and endpoint (right) clinical measure of the same patient. There is a 4mm pocket depth reduction and clinical attachment gain with no change in gingival recession.*

4. *Probing bone level of CEJ-BD* (distance between: cemento-enamel junction and bottom of the intrabony defect) *and CEJ-BC* (distance between: cemento-enamel junction and bony crest. The latter is the most coronally located point of the surrounding still existing bony wall of the defect.) CEJ is regarded here as a fix reference point on the tooth.

5. *Intrabony component* (IC) of the defect: the distance from the BC until the BD. While both of these reference points can vary through the healing period, we need to calculate the intrabony component respective to an independent fix point according to the probing bone levels. Therefore we need to extract the two probing level from each other to get the intraosseous component: (CEJ-BD) – (CEJ-BC), (whereas CEJ: cemento-enamel junction, BD: bottom of the defect, BC: bony crest) [Cortellini et al 1993].

From these variables the changes (Δ) can be calculated by extracting the baseline from the endpoint parameter. The percentage of the *intrabony fill* can be calculated by dividing the change of the intrabony component with the baseline intraosseous component and multiplying it by one hundred. Probing bone level measures are illustrated under *Figure 2.*

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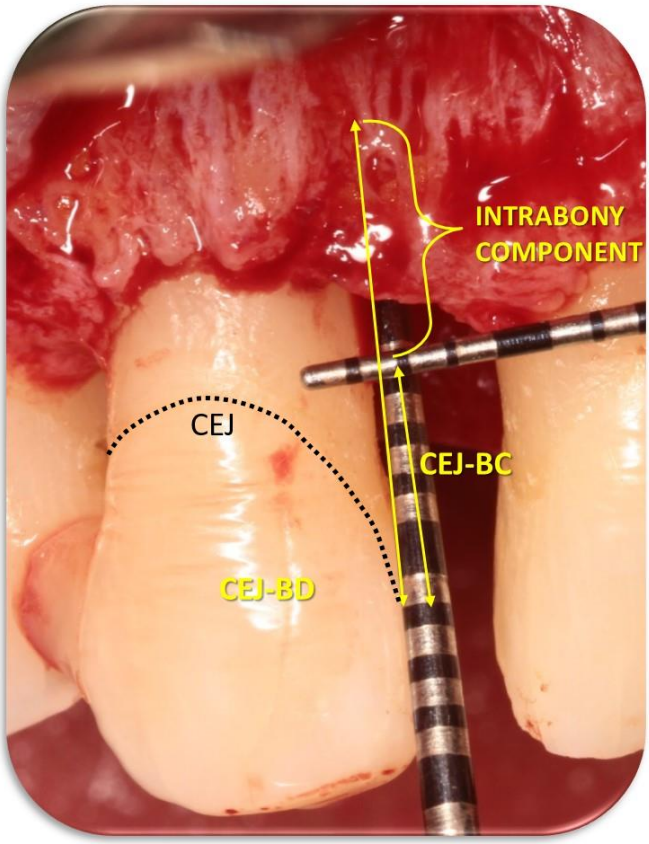


***Figure 2.*** *Probing bone levels of the intraoperative measures and the calculated intrabony component.*

Radiographic examinations are performed with standardized periapical X-rays made with the parallel long-cone technique. In order to use the same position for different time point X-ray recordings, standardization is done with the help of customized radiographic stents. To customize the process a plastic film holder covered by flow silicon material was applied, on which the patient bites during exposition A manual periodontal probe, where the millimeter notches are visible thanks to a grinding marking, has to be positioned next to the tooth. The distance on the probe between two marks serves as an independent distance, which is required for the calibration of the digitalized analog film. Further measurements are done in the digital software mentioned above (ImageJ). The following radiological parameters are recorded at baseline and at endpoint (before reentry procedure):

1. *Intrabony depth* of the defect: measured as the vertical distance from the bone crest to the most apical extension of the defect where the periodontal ligament space was considered to have a normal width [Tonetti et al 1993]. In order to

measure the smallest distance between these two points, we need to use the perpendicular projection of the most coronally located bone crest surrounding the defect to the root surface. This projection is connected with apical extension of the defect resulting in the radiological intrabony component, which is parallel with the root surface (see *Figure 3*.).

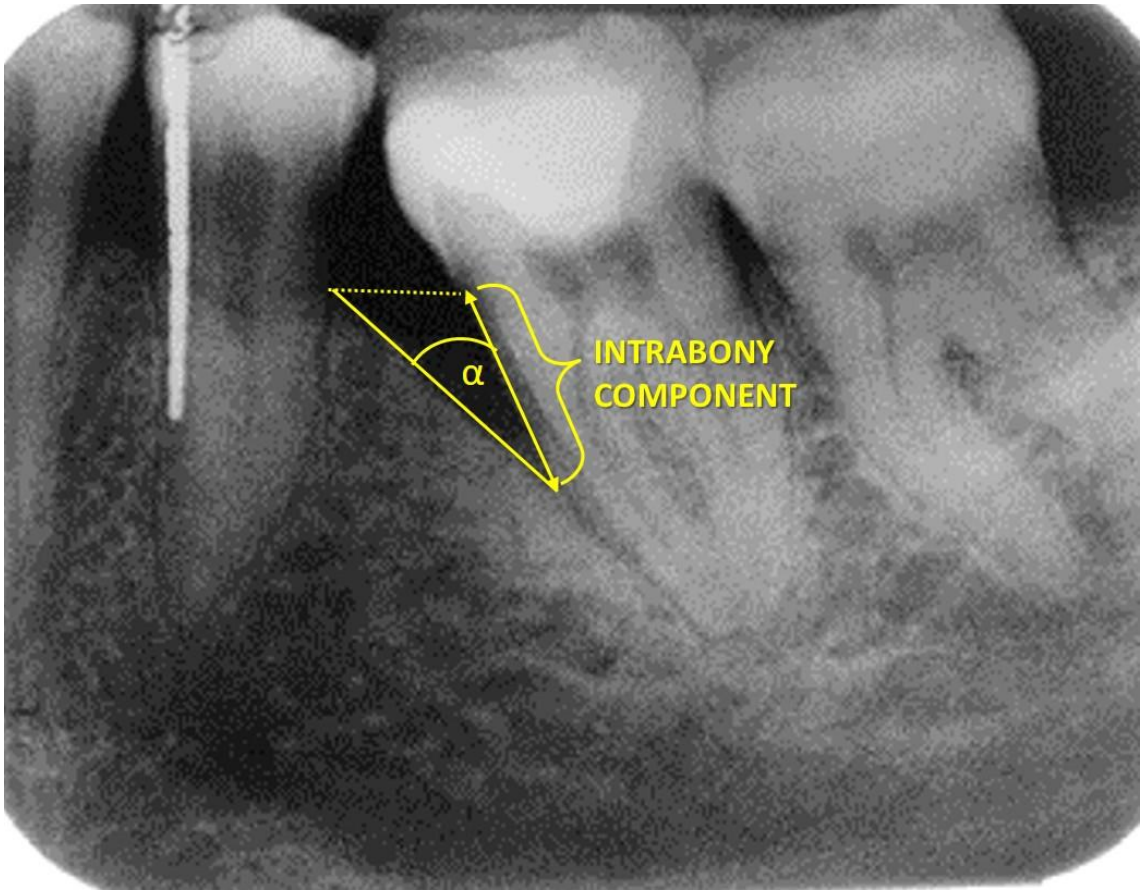


***Figure 3.*** *Intrabony depth and the radiologic angulation (α) of the defect.*

The following parameters will additionally be recorded just at baseline to proof if these meet inclusion criteria:

1. Full Mouth Plaque Score (FMPS): it records the amount of teeth surfaces representing plaque in a dichotomous score (plaque is present/absent), which is divided by all the measured teeth surfaces and multiplied by 100 [O`Leary 1972].

2. Full Mouth Bleeding Score (FMBS): it records the amount of teeth surfaces representing bleeding on probing in a dichotomous score (bleeding is present/absent), which is divided by all the measured teeth surfaces and multiplied by 100 [O`Leary 1972].

3. Radiological angulation (in degrees) of the intrabony defect: determined as the angulation between the root surface and the hemiseptal bony wall of the defect (which is the line connecting the radiological bottom of the defect and the coronal extension of the bone crest located at the adjacent tooth's root surface). (*Figure 3.)*

Following 9 months of healing after the GTR procedure, a reentry surgery is performed. Arbitrary bone quality evaluation and bone sample harvesting for histology is done during this intervention (see chapter 3.5.). Arbitrary bone quality examination means a cautious scraping of the newly formed bone at the previous defect site. The bone is then qualitatively classified based on all or nothing principles:

- +: There are hardly associated DBBM particles to the bone (supposed that embedded just in connective tissue), which are easy to remove with the scraper.
- - : There are no easily removable DBBM particles at the previous defect site.

This is intended to be done in order to possibly find any correlation between the crestal integration of the graft material and the postoperative healing pattern (with or without OTM) to further support histological findings.

During the healing period adverse events are noted: membrane exposure due to gingival dehiscence, root resorption due to excessive OTM or inflammatory process [Killiany 1999], lack of orthodontic tooth movement, etc.

### *5.3. SURGICAL PROCEDURE AND RANDOMIZATION*

Before surgical procedure two acrylic stents are made on the occlusal or incisal surface of the study tooth from a composite material. One stent serves as a base to connect the periodontal probe during surgery with a flowable composite, while the other is responsible for holding a microtrephine. Both are necessary in order to standardize the intraoperative measurements and for the correct position of the later biopsy. Both instruments have to point toward the bottom of the defect while fixing them with the flow composite to the stents. Every patient's stents are stored after the first surgery until the reentry visit.

According to the author's opinion, the coronally advanced flap design, described by Zucchelli and De Sanctis, is one of the best choices in the treatment of unfavorable vertical bony defects. The extended flap is preferable not only because of the defect

morphology, but because of the larger surgical accessibility due to the membrane handling according to the rules of the GTR technique. The further advantages is of this flap type are described in the introduction (see chapter 1.). Therefore the modified CAF intended to treat infrabony defects are utilized through our clinical trial, whose main steps are described below [Zucchelli and De Sanctis 2008].

Surgeries are performed under local or mandibular block anesthesia (articain 4% with epinephrine 1: 100 000, Ultracain DS Forte, Sanofi-Aventis, Paris, France). An envelope- type flap is elevated at the buccal and palatal/ lingual aspects to gain access to the intrabony defect. The gingival flap was prepared with an intrasuclular incision, while the papilla is dissected with the simplified or modified papilla preservation technique (SPPT or MPPT) [Cortellini et al 1995 and 1999] over the intrabony defect. The papillae mesial and distal to the defect are dissected with submarginal incisions (*Figure 4*.). If there is a multiple type defect affecting neighboring interdental areas, the incisions and the flap extension have to reach the nearest approximal area, where the interdental bone is originally maintained. No vertical releasing incisions are performed not to compromise the blood supply of the flap. Over the defect the palatal flap contains the interdental full thickness soft tissues. In those cases, where interdental bone is maintained (mesial and distal to the defects), the soft tissues remain in place for the anchorage of the sutures (*Figure 5*.). Buccal flap supracrestally at the level of the papillae starts with a partial thickness (surgical papillae), once the flap reaches the alveolar bone, it continues with a full thickness manner. When flap elevation goes beyond the mucoginigival junction, a periosteal incision is performed in the inner aspect of the flap. A blunt dissection is used into the vestibular lining mucosa to eliminate muscle attachment, while the flap's deposition continues with a superficial mucosal partial thickness layer. Therefore the buccal flap design is an extended envelope type one, where the split-full-split thickness design makes it extendible in a coronal position (*Figure 6.*). This is required for a tension free wound closure. The palatal or lingual flap is a full thickness flap, which is extended only until gaining enough access to the defect cleaning and handling with the membrane.

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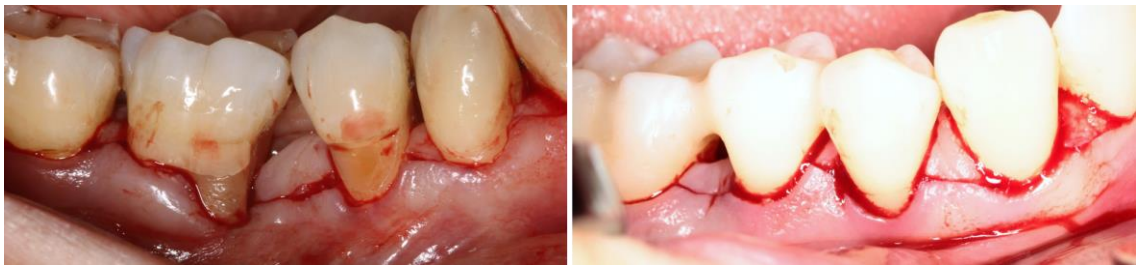

***Figure 4.*** *The incision lines can be inspected in two cases. There are intrasulcular incisions around the teeth, while over the intrabony defects papilla preservation techniques are utilized. The papillae mesial and distal to defect are dissected with submarginal incisions, furthermore no vertical releasing incisions are done.*

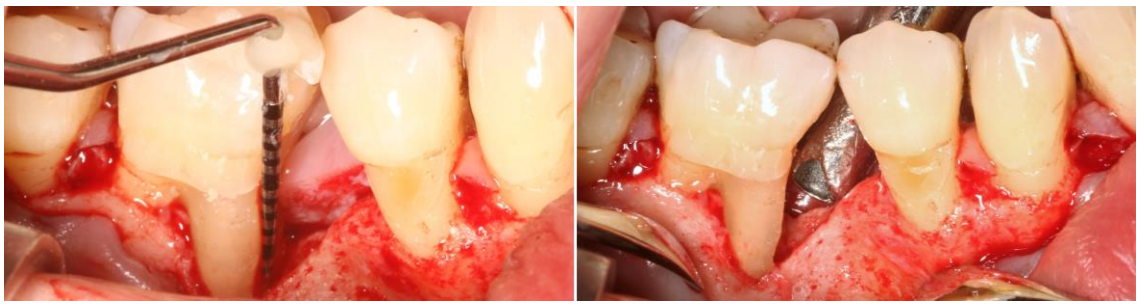

***Figure 5.*** *After papilla elevation the lingual flap contains the full thickness soft tissues over the defect, while mesially and distally the interdental soft tissues remain in place to anchor the sutures.*

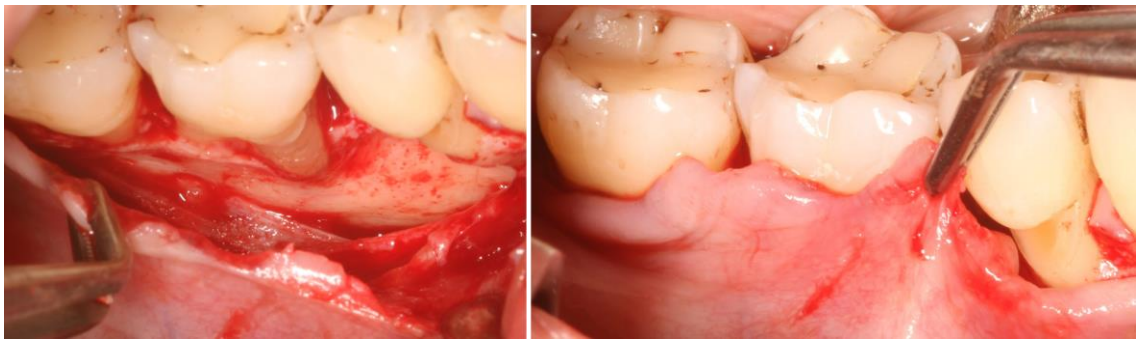

***Figure 6.*** *Flap design is an extended envelope type, where the buccal is a coronally advanced one (split-full-split thickness), and the oral is a repositioned full thickness flap.*

A thorough granulation tissue removal from the defect, scaling and root planing of the exposed and contaminated root surfaces follow the flap elevation. Once the defect

and the root surface are considered to be clean, intraoperative standardized measures are done (*Figure 7.*) with the help of two UNC-15 probes (the vertically positioned probe is fixed to one of the stents). The other prefabricated stent is utilized to position the trephine facing the deepest point of the intraosseous defect (*Figure 8.*). The trephine together with the stents are sterilized and stored until the reentry session.

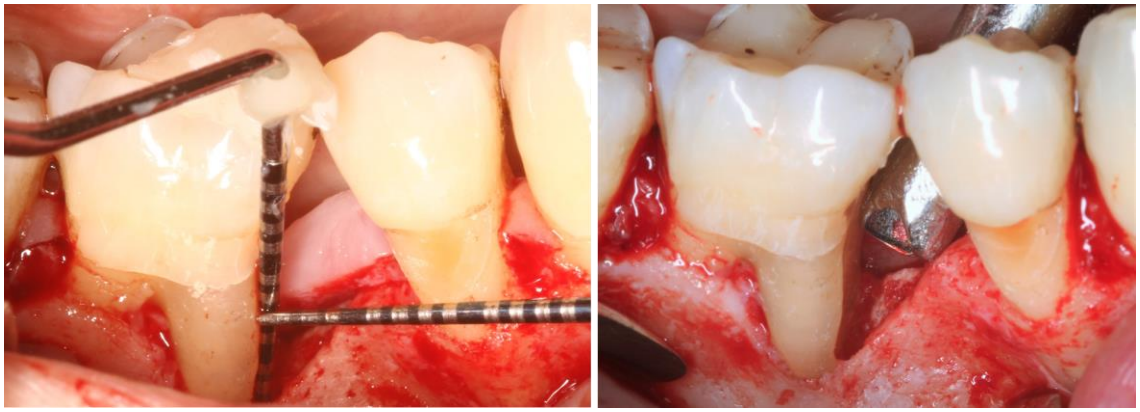

***Figure 7.*** *One the left site picture the intraoperative probing bone measures can be visible with a 7 mm intrabony component. Right site represents a cleaned root surface and a mainly one-wall periodontal defect.*

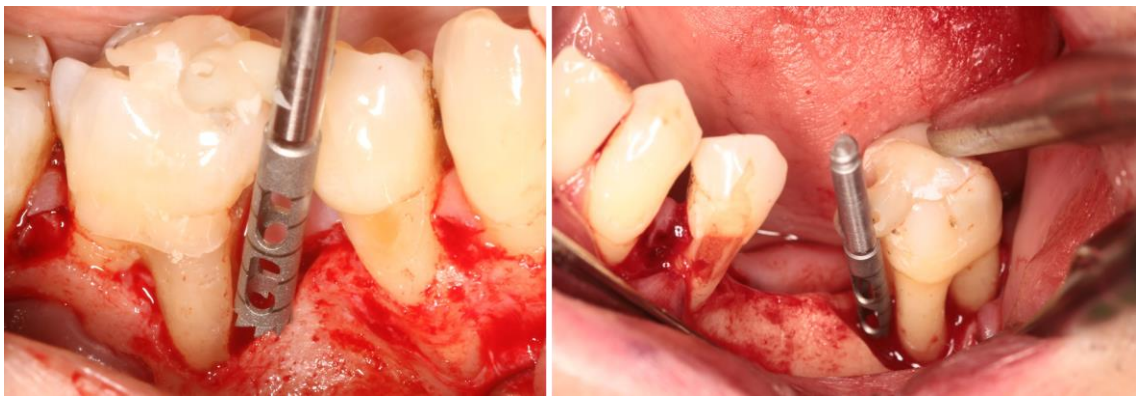

***Figure 8.*** *The trephine is attached to the acrylic stent in order to standardize the place of the later biopsy in two consecutive cases.*

Regenerative strategy is the guided tissue regeneration technique, where we cover the defect with a resorbable bilayer native collagen membrane (Geistlich Bio-Gide®, Wolhusen, Switzerland). Membranes are trimmed according to the extension and location of the defects (*Figure 10.*). In order to better stabilize the membrane, a double layer technique is used in case of missing buccal bony walls (*Figure 11.*). Since

collagen membranes are flexible with improper space maintenance property, the defect is filled with lightly packed xenogeneic DBBM (Geistlich Bio-Oss®, Wolhusen, Switzerland) in order to prevent the collapse of the membrane (*Figure 9.*).

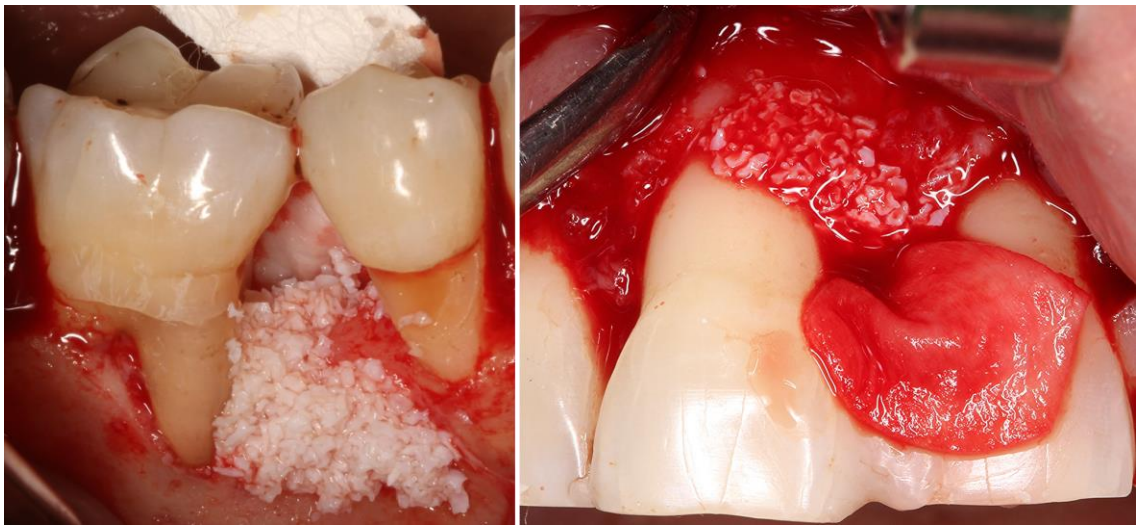

***Figure 9.*** *Filling the intrabony defects with lightly packed DBBM particles. Note that root surface almost outside of the bony envelope is not covered with biomaterial due to the bad regenerative capacity (left site). On the right site the denudated root surface is inside the alveolar bony housing, therefore covered by particles to mimic the original architecture of the alveolus. The collagen membrane is already adapted from the lingual aspect to prevent the efflux of the grafting agent.*

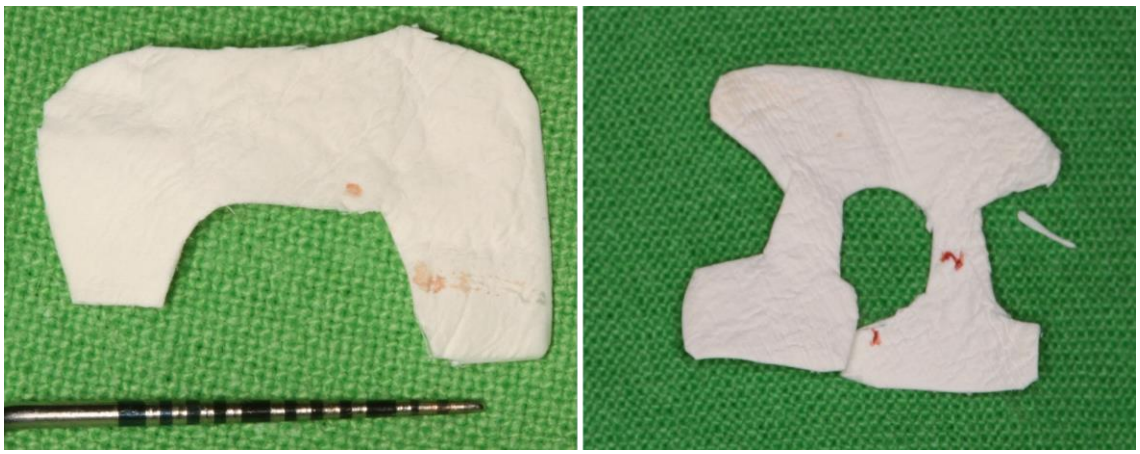

***Figure 10.*** *Resorbable bilayer native collagen membranes are trimmed to proper shape and size in order to cover the defects according to the principles of the guided tissue regeneration.*

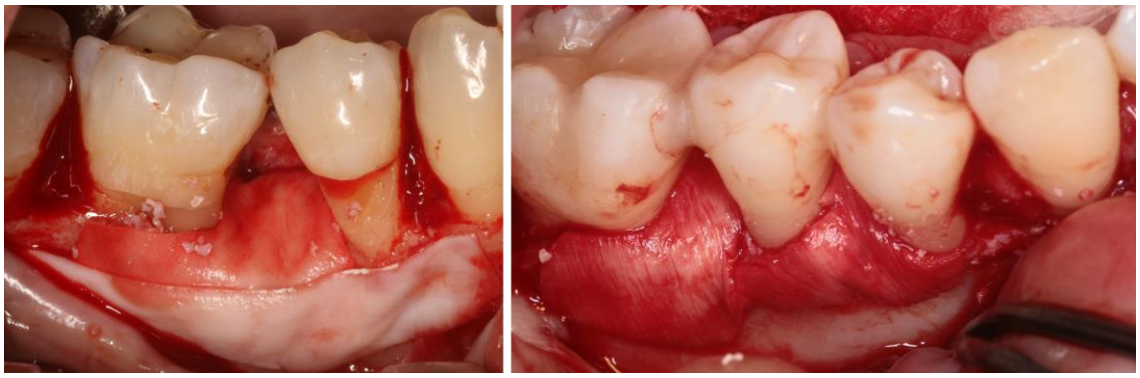

***Figure 11.*** *Defects after grafting are covered with the preshaped collagen membranes.*

The remaining untouched facial portion of the papillae (anatomical papillae) are desepithelialized in order to gain a well vascularized connective tissue bed for the surgical papillae, which are coronally advanced and secured on top of the anatomical ones with the help of sutures. Over the defect, double internal mattress sutures are applied. The first apical layer is a horizontal internal mattress suture connecting the oral flap with the buccal periosteum (*Figure 12.*). This further helps to stabilize and fix the collagen membrane. The second coronal layer is a modified vertical mattress suture. Neighboring interdental areas are closed by sling circumdental sutures, which play an important role in the coronal advancement of the flap (*Figure 13.*). The first layer of suturing material is a 5-0, while the second one is a 6-0 non-absorbable monofilament (Dafilon, B.Braun, Melsungen, Germany).

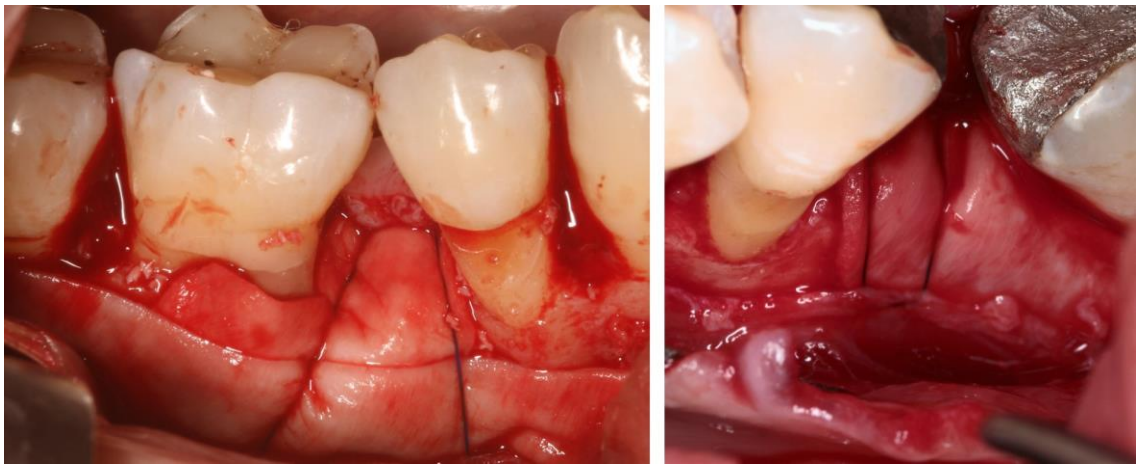

***Figure 12.*** *The most apical layer of suture is a horizontal mattress connecting the lingual flap with the buccal periosteum. It also plays a role in the fixation of the membrane.*

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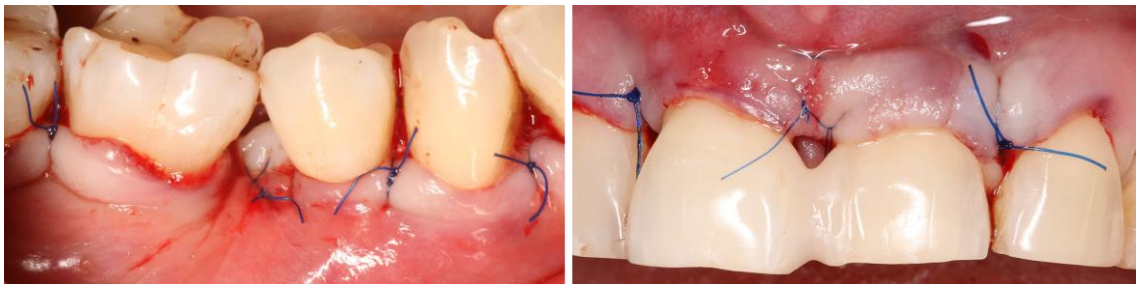

***Figure 13.*** *Coronal advancement of the buccal flaps with the help of the superficial sutures. Primary tension free wound closures are achieved.*

In order to standardize the procedure, all surgeries are performed by the same periodontist, who is the author of this PhD thesis.

The randomization of the individuals is done only after the regenerative surgical procedure in order to avoid possible bias regarding the quality of the surgical intervention. A sealed and labelled envelope is selected by the patients from a basket, which determines their allocation into one of the following groups:

**A. Control:**

Patients in control group (C) do not get orthodontic appliance for the healing and survey period (until 9 months). The composite temporary splints remain in place until the reentry procedure, or in case of a debonding they are changed and strengthened to an extracoronally anchored fiber reinforced composite splint. If the patient is willing to continue with orthodontic treatment, then it can be done only after the reentry procedure is accomplished.

**B. Test:**

If the patient is allocated in the test group (T), early (7 days postoperatively) orthodontic tooth movement is performed at least on the study tooth, or on the entire arch. Subjects within the test group are further divided into two subgroups regarding the direction of tooth movement related to the location of the defect. If tooth movement is directed away from the previous defect (T1), it will possibly exert tensile force to the healing site. Once the tooth is moved toward the periodontal defect (T2) applying pressure forces to healing area, it is considered another biological phenomenon. The

continuous and low level of orthodontic forces are mandatory, and controlled by an orthodontist (see the next chapter).

### *5.4. POSTOPERATIVE PROTOCOL*

Subjects receive antibiotic (amoxicillin and clavulanic acid) therapy (625mg thrice a day) for 7 days, supplemented with non-steroid pain killers (diclofenac 50mg) until the pain is gone. Local chemoprophylaxis (0.2% chlorhexidine including hyaluronic acid, Curasept ADS® Implant, Curaden AG, Kriens, Switzerland) will be given once a day for 2 weeks. Sutures are removed 14 days after surgery. Mechanical tooth brushing at surgical site is forbidden until 2 weeks. At the 3rd week tooth brushing according to the modified Stillman's method is allowed with a soft surgical toothbrush for an additional 4 weeks [Bergenholtz et al 1984]. Interdental tooth cleaning is also skipped over the regenerated area for 6 weeks. Patients are recalled for professional cleaning at the 1st, 2nd, 4th and 6th weeks, then restitution of the original at home oral hygiene regimen is recommended.

In the test group a multibond orthodontic appliance is inserted by an orthodontist 7 days after surgery (*Figure 14.*). The type of the appliance cannot be the same with every candidate, due to the different tooth movement modalities, however there is a routine wire sequence, which can be used in most of the cases. Gentle OTM is performed with 0.014 round NiTi wire, with 0.016 x 0.016 stainless steel overlay wire fixing neighboring teeth, in order to prevent their movement. Usually after one month the archwire can be changed to a 0.016 NiTi type one. Around the 3rd month a 0.016 x 0.022 Niti follows the sequence, which is continued generally in the 4-5th month with a 0.020 x 0.020-as Bioforce type wire (GAC Dentsply Sirona, York, USA). Appliance adjustments in combination with professional periodontal care happen monthly. Treatments in general are finished with different diameters of steel archwires. The estimated time of orthodontic treatment will be a minimum of 9 months or even more. If the OTM finishes before the scheduled reentry visit, then the appliance will remain on the teeth as a retainer, or will be removed right before the reentry procedure.

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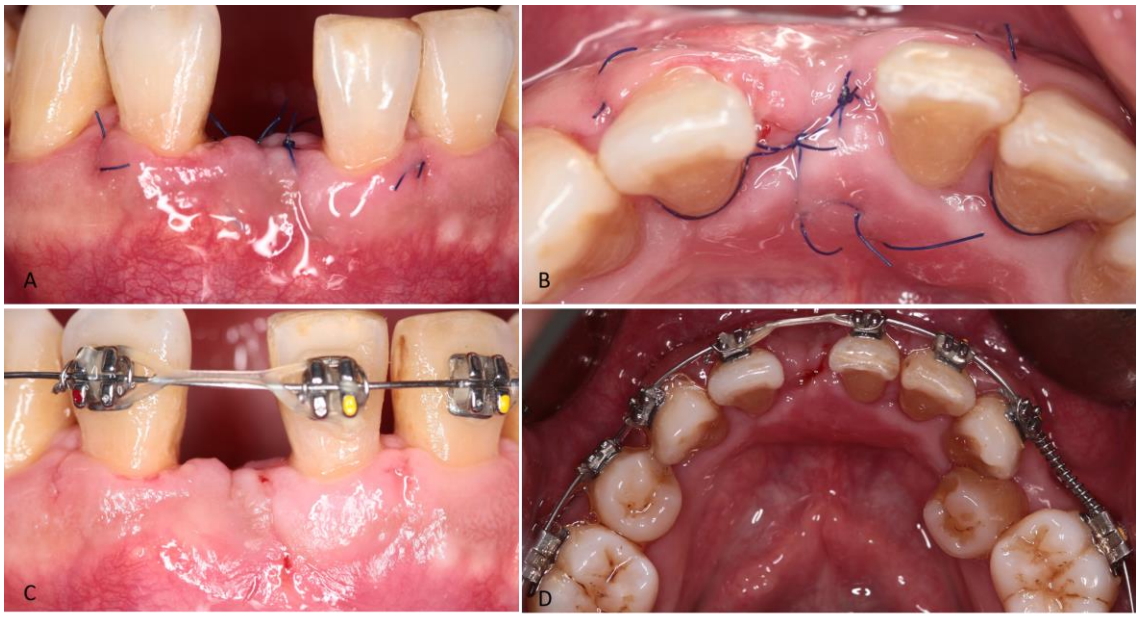


***Figure 14.*** *Image A and B show the one week postsurgical phase of a test subject right before multibond appliance insertion. Picture C and D are the two weeks postoperative conditions after suture removal.*

### *5.5. REENTRY PROTOCOL AND HISTOLOGICAL PROCESSING*

The reentry procedure is scheduled at the 9 months endpoint visit, when the same clinical and intraoperative parameters are measured. Reopening of the former defect is performed under similar local anesthesia, and a minimal invasive full-thickness flap is elevated only at the interdental site (*Figure 15. and 16.*). The flap contains a barely elevated buccal and lingual mucosa, which is very similar to a MIST approach [Cortellini 2012]. Once the bone is visible, an arbitrary measurement serves to find out integration level of the graft material to the surrounding bone. Therefore a cautious scraping of the bone at the previous defect site is performed before the bone sample harvesting. The newly formed bone is classified according to the all or nothing principles (see chapter 3.2.). Furthermore, the possibility of eliminating the remaining residual pockets during this surgical intervention is also given, which confirms its validity form an ethical point of view.

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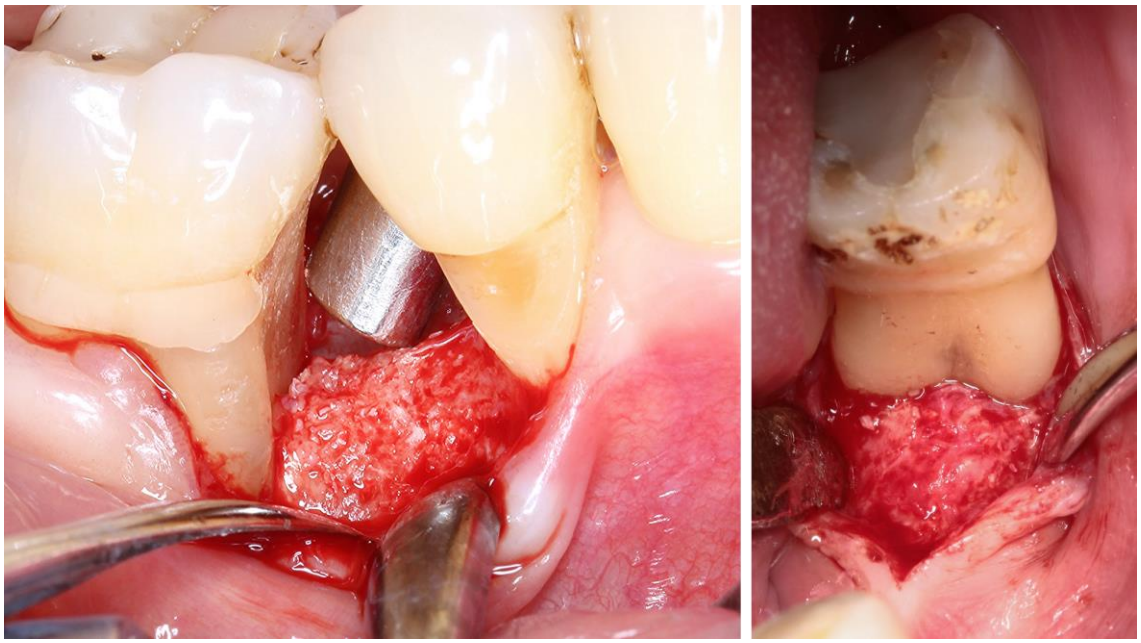

***Figure 15.*** *Minimally invasive flap elevation during reentry procedure with a control (left) and a test (right) patient. It seems that there is less visible DBBM particle on the surface of the bone with the latter (test) subject.*

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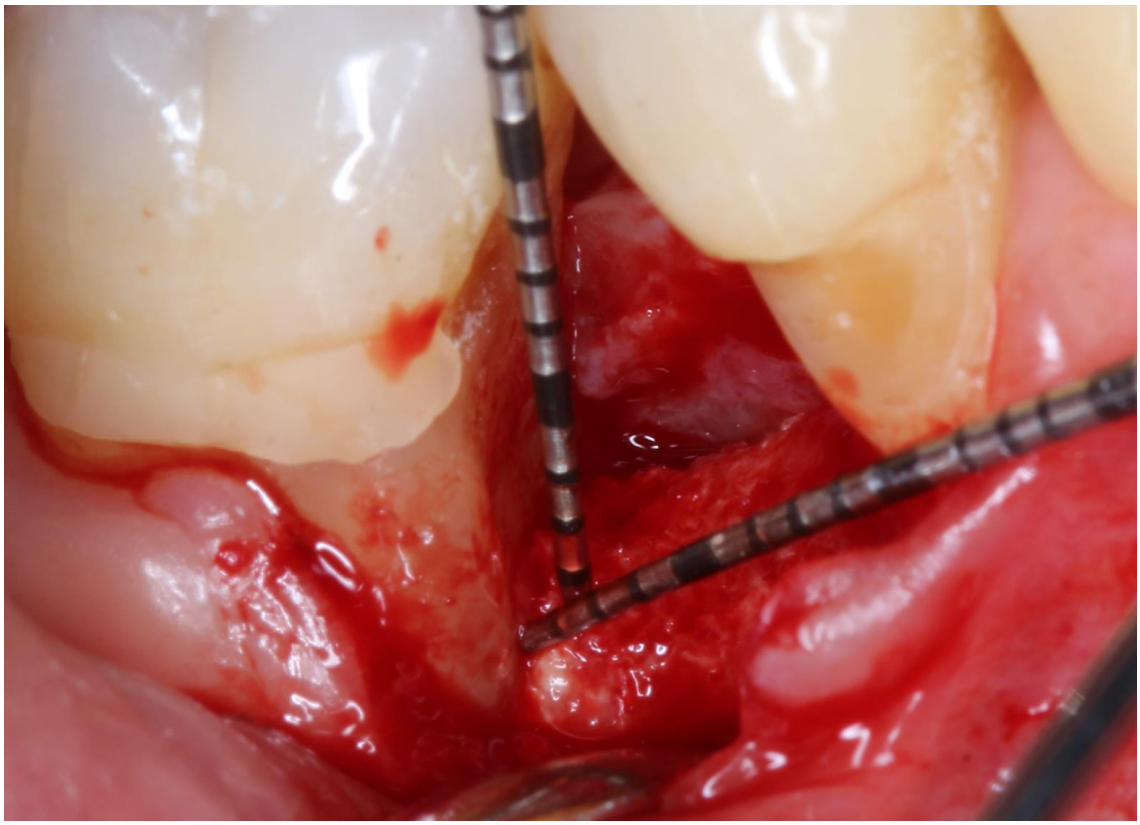

***Figure 16.*** *The intraoperative probing bone level demonstrates a complete bony fill of the former intrabony defect.*

However, the main aim of the reentry is to harvest a piece of bone from the place of the original defect site, representing sample for bone quality and quantity evaluation. Therefore only a microbiopsy is indicated for histological analysis, which has to be as close to the root surface as possible, but in avoidance of harming the potentially newly-developed periodontal attachment. This means that mainly due to ethical considerations, the tooth is not removed with a bony block for a full periodontal regeneration assessment. Only a bony sample is harvested with a trephine (internal diameter 2,0mm) (Meisinger, Neuss, Germany) from the previous defect site to evaluate the behavior of the DBBM particles. In order to standardize the correct place of the biopsy, which after a possible tooth movement has to be at the same site of the former intrabony defect, a stent is fabricated on the study tooth prior to first surgery. This stent, made of an acrylic agent, can be snapped on the tooth and the micro trephine can be attached to it during the regenerative surgery. The trephine has to look toward the bottom of the intrabony defect (*Figure 17.*). Every attempt is made to position the trephine's outer rim

approximately 0.5 mm in distance from the root surface (*Figure 18.*) [Nagy et al 2019]. This approach can be considered as a “semi- invasive” harvesting technique compared to the block segment removal of tooth used in most human periodontal histological studies. The stents together with the fixed trephines are stored and used once again at the reentry. The biopsy is performed under copious irrigation with a rotation speed of 1000 rpm. The depth of the drilling determining the length of the core is crucial, because it should not contain any bones from the still-intact apical periodontal structures. Therefore, the drilling depth is specified after calculating the bony fill of the defect (baseline – endpoint intrabony component). Bony cores have to remain inside of the trephine, so every harvesting procedure is done with a new device. From an ethical point of view, this microbiopsy is not considered to cause an irreversible destruction to the tissues; namely, it causes a narrow defect surrounded by bony walls, which is considered to have innate regenerative potential.

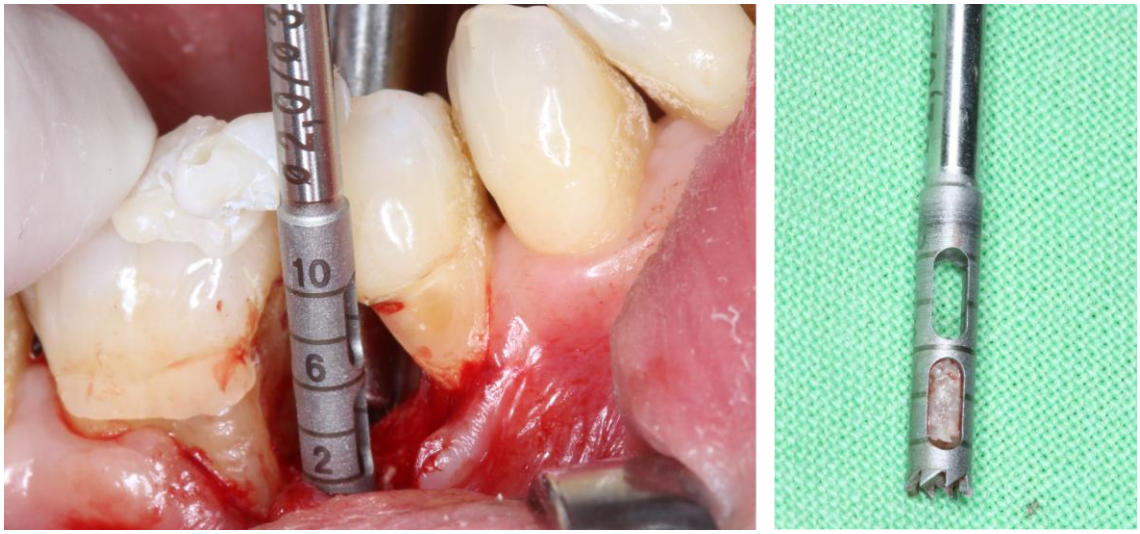


***Figure 17.*** *Bone core harvesting with the trephine. Until the drilling is not initiated, the acrylic stent dictates the correct position of the biopsy. Bony sample should stay within the cavity of the trephine.*

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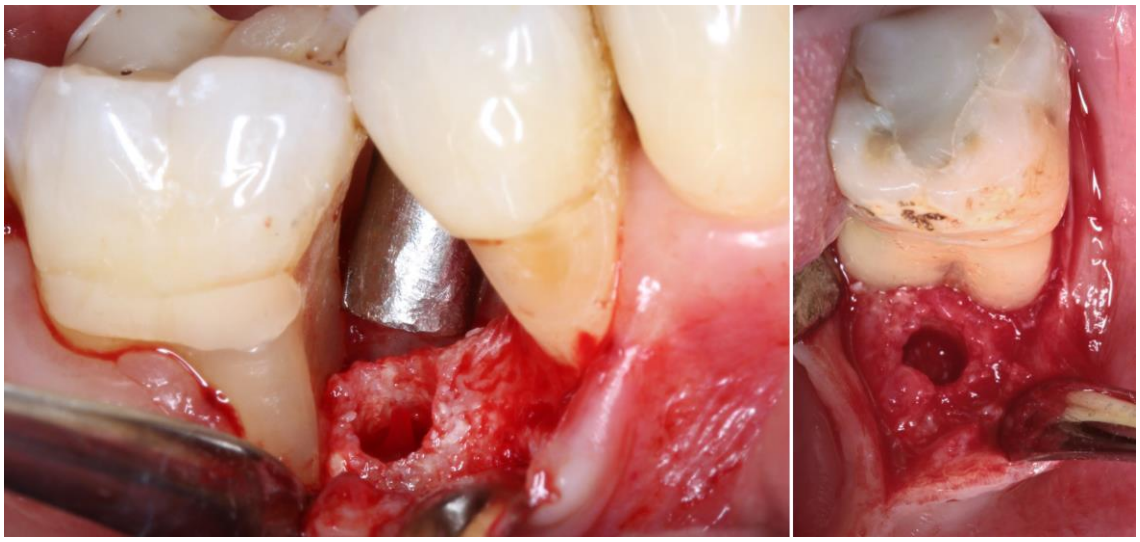

***Figure 18.*** *The site of the biopsies in case of the two patients showed under Figure 15. Every attempt is made in order not to harm the potentially developed new attachment.*

Bony samples are sealed in a vial filled with 10% buffered formalin (pH 7-7.5) and kept there for 48 hours at room temperature. The volume of fixative is important. There should be at least a 10:1 ratio of fixative to tissue. After 2 days the trephines holding the samples are washed in running tap water for 2 hours to remove the formalin solution, then wrapped the in a piece of gauze and placed in a new vial containing 70% ethanol. The vials are further sealed with a flexible and highly waterproof sheet material (Parafilm®, Sigma-Aldrich, Saint Louis, USA) to make sure that it is absolutely tight, and are labelled according to the laboratory's (Schupbach Ltd, Service and Research Laboratory, Thalwil, Switzerland) instructions. Vials are packed in boxes and sent by courier service to the mentioned lab facility. Samples are there further dehydrated in ascending ethanol series and embedded in a methacrylate- based resin medium (Technovit 7200 VLC, Kulzer, Hanau, Germany) according to standard procedures. 60 micron–thick ground sections are prepared through the center of the trephine [Donath and Breuner 1982], stained with rapid bone stain (Sanderson's RBS, Dorn and Hart Microedge, Loxley, USA) and counterstained with acid fuchsine (Sigma-Aldrich, Saint Louis, USA).

### *3.6. STATISTICAL ANALYSES*

The independent examiner is tested for intra-examiner reliability according to the agreement between different time point full mouth clinical measures (PPD and GR) in the same individual. Unweighted kappa score statistics revealed for PPD and GR: 0.79 and 0.88 respectively, which is considered “very good” [Regier et al 2012].

After a prior sample size calculation we need to enroll a minimum of 18 patients per group to reach a study power of a minimum 80% (probability of Type I error $\alpha = 0.05$). (Within group standard deviation /expected background standard deviation/ = 0.58, based on data from a previous study involving similar protocol [Cardaropoli et al 2006].)

Means and standard deviations are calculated from baseline (regenerative surgery) and endpoint (at 9th month reentry) parameters for statistics. Statistical software (SAS, SAS Institute, USA) is utilized to compare the results. Wilcoxon matched pairs test is used to compare the means of baseline and endpoint parameters (intragroup variables). The changes of the means (from baseline to endpoint) between test and control group (intergroup changes) are analyzed with the Mann- Whitney U test. The latter test is also used to assess baseline homogeneity of the two groups. A regression analysis (Bland-Altman plot) is used to identify the correlation or agreement between the two different methods used to measure the same parameter, which is the intrabony component (intraoperative versus radiographic measures). The comparison of the histomorphometric data between the two groups is assessed with the help of a paired T-test. The level of significance is set to 5% ($P \leq 0.05$) and expressed with 95% confidence intervals in cases of every statistical tests.

### *5.7. RAMAN SPECTROSCOPY METHODS*

The Raman device (BTR111- 785 RAMAN spectrometer, StellarNet, Inc., US) consists of a handpiece, which releases the laser beam ($\lambda$=785 nm, output power P =300 mW and spectral resolution as fine as 5 $cm^{-1}$), detects the reflections and is connected to a computer. The head of the handpiece has to be in a close contact with the examined tissue (*Figure 19.*). The device creates a so called Raman spectrum after a measurement representing the chemical composition of the tissue. In a case series study a total of 8 individuals are selected from the patients seeking care under the Department of Periodontology, Semmelweis University. Half of these subjects are affected by

periodontitis, half of them are considered to be periodontally healthy, but all of them are selected for a maxillary sinus floor augmentation (lateral window technique) on maxillary edentulous areas. The sites serving as a candidate for augmentation are opened by mucoperiosteal flaps, and small samples (initial, original bone) are removed from the bony wall of the sinus floor's prepared lateral window. DBBM (Cerabone®, Botiss, Zossen, Germany) is utilized as a grafting agent during the procedures. After 8 months of healing bony cores (with trephines with a diameter of 2,5mm, Meisinger, Neuss, Germany) were harvested from the former place of the lateral window from the augmented bone (final, regenerated bone) as illustrated on *Figure 19*. Bone specimens harvested before and after the bone augmentation procedure are examined in vitro. In addition to acquired Raman spectra, Energy Dispersive X-ray Spectroscopy (EDS) and Scanning Electron Microscopy (SEM) measures are also obtained to testify and endorse the corresponding Raman results. Harvested bone samples obtained from the 8 patients as the field of interest are examined with all of the above mentioned devices. Each individual signed the informed consent, while the study protocol was approved by the Regional and Institutional Committee of Scientific and Research Ethics of the University (Nº 234/2015).

Before the in vitro investigation the following reference calcium phosphates compounds and its precipitations (*octacalcium phosphate*- OCP, amorphous *hydroxyapatite*- HAP and crystalline HAP from Sigma- Aldrich Chemicals Company, US) together with the used DBBM grafting agent are analyzed with the Raman device in order to identify their specific spectra. Differences in the shape of the spectra and in peak intensity reflect the variation in the chemical components' quantity.

In order to assess the differences between the bone of a periodontitis patient and a healthy control and also between the initial and the regenerated bone, we need to highlight the peaks (Raman shift) of the main bone components. Relevant Raman shifts determined by the chemical compounds (identified in the reference calcium phosphates) are assigned to the following type of components: extensive mineral immature bone (b-type carbonate substituted apatite related to amorphous phase of calcium phosphates), mineral mature bone (crystalline phase of HAP), inorganic pyrophosphate (PPi) and collagen (CO). The materials and methods of this approach is discussed in details in the original article [Gatin et al 2019].

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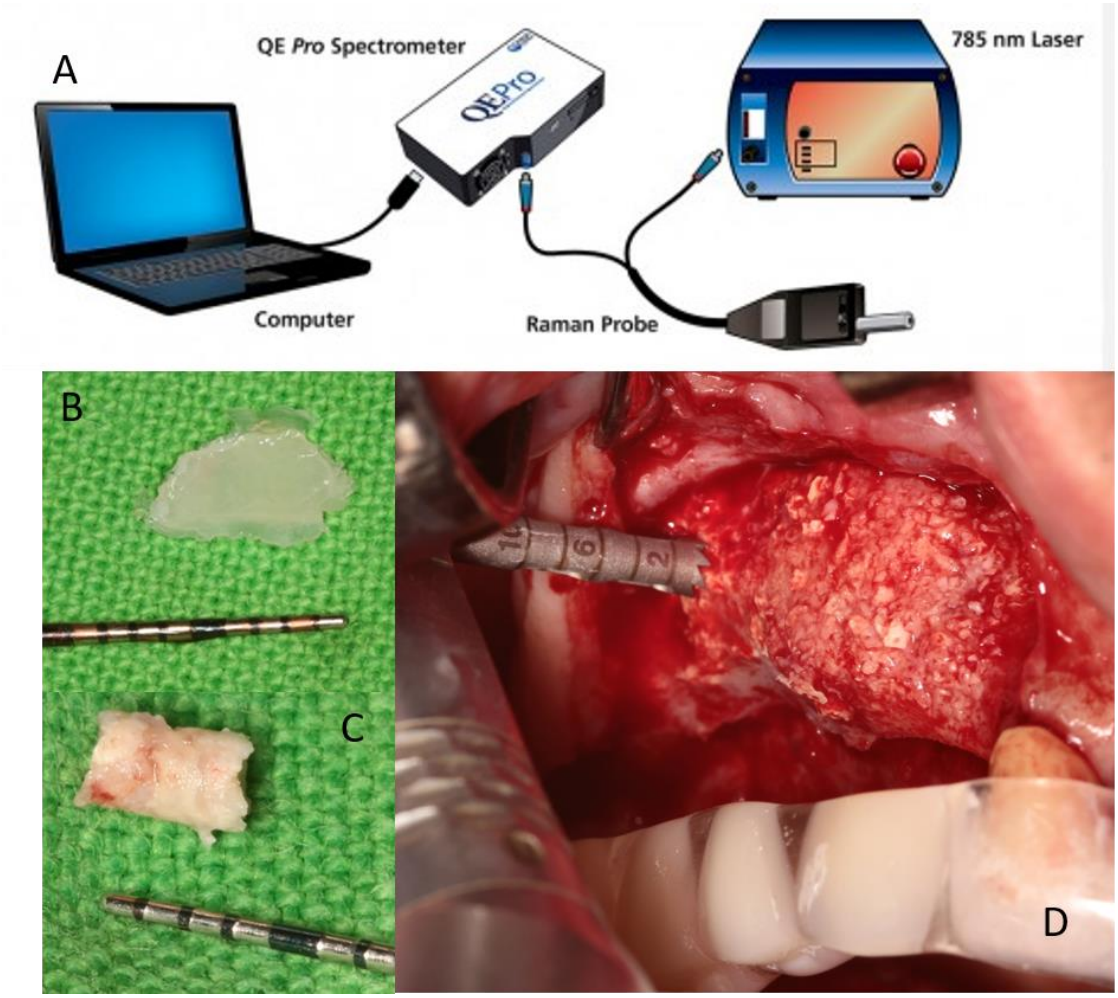


***Figure 19.*** *Picture A depicts the components of a Raman device. On the lower left images (B and C) an initial and an augmented bone after the biopsy are visible, respectively. At last, part D shows the bone core harvesting from the healed site of the augmented area.*

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# 6. RESULTS

## *6.1. OVERVIEW*

There are 27 patients enrolled so far in the present randomized controlled clinical study according to the inclusion criteria. All of these individuals benefited initial cause-related periodontal therapy in the Department of Periodontology of Semmelweis University. After reaching the relative non-inflamed periodontal conditions, every subject successfully went through the guided tissue regeneration surgery in the case of the eligible study tooth. After the GTR approach, the randomization process allocated 14 candidates to the test and 13 to the control group. All patients belonging to the test group received orthodontic appliance 7 or at latest 10 days postoperatively according to the protocol. The observation period of 9 months has elapsed in case of 24 out of the 27 patients, which means, that at the time of the thesis submission 3 patients are still in the healing period. So far, a total of 24 patients (13 in the test and 11 in the control) serve data for statistical analyzes of the parametric data of clinical measures. The author had a limit for histological evaluation due to financial reasons (see acknowledgements), therefore only 20 samples present information about the healing of the bony defects (11 in the test and 9 in the control group). The results of the author are mainly non-published data yet, because only a case series representing preliminary data has been published so far [Nagy et al 2019].

Complications were observed with 5 patients (in the case of 3 control and 2 test subjects) during the healing period. All of these occurred during the early healing phase, whereas a gingiva dehiscence developed interdentally over the membrane at the defect site, where the papilla preservation incision had been utilized (*Figure 20.*). These complications healed with secondary wound healing around the 4$^{th}$ week. Adverse events neither during the later healing period, nor any correlated with the orthodontic treatment were observed.

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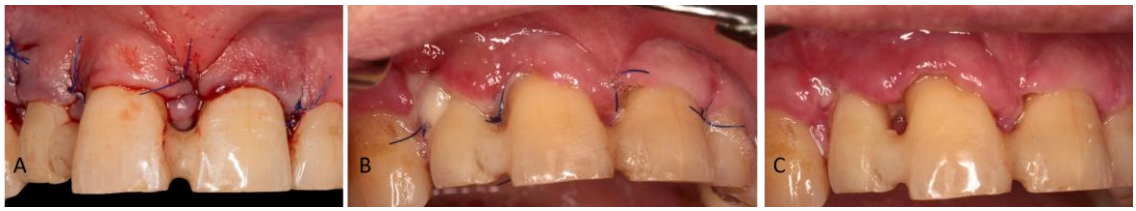


***Figure 20.*** *And early wound failure with a secondary wound healing is presented in case of a control patient. Wound closure, 1 and 2 weeks postoperative conditions are visible on picture A, B and C, respectively.*

During the reentry procedure, a cautious scraping of the crestal part of the former defect was performed according to the described arbitrary bony evaluation. In cases of 11 out of the 24 patients there were easily removable DBBM particles (7 in control and 4 in test group).

### *6.2. CLINICAL PARAMETERS*

The changes of the clinical parameters from baseline until endpoint are the secondary outcome variables. These are expressed by calculating means and standard deviations (mean ± SD). Both test and control groups show statistically significant pocket probing depth reduction (ΔPPD), clinical attachment level gain (ΔCAL) and intrabony component fill according to the Wilcoxon matched pairs test. However, the parameters evaluating the marginal gingiva and the crestal bone levels (GR and CEJ-BC) do not reveal significant deterioration through the observation period (*Diagram I.*). ΔCAL and the change of the intrabony component are -3,3 (± 2,2) mm and -4,9 (± 1,75) mm in test and -5,1 (± 1,8) mm and -5,4 (± ,7) mm in control group, respectively. Expressing the percentage of intrabony fill, there is an improvement of 70% in the test and 67.5% in the control group. The changes of the further clinical parameters can be inspected in *Table II and on Diagram I.*

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***Table I.*** *The changes of the clinical parameters from baseline to endpoint in case of test and control group. On the left side the p values can be observed after the Mann-Whitney U test, which does not reveal any significant difference for all parameters between the two groups (intergroup changes.)*

Significance level: p<0.05

| | P≤ |
|---|---|
| PPD | 0,167 |
| GR | 0,955 |
| CAL | 0,072 |
| CEJ-BD | 0,252 |
| CEJ-BC | 0,459 |
| Intrabony comp. | 0,649 |

Mann- Whitney U test

| | | Groups | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | | | Test | | |
| | | Δ (endpoint - baseline) | | | Δ (endpoint - baseline) | | |
| | | Number | Mean | Standard Deviation | Number | Mean | Standard Deviation |
| Para-meter | ΔPPD | 11 | -5,7 | 2,7 | 13 | -4,2 | 1,5 |
| | ΔGR | 11 | 0,6 | 1,4 | 13 | 0,9 | 2,1 |
| | ΔCAL | 11 | -5,1 | 1,8 | 13 | -3,3 | 2,2 |
| | ΔCEJ-BD | 11 | -4,9 | 2,2 | 13 | -3,7 | 2,0 |
| | ΔCEJ-BC | 11 | 0,45 | 0,7 | 13 | 1,2 | 2,1 |
| | ΔIntrabony component | 11 | -5,4 | 2,3 | 13 | -4,9 | 1,75 |
| Intrabony fill: | | | 67,5% | | | 70% | |

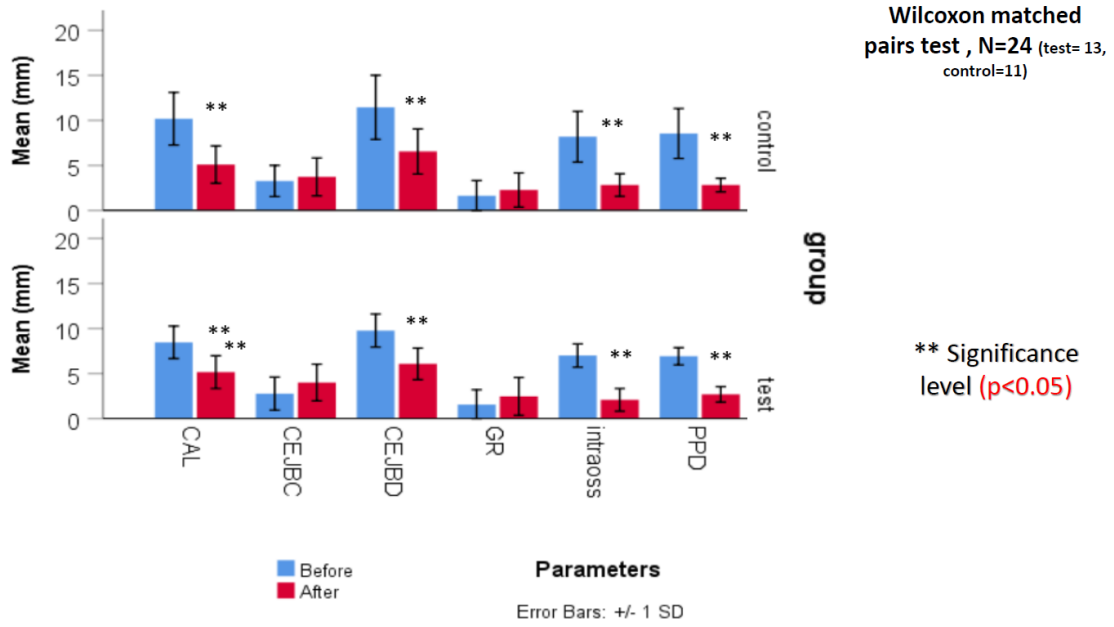


***Diagram I.*** *The intragroup changes of the clinical parameters from baseline until endpoint. Significance level is tested with the Wilcoxon matched pairs test.*

Baseline homogeneity of the two groups is also evaluated, and none of the measured clinical parameters reveal significant difference between test and control according to the Mann-Whitney U test (*Diagram II.*).

Mann- Whitney U test

| | |
|---|---|
| CAL | 0.119 |
| CEJ-BC | 0.392 |
| CEJ-BD | 0.252 |
| GR | 0.910 |
| Intrabony component | 0.459 |
| PPD | 0.150 |

* Significance level (p<0.05)

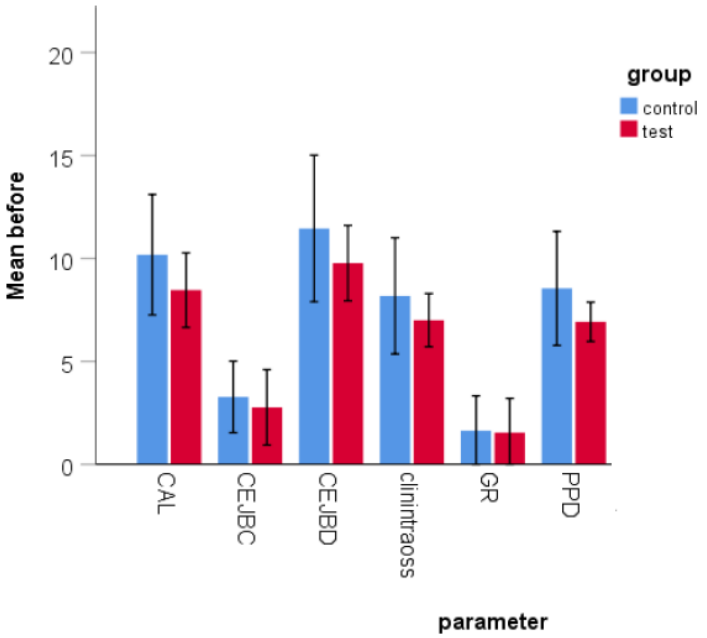


***Diagram II***. *Baseline intergroup homogeneity. None of the measured parameters reach the significance level between the two groups, ergo they are considered to be homogenous.*

The same test is used to analyze the interactions between the groups (intergroup changes). Although the control group demonstrates better improvement in clinical attachment level and intrabony component fill and higher reduction in pocket probing depth, neither variable is considered to be statistically significantly better than the test group's (*Diagram III.*). This ascertainment is also true for ΔGR and ΔCEJ-BC, with an apparent less setback in control group.

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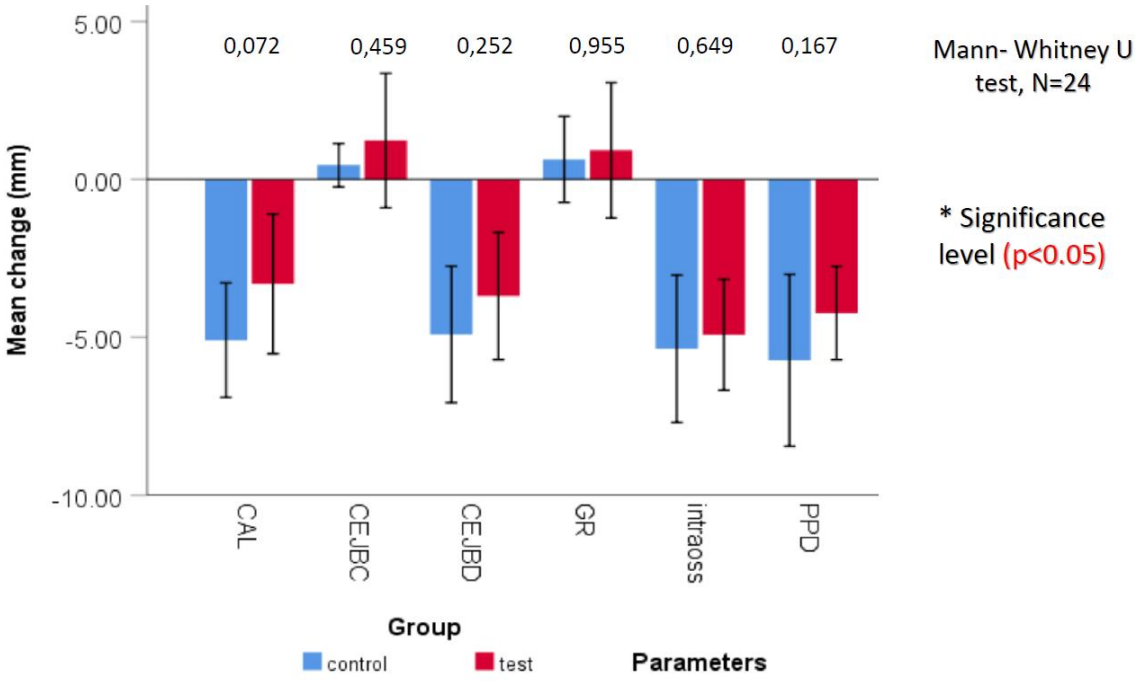


***Diagram III.*** *Intergroup changes. There is no significant difference in any change of the parameters between control and test group. P values are highlighted over the columns.*

According to the Bland- Altman plot the mean difference (in case of extracting the intraoperative from the radiographic parameters) between the two methods is -1.03 mm with a coefficient of repeatability (1.96 X SD) 1.27 mm. With this values the 95% limits of agreement are between 0.24 and -2.30 mm (*Diagram IV.*).

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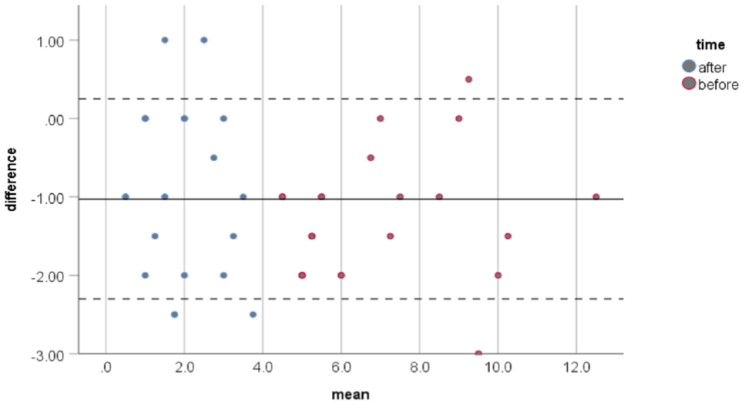
| | |
|---|---|
| Mean difference (bias) | -1.03 mm |
| CR (1.96xSD) | 1.27 mm |
| LoA- | 0.24 mm |
| LoA+ | -2.30 mm |




Bland- Altman plot

***Diagram IV.*** *Regression analyses utilizing the Bland- Altman plot. The radiographic measures under calculate the distances compared to the intraoperative values with a mean difference of 1 mm.*

### *6.3. HISTOLOGICAL ANALYSES*

The primary outcome variables are histological and histomorphometry analyses of the biopsies. Samples are described and grouped according to the different post healing patterns from the defect point of view. Control (C), and test samples with tension and pressure sites (T1 and T2 respectively) are distinguished.

Most samples in the control group show the osteoconductive property of DBBM. Particles serve as a scaffold for new bone formation, which is mainly observed in the apical and middle thirds of the previous defect. The bone substitutes in the coronal third of the samples are rather embedded in connective tissue (*Figure 21. and 22.*). A higher magnification from the middle-apical part of the core discloses favorable framing and interlinking of the bone substitute materials by new bone (*Figure 23.*). Ongoing bone formation is also present in case of a (C) sample, which is characterized by osteoblast cell activity, osteoid production and the development into newly formed woven type bone. It seems that this formation is going to bridge the space between two cross section of de novo bone, evolving a trabecular structure (*Figure 24.*). Histomorphometry results expressed in means (± standard deviation) in control subjects show the presence of 33,2 (± 3,7)% graft particles with a combination of 17,4 (± 9,2)% de novo bone formation, while the remaining part was filled with soft tissue components (49,4 ± 11,4%) measured in the whole core. It is highlighted that the previous percentage ratio of the

different tissue elements represent data about the entire entity or length of the sample. While the distribution of the DBBM particles among the core seems to be consistently proportional, soft tissue elements tend to be more frequent in the coronal third. In 3 cases the bone substitute material displays a connective tissue encapsulation among almost the whole apico-coronal dimension of the core (*Figure 25.*).

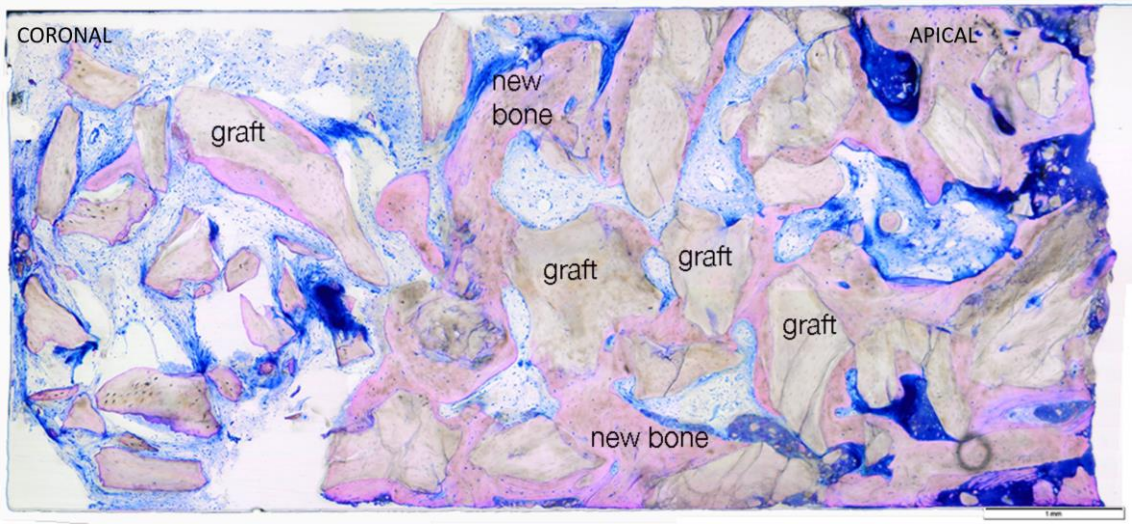



***Figure 21.*** *Core No. 12 of a control patient. Note the encapsulation of the coronally located particles into connective tissue [Nagy et al 2019].*

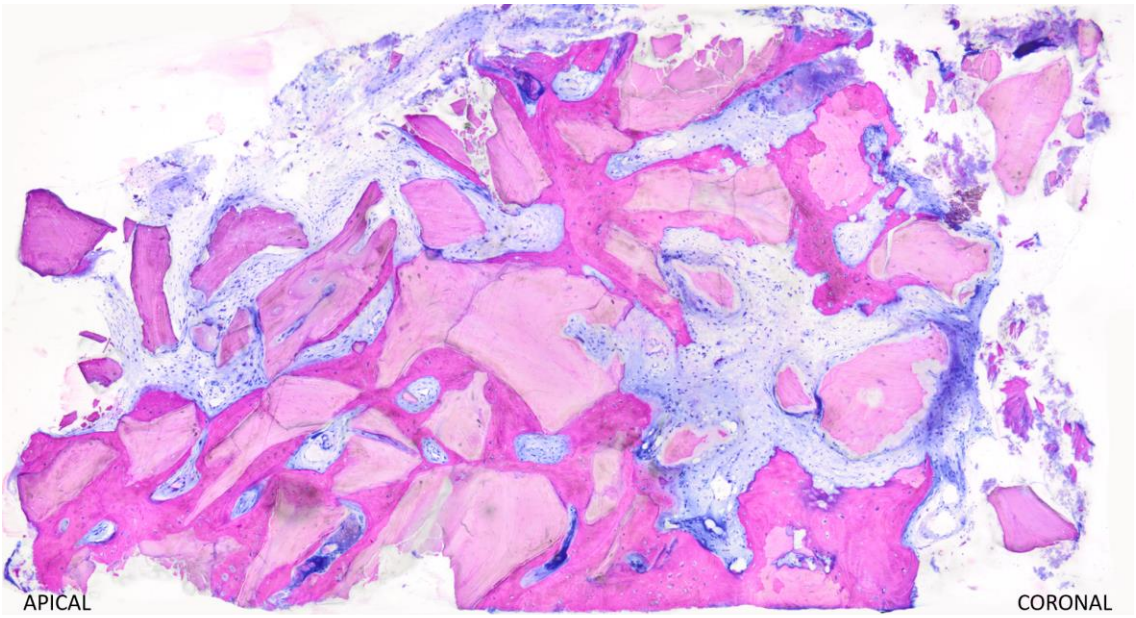



***Figure 22.*** *Sample of another control patient (No. 09) represents the same finding such as No.12.*

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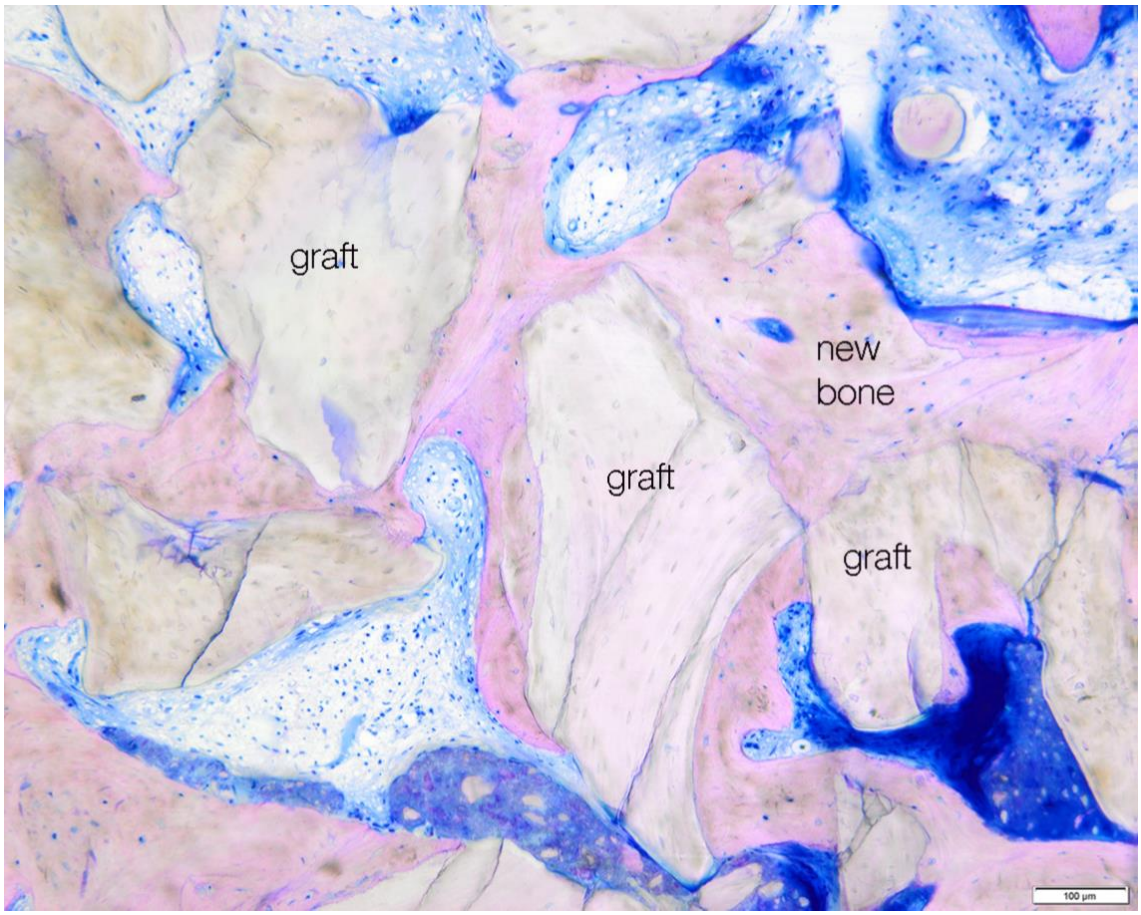


***Figure 23.*** *An enlarged picture from the middle part of core No. 12 expresses nice incorporation of the graft material into newly formed bone [Nagy et al 2019].*

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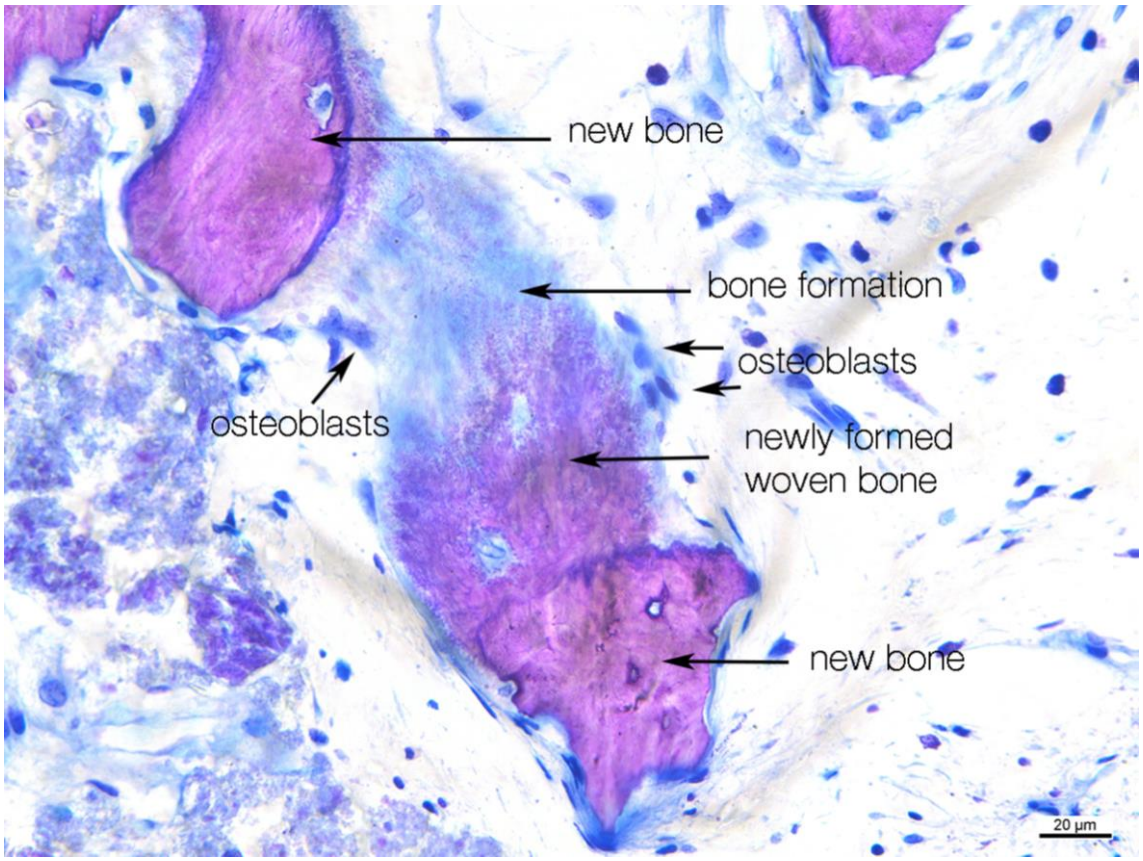


***Figure 24.*** *Ongoing bone formation is observed, characterized by osteoblasts, secretion of osteoid and maturation into woven bone.*

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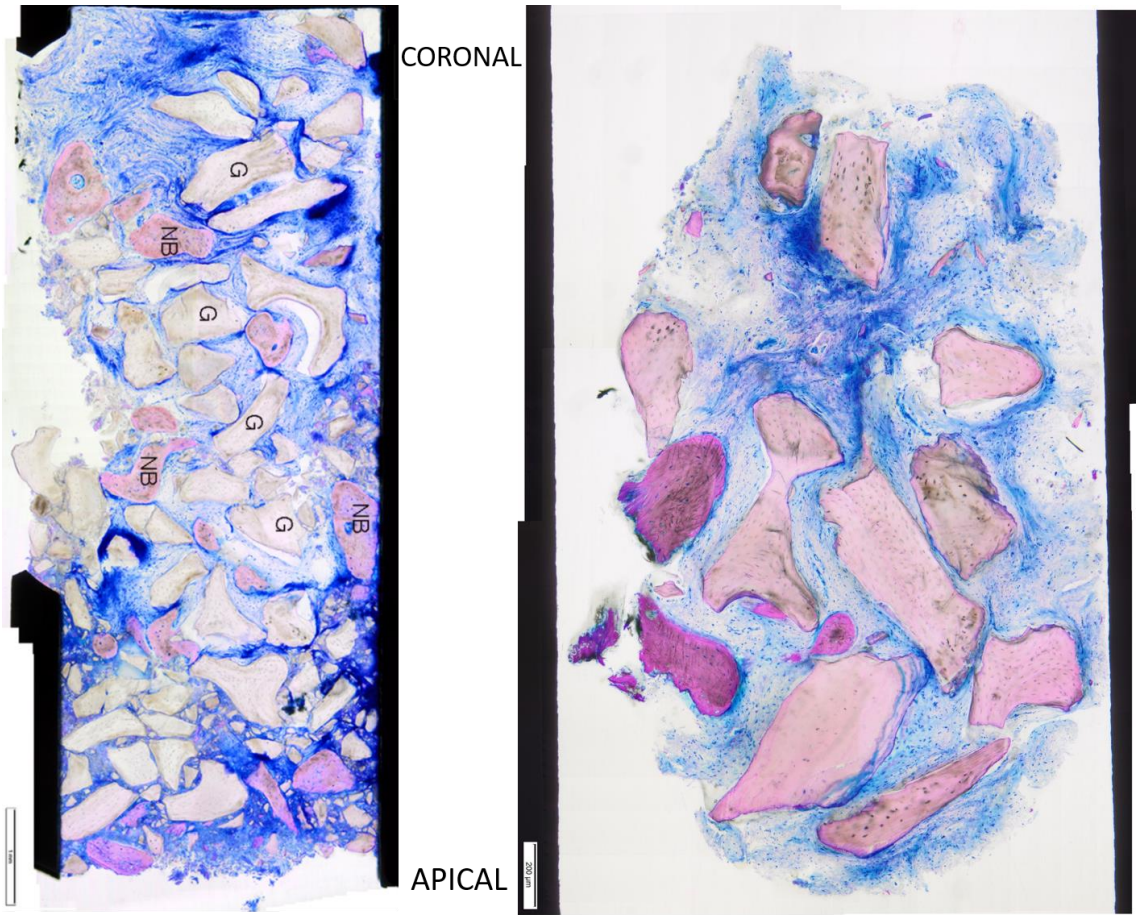


***Figure 25.*** *Cores No. 13 and 22 almost lack new bone formation, while graft materials are embedded into connective tissue.*

The overall histomorphometry results of the test group is as follows: 36,4 (± 12,3)% new bone, 15,9 (± 9,2)% graft material and 47,7 (± 11,7)% connective tissue elements. Due to the qualitative evaluation the test group is further described according to the type of OTM in correlation with the defect location (T1 and T2).

The first subgroup (T1) comprises of 6 patients, in which the study teeth were moved away from the periodontal defect. One out of the six samples was damaged during biopsy, tissue particles had fallen apart, and therefore the histologic result of this is not acceptable. The remaining five cores shows similarities with nice incorporation of the grafted areas into newly formed bone (*Figure 26.*). There is an intimate contact of de novo bone with the graft particles observed on the enlarged pictures. Higher magnifications also proves the process of ongoing bone formation, where osteoblasts, their secretion of osteoid and the maturing woven bone are also present. New bone is bridging the space between the graft particles, while the bone marrow spaces are

enriched with the cross sections of blood vessels (*Figure 27. and 28.*). In the case of one patient few images demonstrate the presence of the so-called cutting cones. Within these "corn-cob" like configurations the ongoing anabolic cellular activity of osteoblast with left behind remineralization zones are also evident (*Figure 29. and 30.*). Two samples show in the apical one half of the section also the presence of local bone, where no graft particles can be detected (*Figure 31.*). The histologic finding of an individual is connective tissue development on the coronal portion of the core, while the apical part is comparable to those areas, where DBBM is embedded in new bone (*Figure 32.*).

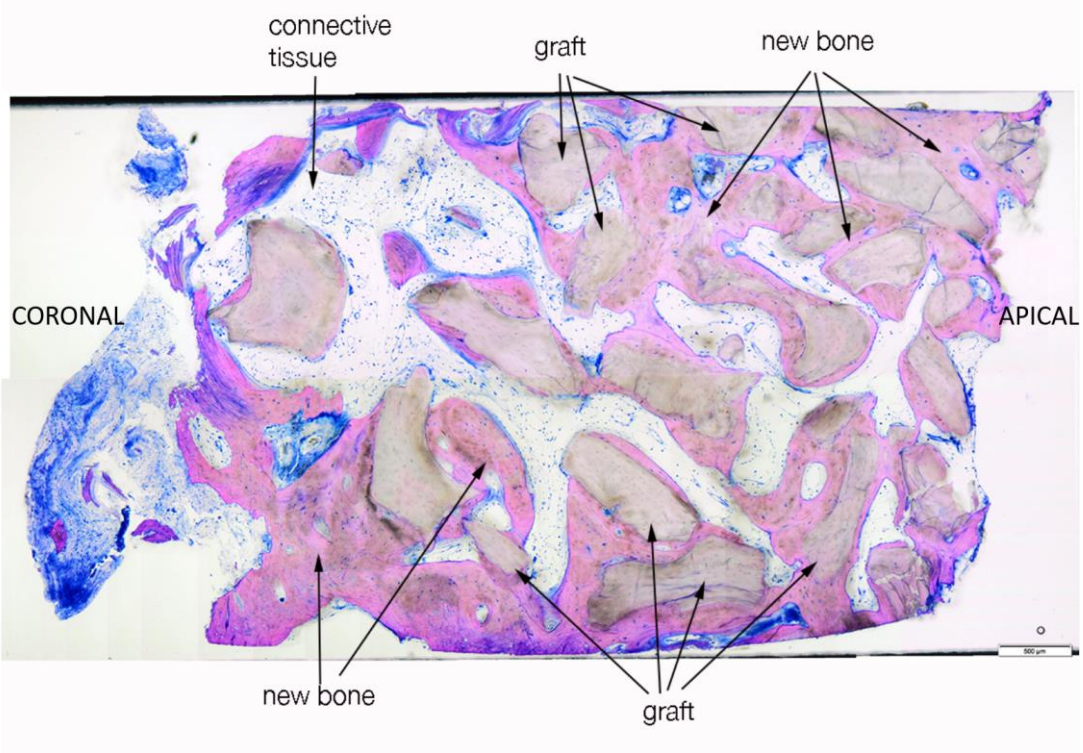


***Figure 26.*** *Patient No. 11's sample represents tension site, where the distribution of newly formed bone, graft and soft tissue components with bone marrow spaces is ideal. Note that there is a tendency of less graft remnants' presence in the coronal part of the core [Nagy et al 2019].*

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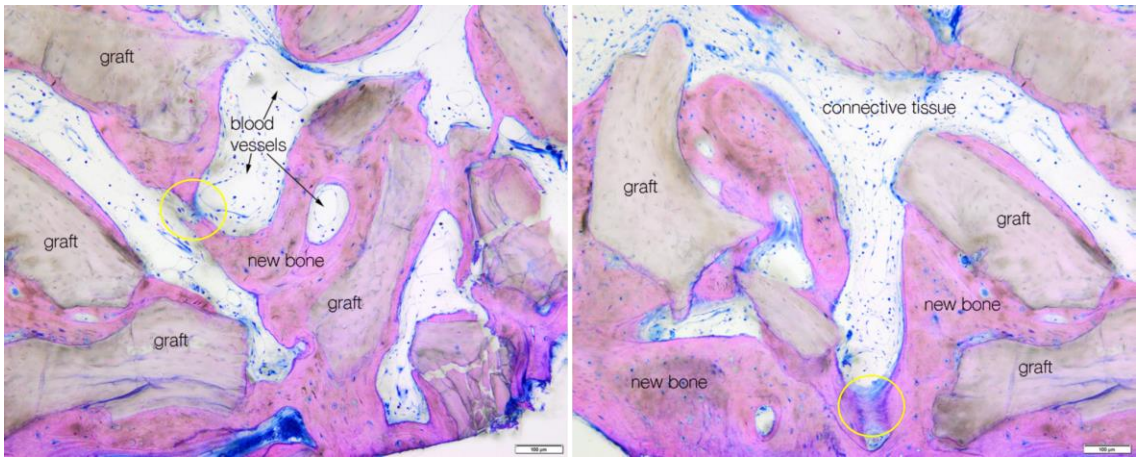


***Figure 27.*** *Higher magnification encloses nice incorporation of the graft biomaterial into de novo formed bone in sample No. 11. Bone marrows are enriched with blood vessels. Yellow circles express the interlinking of the bone between two DBBM particles [Nagy et al 2019].*

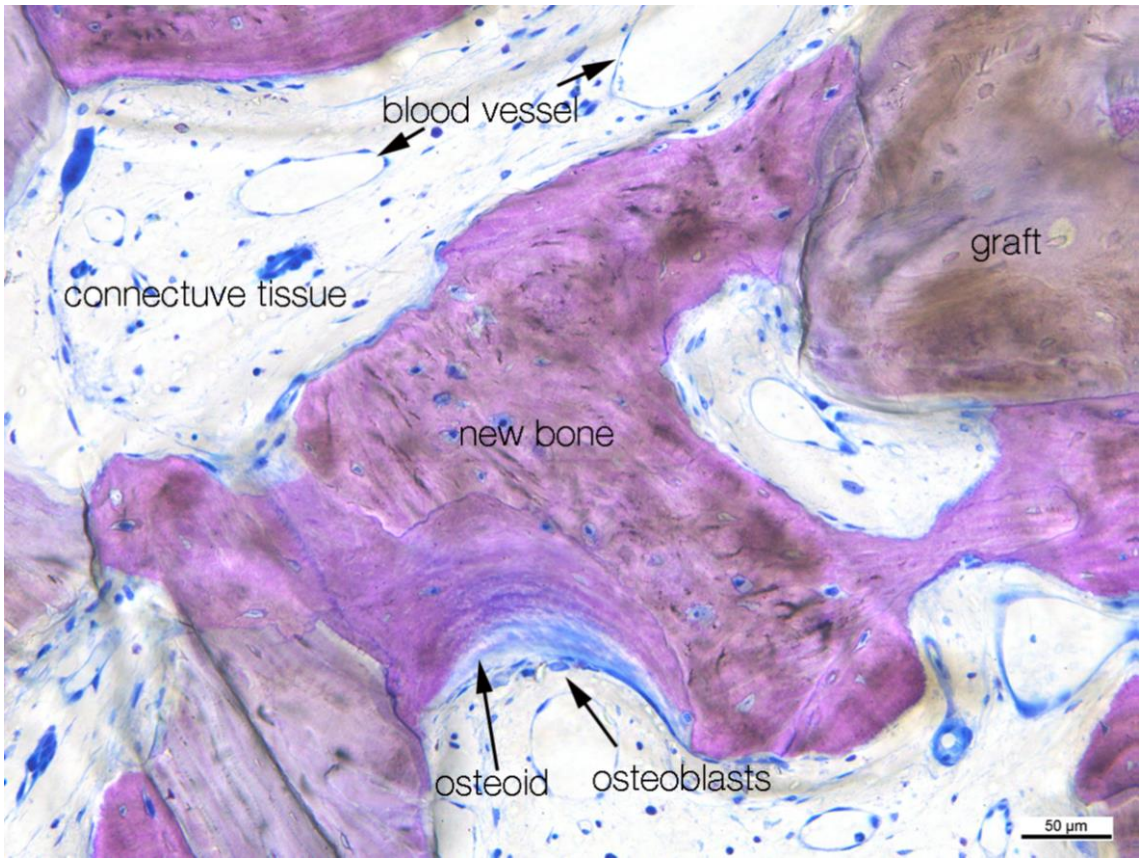


***Figure 28.*** *Ongoing bone formation between the cross sections of the bone substitute and the newly formed trabeculae can be observed on core No. 5.*

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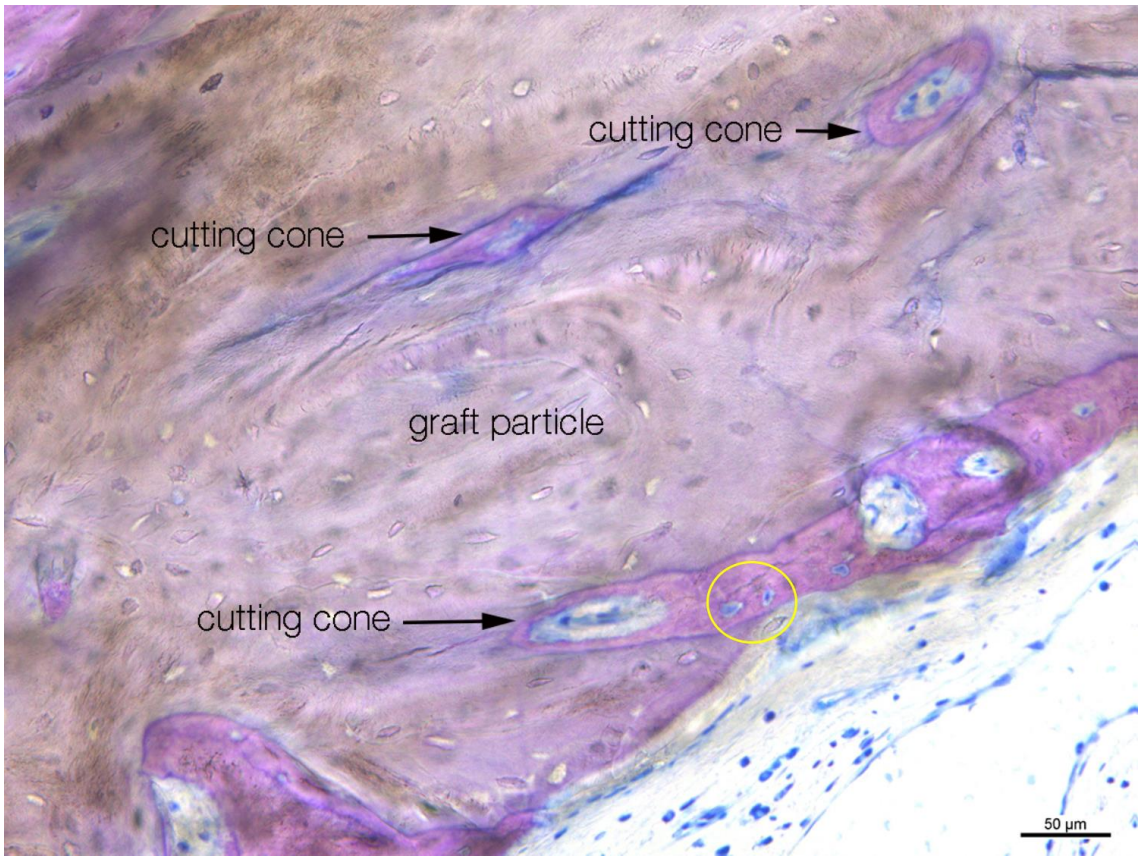


***Figure 29.*** *Ongoing remodeling of a graft particle is evident by the presence of cutting cones, which are filled with newly formed bone and trapped osteocytes (inside yellow circle). On the right upper side of the image a cross section through a remodeling part of a cutting cone is also visible. (T1 group, biopsy No. 5)*

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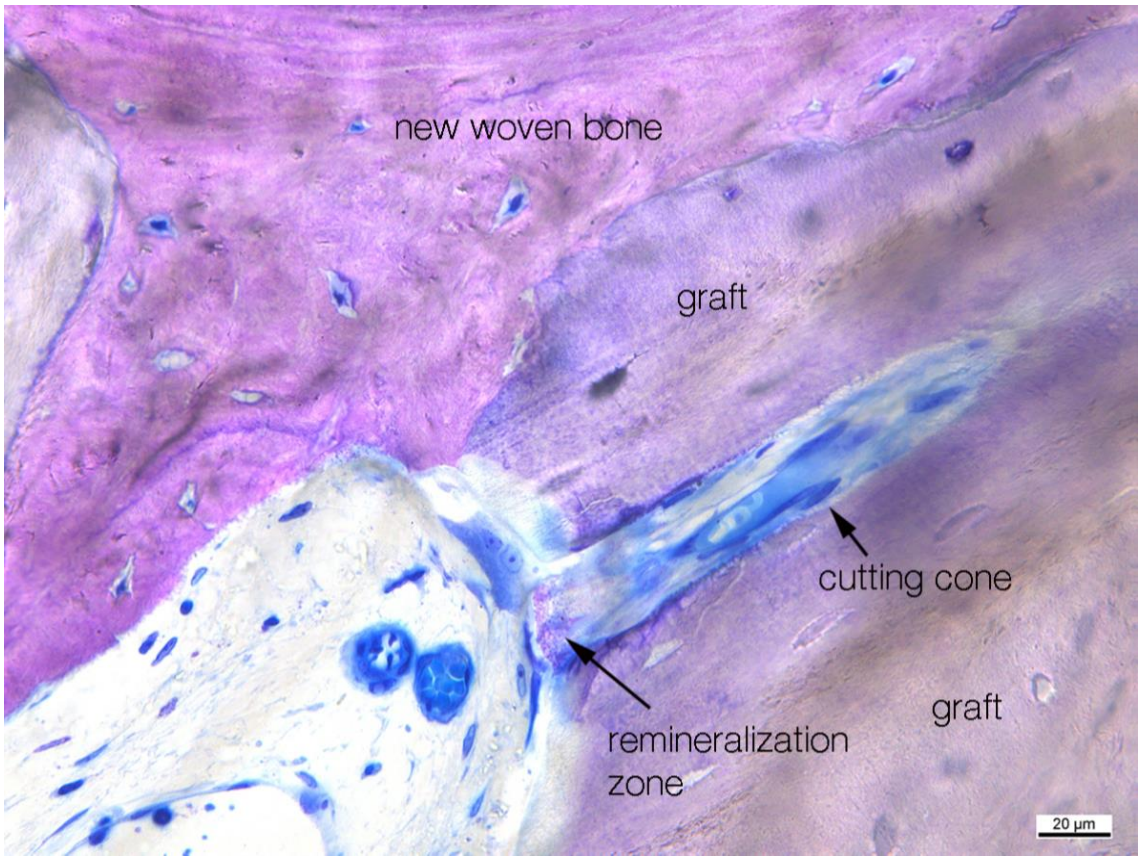


***Figure 30.*** *Sample No. 5 represents on the left side an osteoblast activity with left behind remineralization zones inside the graft particle.*

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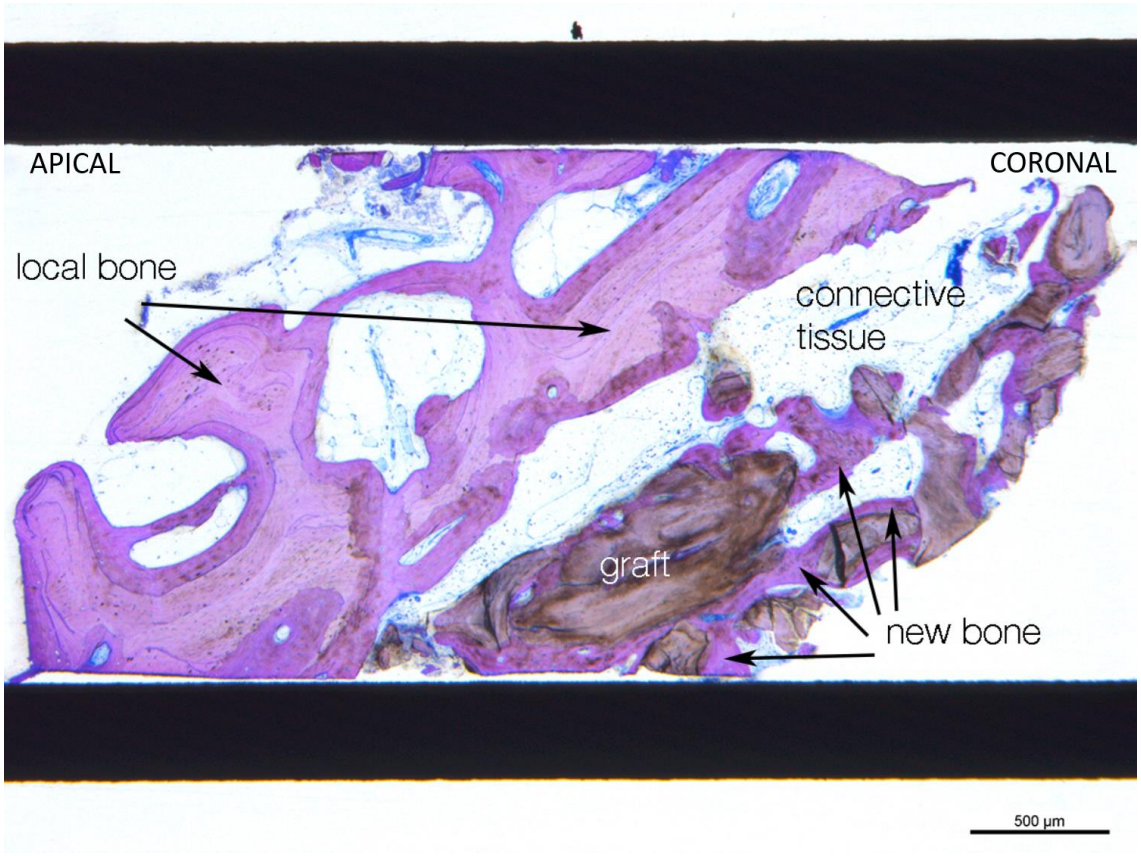


***Figure 31.*** *Apical one half of the core No. 5 reveals the presence of local bone. Graft can be detected only in the coronal part.*

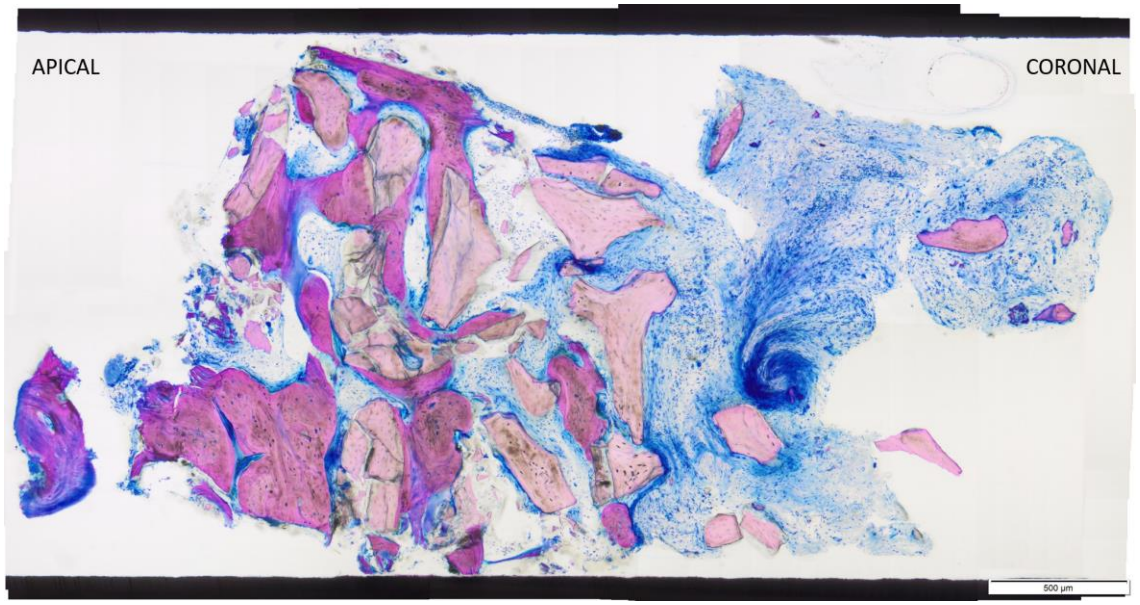


***Figure 32.*** *Biopsy No. 23 displays connective tissue encapsulation on the coronal aspect of the core. Apical region's bone regeneration is acceptable.*

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Five patients serve data for histologic analyses in T2 subgroup. Beside de novo and still-ongoing bone formation proved by secreted osteoid of osteoblasts, the active remodeling phenomenon of xenograft bone substitute are also observed. The osteoclastic cell function creates resorption lacunae on the surface of the DBBM particles (cutting cone formation), in which the replacement anabolic formation takes place characterized by osteoblast cells and osteoid formation (*Figure 33. and 34.*). In a higher magnification we can see a multinucleated giant cell, which interferes with a particle and proves the resorption process of it (*Figure 35.*). Image amplification also reveals a bone ingrowth into the graft surface which was affected by previous resorption (*Figure 36.*). In the case of an individual, where the previous periodontal defect remarkable narrowed due to the OTM, the graft material almost completely disappeared. On the other hand, ongoing bone formation was still present at the 9 months biopsy (*Figure 37.*). Few samples in T2 group also contained local bone. Connective tissue encapsulation of the DBBM is also present in one biopsy belonging to this subgroup (*Figure 38.*).

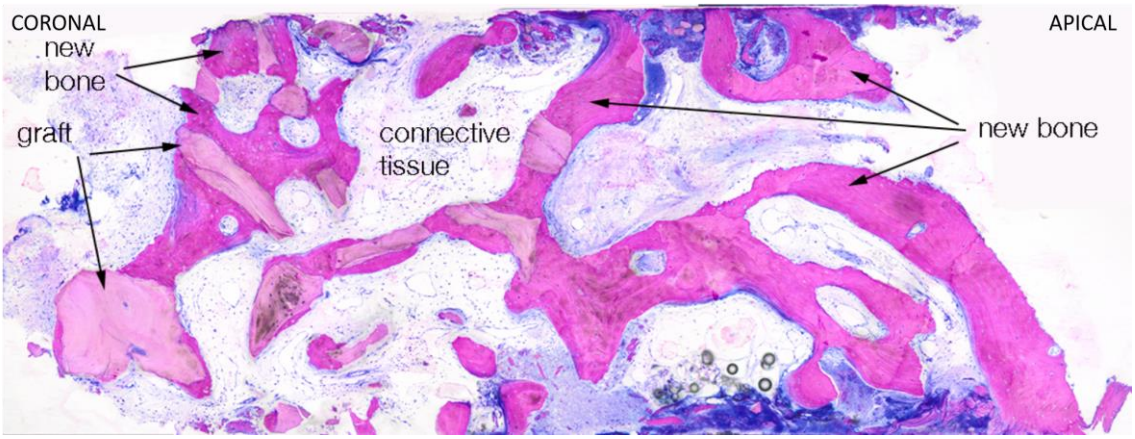


***Figure 33.*** *Core No. 10 represents T2 subgroup, where former defect is a pressure site. DBBM particles are mainly located at the coronal aspect with nice incorporation into newly formed bone [Nagy et al 2019].*

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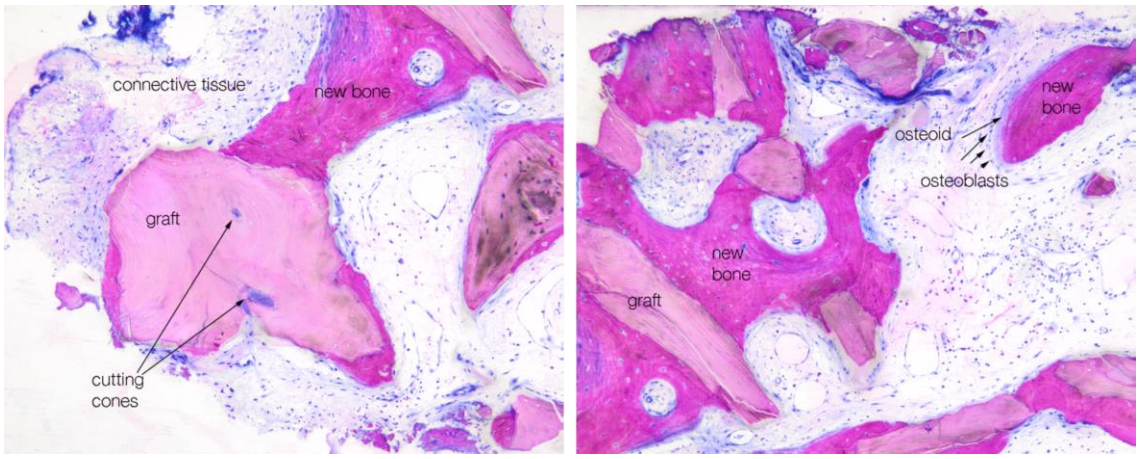


***Figure 34.*** *Two images in a higher magnification from sample No. 10. Ongoing bone formations are present on both pictures (on the left side inside a cutting cone, while on the right side in close proximity of a graft material) [Nagy et al 2019].*

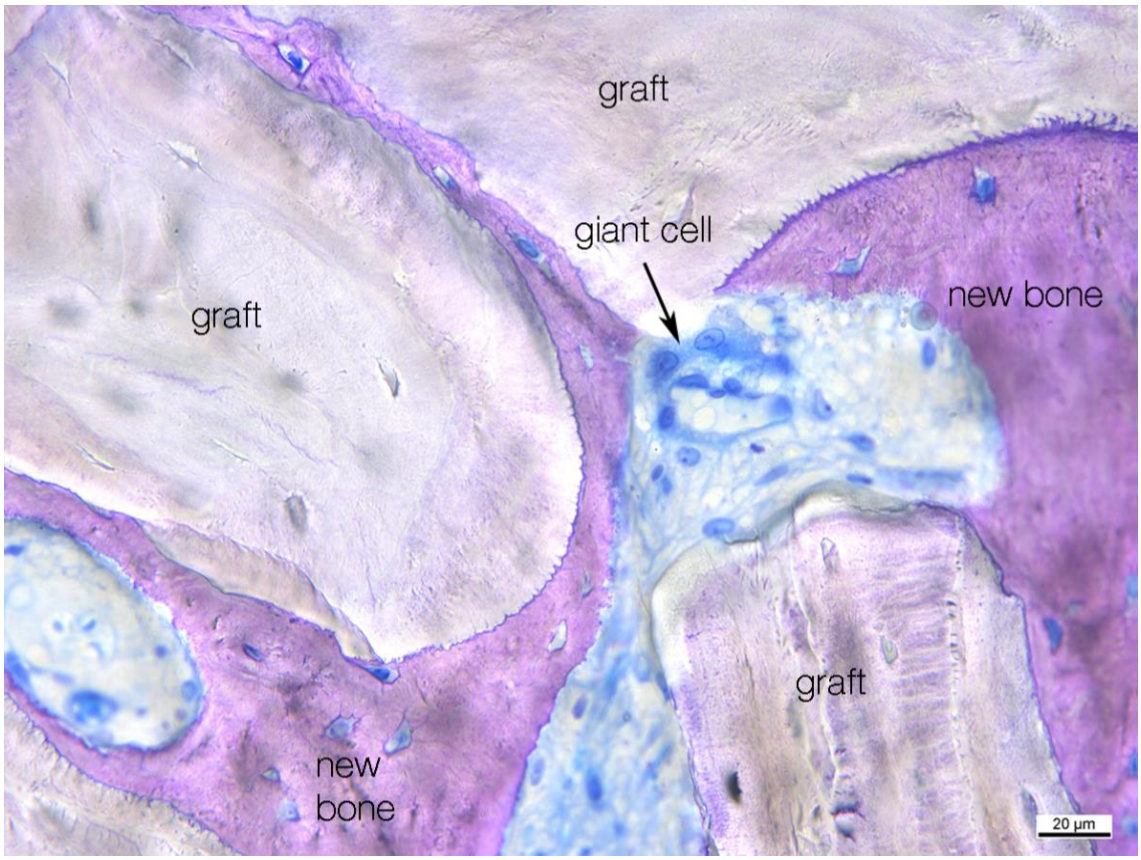


***Figure 35.*** *A unique phenomenon of graft remodeling is demonstrated with the presence of a multinucleated giant cell on a highly enlarged picture of sample No. 2.*

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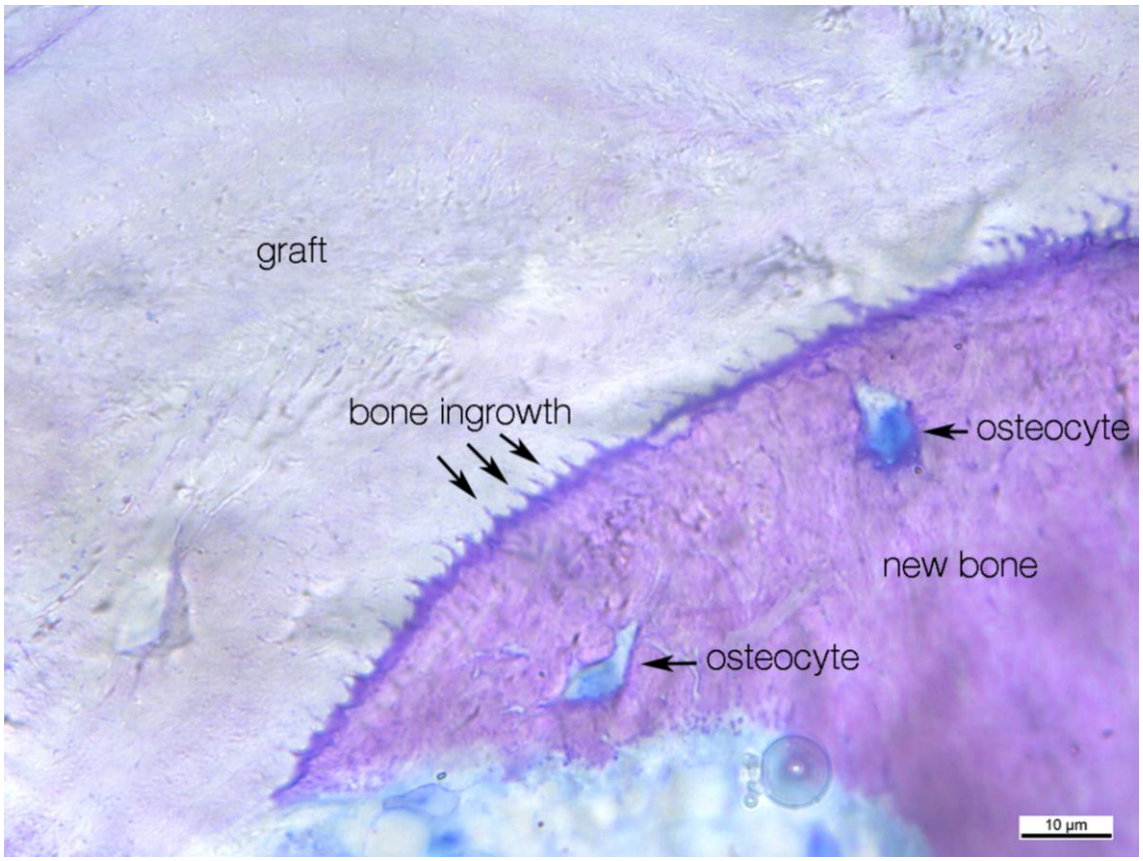


***Figure 36.*** *The previous image with a further amplification presents the bone ingrowth into the surface of the biomaterial, which might have been previously in contact with the osteoclast.*

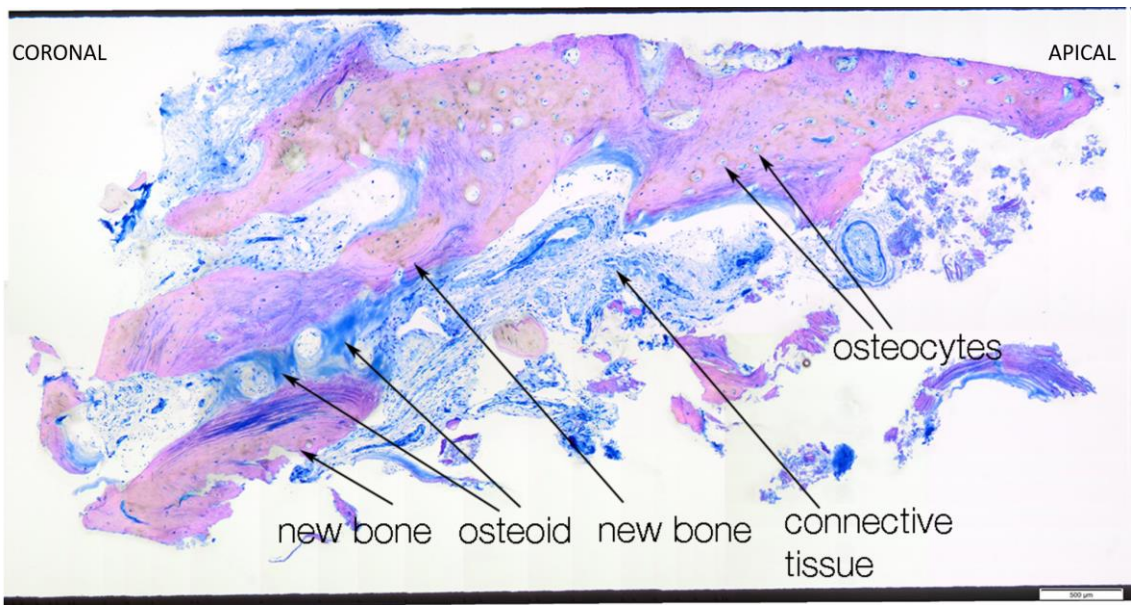


***Figure 37.*** *The study tooth of this patient (No. 4) almost closed the previous defect due to OTM, therefore there are hardly visible graft remnants on the image.*

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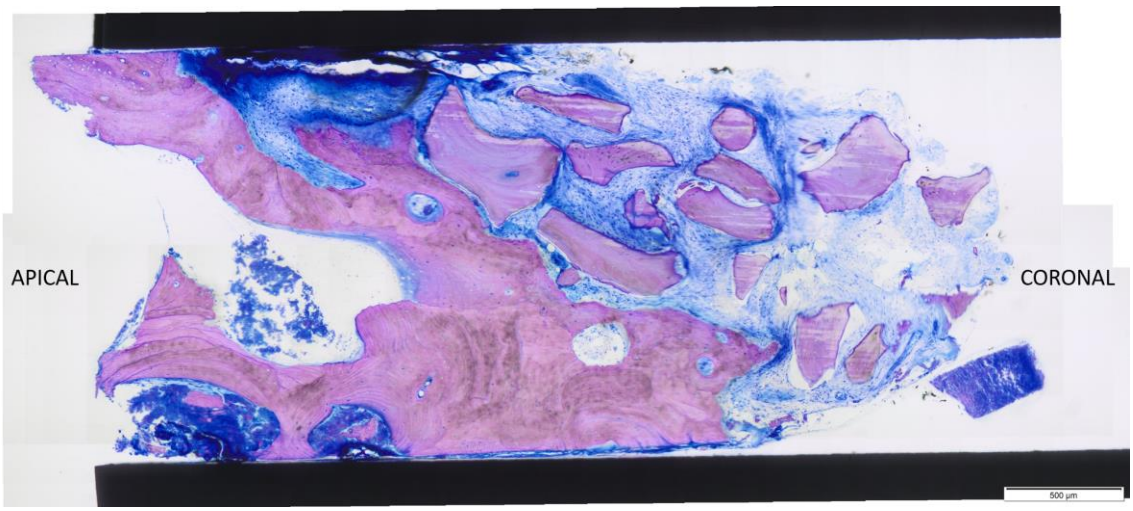


***Figure 38.*** *The unfavorable connective tissue healing on the coronal portion of sample No. 15. Apically local bone is present.*

The histomorphometry dispersion of de novo bone, graft particles and connective tissue is 37,1 (± 17,1)%, 19,7 (± 3,5)%, 43,2 (± 16,6)% and 31,4 (± 11,8)%, 13,7 (± 11,8)%, 54,9 (± 7,3)% in T1 and T2 subgroups, respectively (*Table I*). Statistically significant differences are found between control and all test groups (overall and subgroups' results) in respect of the graft's and the newly formed bone's percentage. The available sample sizes representing data for histomorphometry are as follows: control N= 9; tension side N=5; pressure side N= 5.

***Table II.*** *The overall histomorphometry results of the different groups. Statistically significant differences are highlighted with red between the control group and the test overall- and subgroups.*

| Group / Tissue | Control (Mean ± SD) | Test (Mean ± SD) | |
|---|---|---|---|
| | | T1 (tension side) | T2 (pressure side) |
| De novo bone (%) | 17,4 ± 9,2 | 36,4 ± 12,3 (p=0,0036) | |
| | | 37,1 ± 17,1 (p=0,024) | 31,4 ± 11,8 (p=0,026) |
| Graft (%) | 33,2 ± 3,7 | 15,9 ± 9,2 (p=0,0002) | |
| | | 19,7 ± 3,5 (p=0,0001) | 13,7 ± 11,8 (p=0,001) |
| Soft tissue (%) | 49,4 ± 11,4 | 47,7 ± 11,7 (p=0,76) | |
| | | 43,2 ± 16,6 (p=0,46) | 54,9 ± 7,3 (p=0,36) |

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### *6.4. RESULTS OF RAMAN SPECTROSCOPY, SEM AND EDS*

Raman spectra for reference calcium phosphates compounds and for the DBBM agent are illustrated in *Diagram V.*, while obtained results in case of two patients' (#1 periodontally healthy and #2 with periodontitis) bone samples are depicted in *Diagram VI*. The results of the specific Raman peaks (assigned to the matching chemical compound) and its intensities from the reference calcium phosphate compounds and from the mentioned two patients (#1 and #2) are summarized in *Table III*.

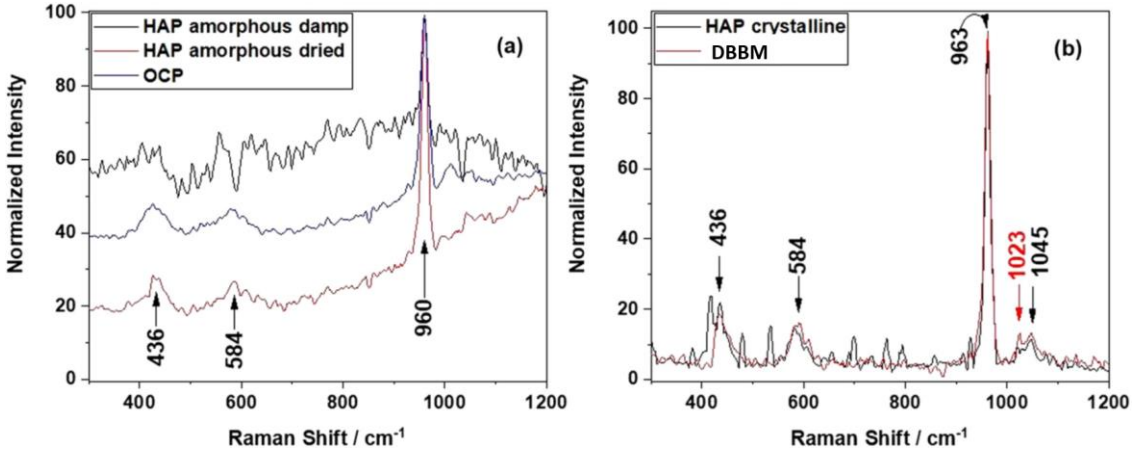


***Diagram V.*** *The Raman spectra of the calcium phosphate compounds: (a) amorphous phase compounds, (b) crystalline phase compounds. [Gatin et al 2019]*

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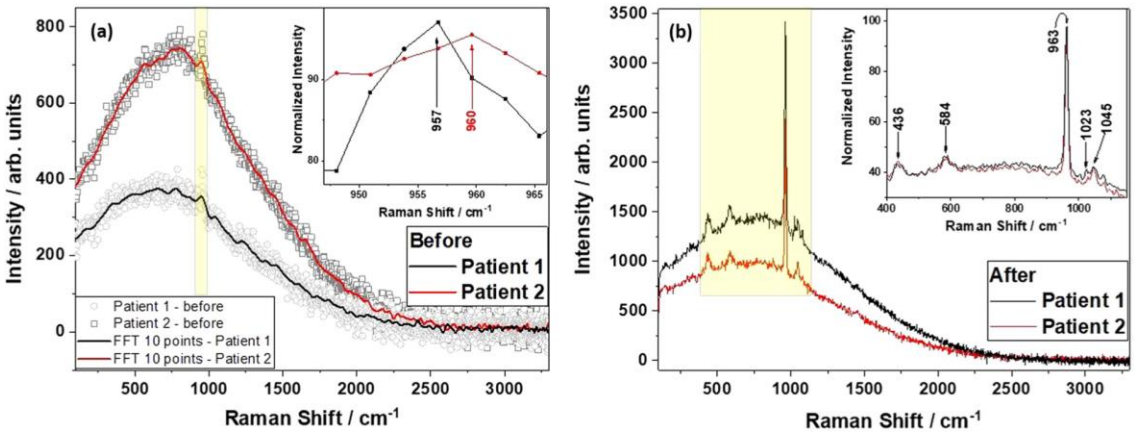


***Diagram VI.*** *The Raman spectra of patients' (#1 and #2) bone specimens: (a) initial condition, (b) after the healing of the augmentation. Insets magnify the shaded areas. [Gatin et al 2019]*

***Table III.*** *The Raman spectra of the reference calcium phosphate compounds and the bone samples. Corresponding wavelengths and peak intensities are listed according to the Raman shifts. [Gatin et al 2019]*

| Sample | | Raman shift ($cm^{-1}$) / Normalized intensity (%) / SD | | | |
|---|---|---|---|---|---|
| OCP | | 436 $cm^{-1}$ / 46.27% / **0.651** | 960 $cm^{-1}$/ 100% / **0.000** | 965 $cm^{-1}$/ 0% / **0.000** | 1023 $cm^{-1}$/ 0% / **0.000** |
| HAP – amorphous air dried | | 436 $cm^{-1}$ / 27.01% / **0.573** | 960 $cm^{-1}$ / 100% / **0.000** | 965 $cm^{-1}$/ 0% / **0.000** | 1023 $cm^{-1}$/ 0% / **0.000** |
| HAP – amorphous damp powder | | 436 $cm^{-1}$ / 65.10% / **0.601** | 960 $cm^{-1}$ / 100% / **0.000** | 965 $cm^{-1}$/ 0% / **0.000** | 1023 $cm^{-1}$/ 65.73% / **0.674** |
| HAP – crystalline (Sigma Aldrich) | | 436 $cm^{-1}$ / 28.59% / **0.456** | 960 $cm^{-1}$ / 0% / **0.000** | 963 $cm^{-1}$ / 100% / **0.000** | 1023 $cm^{-1}$/ 0% / **0.000** |
| Cerabone | | 436 $cm^{-1}$ / 17.34% / **0.643** | 960 $cm^{-1}$ / 0% / **0.000** | 963 $cm^{-1}$ / 100% / **0.000** | 1023 $cm^{-1}$/ 14.15% / **0.842** |
| Patient | | | | | |
| PATIENT (#1) | Before | 436 $cm^{-1}$ / 87.20% / **1.845** | 960 $cm^{-1}$/ 96.67% / **1.789** | 965 $cm^{-1}$ / 84.74% / **1.910** | 1023 $cm^{-1}$/ 73.04% / **2.014** |
| | After 8 months | 436 $cm^{-1}$ / 44.54% / **2.023** | 960 $cm^{-1}$/ 0% / **0** | 963 $cm^{-1}$ / 100% / **0.000** | 1023 $cm^{-1}$/48.76% / **2.010** |
| PATIENT (#2) | Before | 439 $cm^{-1}$ / 83.16% / **1.956**<br>429 $cm^{-1}$ / 79.84% / **2.212** | 960 $cm^{-1}$ / 98.51% / **1.856**<br>952 $cm^{-1}$ / 95.67% / **1.768** | 965 $cm^{-1}$/ 0% / **0.000** | 1023 $cm^{-1}$/ 81.22% / **1.965** |
| | After 8 months | 436 $cm^{-1}$ / 41.14% / **1.984** | 960 $cm^{-1}$/ 0% / **0.000** | 963 $cm^{-1}$ / 100% / **0.000** | 1023 $cm^{-1}$/38.39% / **1.902** |
| Assignment | | $\nu_2$ $PO_4^{3-}$ b-type carbonate substituted apatite | $\nu_1$ $PO_4^{3-}$ b-type carbonate substituted apatite | $\nu_1$ $PO_4^{3-}$ tetrahedral internal mode | $\nu_S$ $PO_3$, P – O – P bridging |

Results gathered after EDS are summarized in *Table IV*. The table shows the calcium/ phosphates ratio, where a score of 1.33 represents an amorphous phase and 1.66 is a crystalline phase. Values between the two extremities indicate a phase with a balanced mixture. Before treatment both patients present a balanced ratio between amorphous and crystalline phase with a higher value for #1 patient. After healing both patients show higher values.

***Table IV.*** *The calcium/ phosphate (Ca/P) fractions after EDS method in case of the two patients. (Three measurements were performed for each sample expressed in weight and atomic percentage. Standard deviations are highlighted.) [Gatin et al 2019]*

| Ca / P | | Wt. % | At. % |
|---|---|---|---|
| Patient (#1) | | 1.49 | 1.37 |
| | Before treatment | 1.46 | 1.41 |
| | | 1.39 | 1.29 |
| SD | | **0.051** | **0.020** |
| | | 1.81 | 1.48 |
| | After healing, 8 months | 1.87 | 1.58 |
| | | 1.91 | 1.66 |
| SD | | **0.050** | **0.090** |
| Patient (#2) | | 0.95 | 1.18 |
| | Before treatment | 1.45 | 1.32 |
| | | 1.39 | 1.27 |
| SD | | **0.273** | **0.070** |
| | | 1.98 | 1.53 |
| | After healing, 8 months | 2.17 | 1.68 |
| | | 2.03 | 1.56 |
| SD | | **0.098** | **0.079** |

SEM pictures highlight the morphological features of bone specimens of patient #1 and #2 (*Figure 39. and 40.* respectively). Patient #1's initial bone, who is considered healthy, encompasses a lamellar and quite regular structure (tile shape) of mineral bone. On the other hand, patient #2 contains a non- regular and compact original bony structure, which represents an immature bone (less crystalline phase). After healing the samples of the augmented bone are compact with bone substitute, however in the case of sample #1 the particles are surrounded by CO fibrils on larger areas, while in the case of specimen #2 this is not confirmed.

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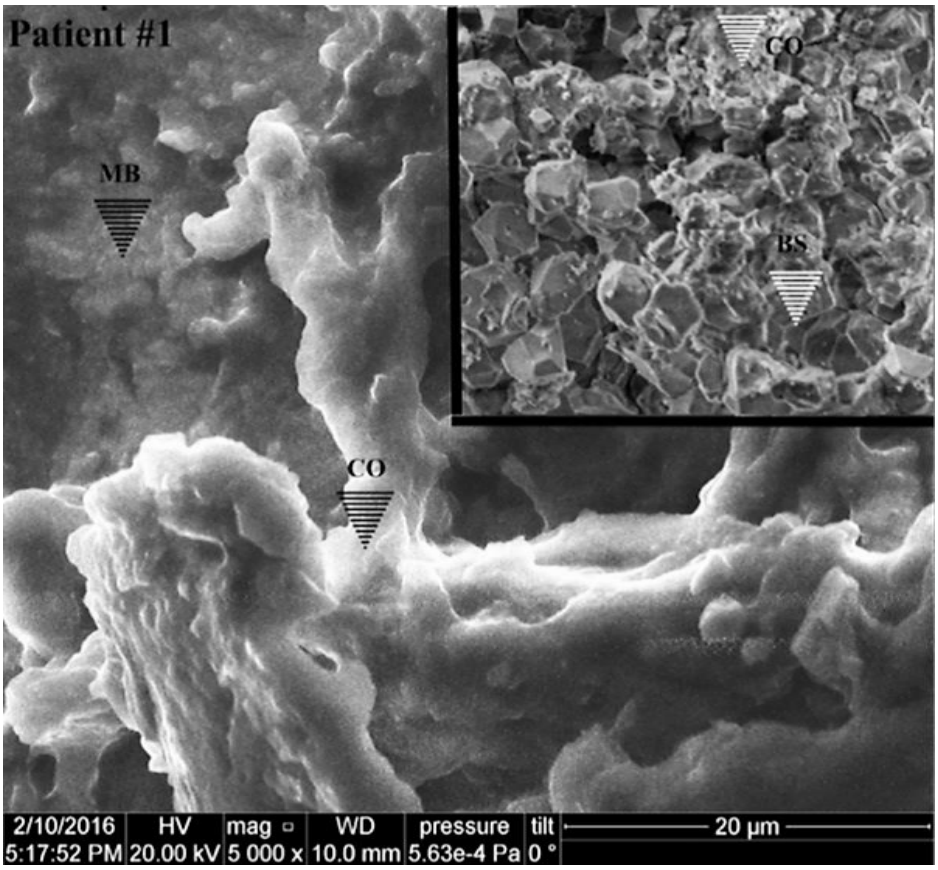


***Figure 39.*** *SEM image of patient #1's bone sample. Initial condition reveals a lamellar and regular bony structure (tile shape) of mineral bone (MB), while right upper inset represents an augmented bony area, which is compact with the bone substitute (BS) surrounded by collagen (CO) fibrils on large areas (X 5000 magnification). [Gatin et al 2019]*

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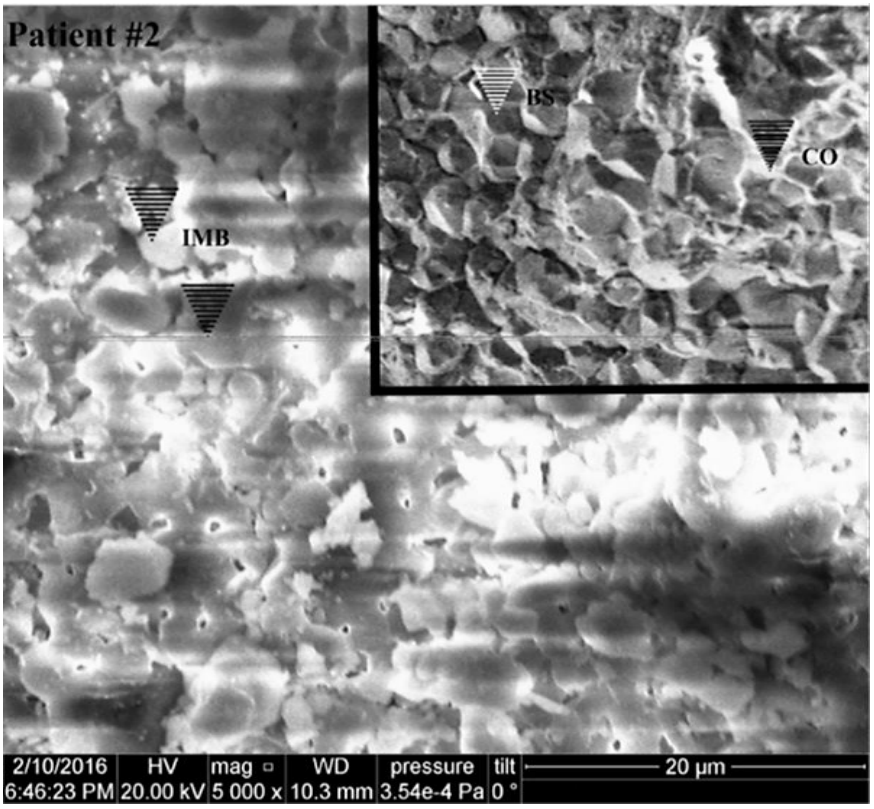


***Figure 40.*** *SEM picture of patient #2's bone. Initial condition expresses a non- regular structure of an immature bone (IMB). Within the inset the healed bone shows also a condensed area with bone substitute (BS); however, it is less surrounded by CO fibrils. (X 5000 magnification). [Gatin et al 2019]*

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## 7. DISCUSSION

In the present randomized controlled clinical trial teeth developing vertical interosseous lesions with a simultaneous pathologic migration are intended to be treated and evaluated. The main focus of interests are the behavior and the morphological changes of the DBBM particles used as a bone substitute material and the newly formed tissue composition of the previous defect site. Further arising questions are the influence of the initiation of OTM on wound healing, the success of the flap design and regenerative strategy utilized during surgery and the clinical improvements in this controlled clinical setting. It is emphasized here that unfavorable, so-called non-containing periodontal defects are selected in the study, which cannot be handled either by minimally invasive flap design, or without the use of a bone substitute material [Cortellini 2012]. The choice of the graft material is also a sensitive issue. The alternatives are negotiated under the introduction, nevertheless the most commonly used material in Europe is probably the xenogeneic one. The disadvantageous periodontal situations can be further aggravated by the pathologic occlusal position of the tooth. Therefore the study protocol is consciously based on common clinical situations, where there are few well- documented guidelines and treatment protocols.

Nowadays there is enough evidence that eliminating the inflammation developed after periodontitis, periodontal care can be safely and successfully supplemented by orthodontic treatment [Antoun et al 2017, Attia et al 2012, Cardaropoli et al 2006, Ghezzi et al 2008, Ogihara and Wang 2010]. There is still no consensus in the literature about the optimal time point of initiating OTM after a regenerative intervention. Attia and his coworkers voted for the immediate application of tooth movement based on both their findings in a randomized clinical trial and in an animal study. The quantity as well as the quality of newly formed bone are improved in their settings [Attia et al 2012]. Another group suggested two weeks of healing after surgery, where orthodontic appliance and early tooth movement did not have any adverse effect, but ended up with improved clinical parameters [Cardaropoli et al 2006]. On the other hand, conservative approaches with a healing period of 6 to 9 months used to be the gold standard due to its safety, but the benefit of bone remodeling during tissue regeneration seems to be lost. The author, considering the different timing possibilities, decided to choose an early

initiation of orthodontics with 7 days postsurgical. The periodontal defects at this time are over the most sensitive healing period (the blood clot formation and vascular phase), where most wound failures happen [Sculean et al 2015]. After 1 week the healing pattern is in transition from the granulation to the regeneration phases, meanwhile getting less susceptible to being jeopardized by orthodontic forces. This recommendation is confirmed by an animal study, where OTM started one week after periodontal surgery, and it had no detrimental effect on soft tissue healing or on diminished but non-inflamed periodontal tissues [Nemcovsky et al 2007]. Nevertheless, cautious tooth movement with low orthodontic forces is mandatory even in our protocol with an early initiation of OTM.

If monitoring the literature, several papers can be found where the regenerative strategy is a guided tissue regeneration in combination with OTM. The favorable findings of these trials are based on clinical improvements as well as on mainly preclinical animal histologic results.

### *5.1. DISCUSSION OF CLINICAL MEASURES*

Under this paragraph the results of other human clinical trials are compared with ours. Ghezzi et al 1 year after GTR therapy proved improvement in clinical parameters and enhanced esthetic outcomes in case of 14 patients with different types of OTM according to the clinical needs [Ghezzi et al 2008]. Cardaropoli et al utilized a similar approach only in 3 individuals, but with an early initiation of orthodontic treatment discussed earlier [Cardaropoli et al 2006]. Almost the same research group observed during a reentry procedure twelve months after the initial surgery a complete filling of the preexisting defect with the presence of bone-like hard tissue. Their results suggest that teeth can be successfully moved and also intruded into bone defects previously augmented with bovine bone substitute material [Re et al 2002]. A case report proved by radiographic and reentry documentation that orthodontic uprighting with simultaneous extrusion can enhance the regenerative potential of GTR [Ogihara and Marks 2006]. The latter 4 trials used bovine bone mineral either with or without a collagen content. A case series and a case report demonstrated the uneventful healing of

periodontal defects, which were filled with allografts and the teeth were moved subsequently toward the defects [Nemcovsky et al 1996, Maeda et al 2005].

The clinical setting described in the PhD thesis proved also positive changes from baseline until endpoint in terms of periodontal parameters, which are in harmony with the findings of the listed researchers. Our test group as well as the control ended up with a statistically significant improvement in ΔPPD, ΔCAL, ΔCEJ-BD and ΔIC. Average PPD reductions are remarkable in both groups (see Table II.). The main goal of pocket surgery is to eliminate the pathologic manifestation of periodontitis, which is the periodontal pockets. Out of the 24 patients who finished the study 22 presented a clinical probing depth of 2 or 3 mm at the endpoint visit, which is considered to be acceptable or healthy [Papapanou et al 2018]. In the remaining two cases (a control and a T2 patient) an early wound failure with corresponding gingiva dehiscence occurred. The $9^{th}$ month PPD values were 4 mm in both patients, which is considered as a moderate residual pocket. The development of this is not surprising in the shadow of the effects of flap dehiscence and its known consequences on wound healing [Polimeni et al 2006]. The histologic samples of these subjects are further discussed in the upcoming chapter (5.2). The achieved intrabony component reductions of the groups can make sense and gain clinical value, if it is compared to the baseline measures. By expressing the percentage of the intrabony fill, a 70% for the test and 67,5% for the control group can be observed. These improvements are in correlation with other trials using GTR approaches in their clinical settings [Needleman et al 2005]. However, it is known that with modified minimally invasive surgical techniques (M-MIST) an 89% of intrabony fill could be achieved [Cortellini and Tonetti 2011]. Nevertheless the M-MIST approach cannot be utilized for those defects selected into our study. The less favorable the defect morphology is and the more extended flap has to be used, the less intrabony fill can be achieved. In light of these, the gained 2/3 filling of our intrabony defects can be concluded to be adequate. Clinicians must accept the fact of a remaining residual intrabony component. This can be handled mainly by resective means through a second surgical intervention.

None of the previously mentioned prospective clinical studies or case reports in this chapter had control groups, where the improvements could be compared with different treatment modalities, which slightly reduces their evidence level. Surveying the

literature only two RCTs were found evaluating the success of GTR in conjunction with orthodontic treatment. Both studies conducted by the same first author used an allograft and EMD combination to treat intrabony defects. In the first trial a combination of EMD and allograft in the treatment of 2- or 3-wall infrabony defects were compared with or without limited orthodontics with extrusive forces. Results did not reveal any significant difference in the changes of PPD and CAL from baseline until 1 year endpoint. The only exception was a significantly better outcome only in the case of 2-wall infrabony defects regarding open probing attachment level gain during a $6^{th}$ month reentry favoring the group with the OTM [Ogihara and Wang 2010]. Meanwhile in the second RCT forced eruption showed better improvements in PPD reduction and CAL gain when allograft was combined with EMD compared with EMD used alone before the OTM [Ogihara and Tarnow 2015].

It might be relevant to further evaluate the changes (from baseline to endpoint) of the parameters between the two examined groups in our study. In order to compare them, it is mandatory that the baseline values be homogeneous. This condition is met within our trial (see *Diagram II.*). Pocket depth and defect's intrabony component are independent measures from the cemento- enamel junction. The changes of PPD and IC are very similar in both groups. ΔPPD in average is -4,2 mm and -5,7 mm, while the mean ΔIC is -4,9 mm and -5,4 mm in the test and control groups, respectively, demonstrating a highly correlating result. It is emphasized here that through an extrusive or intrusive type of OTM, the CEJ can also be transpositioned in an apico-coronal manner. While CEJ is considered to be an independent reference point regularly used in periodontal measurements, in case of OTM, it might interfere with results due to its translocation. In light of this, it is not surprising, that those parameters which are not independent from CEJ, are not showing that close proximity to each other in the test and control group. In the case of ΔCAL and ΔCEJ-BD the control group shows better improvement, while ΔGR and ΔCEJ-BC reveal less deterioration. Both findings can be explained by the type of tooth movement used in some test cases. 4 patients study teeth went through uprighting due to orthodontics, where the appliance forces the teeth to be extruded during distalization. Due to the fact that the angular bony defects are located at the mesial aspect of the tilted teeth, all in these cases the CEJ reference points used for measurements become a more coronal position. This explains the slightly better changes

of the control group inspecting the CEJ- dependent parameters. Despite all this, none of the evaluated changes of the clinical parameters revealed a significant difference in the case of intergroup comparison (interactions), which led us to retain the null hypothesis. This in contrast with Ogihara's finding, where a probing bone level gain was significantly more in the orthodontic treatment group in the case of 2-wall bony defects [Ogihara and Wang 2010].

However, the major drawback of all the positive clinical findings, either in our or in the mentioned similar other studies, are the unrevealed tissue responses and unknown regenerative material behavior. The author highlights here that the results of the present trial do not reach the intended power estimated with the sample size calibration, therefore the enrollment of patients and subject size enlargement are still going on.

Bland- Altman plot is used to compare the radiographic and the intraoperative measures of the intrabony components. The analyses revealed a mean difference of 1 mm between the methods, which means that the intrabony components in the radiographs are underestimated, and radiological measures have a minimal methodological bias. This statement is only true, if we assume that the intraoperative measures, which have a direct visual access, are more reliable and considered as a gold standard. By calculating the 95% limits of agreement, which tells us how far apart measurements by two methods were more likely to be for most individuals, the values are between 0.24 and -2.30 mm. This means, that using radiographs in order to assess intrabony components, it is 95% sure, that the calculated value will be approximately 2 mm below or almost the same, which can be measured intraoperatively by opening or reopening an intrabony defect.

### *5.2. DISCUSSION OF THE HYSTOLOGY*

Several human histologic studies showed the benefits of treating infrabony defects with the GTR approach [Camelo et al 2001, Sculean et al 2004]. These high levels of evidence verified the formation of new cementum with inserting collagen fibers and new bone formation on the surface of the graft particles. This regenerative effect was even more pronounced using the combination of a resorbable membrane with a DBBM [Camelo et al 1998]. However the more apical the location of the graft particles are, the

better the encapsulation is into de novo bone, yet the coronally located graft pieces might be encapsulated into connective tissue [Sculean et al 2004]. Unfortunately there is no human histology evaluation regarding the whole periodontal apparatus in the combined approaches (OTM + GTR). Only partial analysis of the supporting bone is evaluated in our case series [Nagy et al 2019]. Beside this only preclinical studies proved the safety of tooth movement into periodontally regenerated areas. Searching the literature two relevant articles are found with similar design, which could be a benchmark for our study.

In a pilot animal study of Diedrich et al, surgically created intrabony defects were created and colonized by periodontopathogenic microorganisms at premolars. After few months a regenerative procedure was performed with the help of EMD and a resorbable membrane. Investigated teeth underwent either intrusion or translational movement into (pressure side) or away from the defect (tensile side) by orthodontic means. Extensive periodontal regeneration was observed both at the intruded root segments and the tension sides according to histology. These groups expressed approximately a 73-84% cemento- and osteogenesis on the denudated root surfaces, whereas the values for epithelial down growth were low (15-19%). Pressure side yielded significant reduction in terms of bone regeneration, but new cementum formation with a corresponding new connective attachment was comparable with the previous groups' (76%). [Diedrich et al 2003].

Araújo and his coworkers also moved animal premolar teeth into previously augmented extraction sites. OTH started 3 months after the extraction of the neighboring teeth, whose alveolar sockets were augmented at the same time by DBBM. The differences between this and the previous preclinical study are the utilization of a non resorbable graft material and the lack of iatrogenically created periodontal defects with a consequential regenerative surgery. In Araújo's study the main focus was the interference of the bone remodeling with the graft particles (*Figure 41.*). Their histological results testified to the possibility of moving a tooth into or through an area, which had been grafted with DBBM before. Pressure side disclosed partly degraded particles within or in the close proximity of the periodontal ligament zone. There were no graft remnants in contact with the root surface. The area served as tension site revealed no residual biomaterial, however it is emphasized that the tension side (named

after the direction of the OTM) was a previous pressure site from the perspective of the augmented area, because the study teeth were moved entirely through them (*Figure 42.*). It can be also instructive, that the speed of the tooth movement in the grafted (test) area was similar to the movement in the non-grafted area (served as control). The root surfaces facing toward the test grafted area did not show a higher amount of root resorption than the ones in the control site. It is also interesting that histomorphometry results of this study showed that grafted sites either in the proximity of the root (affected by OTM) or harvested from intact (not affected by OTM) area expresses almost the same amount of mineralized bone compared to control, non-grafted sites. The only difference in test and control (grafted vs. non-grafted) is the percentage of the bone marrow and for sure the presence of the graft, regardless of the biopsy site (affected by OTM or not). This means according to the authors' opinion that "DBBM occupies space at the expense of the bone marrow and not at the expense of mineralized bone" [Araújo et al 2001].

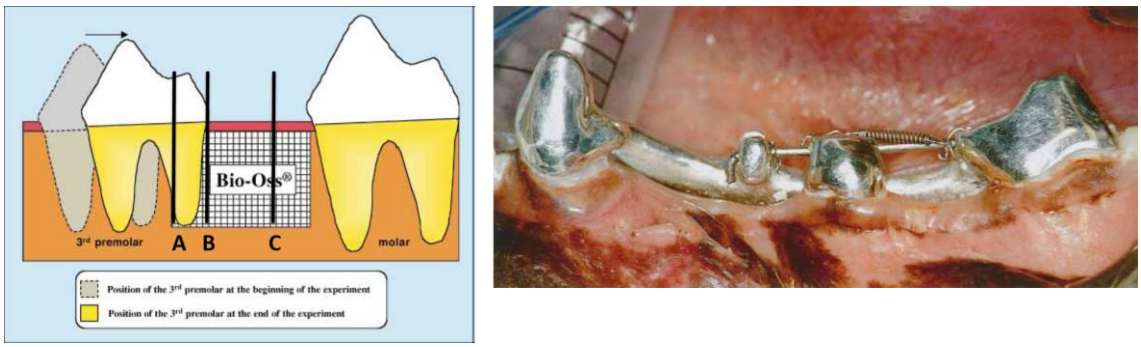


***Figure 41.*** *The schematic drawing and the clinical picture of Araújo's study design regarding the type of OTM [Araújo et al 2001]. A, B and C lines represent the place of the biopsies.*

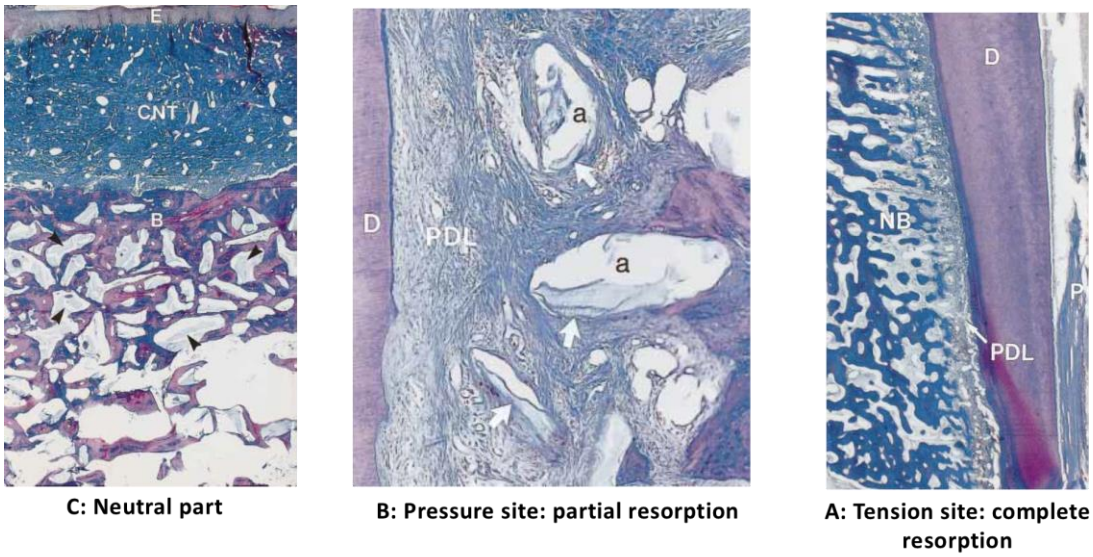


***Figure 42.*** *The histologic images of the biopsies obtained from 3 different places [Araújo et al 2001].*

The present study's investigation is roughly a combination of these latter two (animal studies). First, we allocate true periodontal defects as in Diedrich's, second the biomaterial used as a scaffold for bone regeneration is a xenogeneic one like in Araújo's. However both preclinical studies used a late initiation of OTM, while we are committed to the early initiation due to the reasons described earlier. Due to ethical considerations we do not sacrifice the tooth with the surrounding attaching apparatus block-wise. Only bony cores with the smallest diameter possible under clinical circumstances are harvested and processed for the histologic analyses, thanks to which we must have resigned from the full periodontal evaluation.

*5.2.1. Discussion of the control group's histology*

Observing control patients' samples, different ascertainments can be drawn. In the case of those individuals where the postoperative healing was uneventful, sections express nice healing and incorporation of the graft into newly formed bone, but this is manifested mainly in the apical and middle thirds of the defects. The coronal part reveals rather the encapsulation of the bone substitutes into connective tissue. This is in agreement with other human histology findings utilizing DBBM for defect filling [Camelo et al 1998, Sculean et al 2004]. The authors lack any data about the long-term

behavior and stability of these healed sites, but the question arises as to whether these areas might be more susceptible to a periodontal breakdown in case of oral hygiene deterioration. It is assumed after empirical observation that not well- integrated non-resorbable particles are more exposed to a later bacterial contamination. This leads to the conclusion that the reduced amount of graft ratio would be beneficial in the long term, and would make the former defect more resistant to possible recidivism.

Ongoing bone formation is also present at 9$^{th}$ months old biopsies, which is proved by the cell activity of the osteoblast together with the secretion of osteoid and transformation into an immature form of woven bone. This further increases the trabecular count and plays an important role in the developing structure of the lamellar type bone. In animal histologic studies, by Susin and Wikesjö's review, it is clarified that most new tissue formation in the periodontium is complete within 14 days. It indicates, assuming some delay in humans, that near-final boundaries of the regenerated tissues should be established within 4 weeks of healing. This is in contrast with the assumption of a slowly progressing regenerative process, which is confirmed mainly by clinical and radiographic measures [Susin and Wikesjö 2013]. Our protocol working also with late (9 months) evaluation cannot dissolve this contradiction, but testifies the fact that the maturation of the bony tissues and the remodeling around the graft material is still ongoing at the postsurgical 9$^{th}$ month.

The C group's averaged histomorphometry result is 17,4% of de novo bone; 33,2% of graft and 49,4% of soft tissue elements. Camelo's tissue distribution in regenerated human intrabony defects with the same material used in our study is highly similar to ours (approximately a 25% of new bone, 34% DBBM and 41% soft tissue) [Camelo et al 1998]. If we compare the histomorphometry results of the periodontal defects with sinus floor augmentations, remarkable correlations can be found. In a human multicenter study augmented sinuses were evaluated, where the grafting material was also DBBM. Biopsies showed an average of 19.8% mineralized bone, 37.7% of remaining graft and 40.4% of soft tissue components. In light of the fact that the sinus is considered the gold standard defect for augmentation due to its containing morphology and good blood supply, the author regards the tissue distribution in their non-containing defects to be favorable.

The exception in the control group is the 3 samples representing almost an entire connective tissue down growth into the former defect, which surrounds the graft particles together with the inhibition of the new bone formation. There is positive correlation between the early wound complications mentioned in Chapter 4 and the development of the latter unsatisfying histologic results. In the author's opinion, the more narrow the interdental spaces were, the more common the complication occurred during the early healing phase. Interdentally narrow soft tissues are correlated with low level of blood supply. These areas are more susceptible to a gingiva dehiscence, even if the wound closure was performed tension free and the papilla preservation technique was utilized. Once membrane exposure happens due to a wound failure, the clinician must wait for secondary wound healing. During this period increased attention should be payed to keep the area clean by means of mechanical and chemical plaque control. Even if the infection of the surgical site can be prevented, the resorbable collagen membrane start to disintegrate and molder. This phenomenon somehow interrupts and negatively influences the wound healing processes, explaining the histologic findings of patients having membrane exposure. We know after the histological studies of Melcher, that it is the type of cells whereby the denudated root surface after a surgical intervention will be repopulated, which dictates the healing pattern [Melcher 1976]. Dickinson later described similar phenomenon by identifying pro-regeneration (originating from the PDL and bone marrow) and pro-scar-forming (originating from the gingival tissues) domains that compete to reculture the wound space. The extent to which the innate regenerative potential may materialize is determined by the net effect of these competing cells modulated by local and systemic factors [Dickinson et al 2012]. The described wound failure can be enunciated as a local factor increasing the reparative domain of a wound, which is correlated with a long junctional epithelium among the root surface and fibrous tissue formation at the site of a bony defect. Therefore the healing of a periodontal wound is always considered a mixture of a coronally migrating reparation and an apically originating regeneration.

### *5.2.2. Discussion of the Test 1 group's histology (tension side)*

The former periodontal defects exposed to tensile forces of OTM represent histologic data mainly of perfect de novo bone formation around bone substitute materials. Nice incorporation of the graft particles into new bone is visible in more sections. The presence of blood vessels in the bone marrows and the ongoing bone formation indicate the vitality and the maturing capacity of the regenerated area. This proves that the forces originating either from the mastication or from the orthodontic appliance have a remodeling effect on the surrounding newly formed mineralized tissues. The observed cutting cones in the case of a sample might testify that OTM induced remodeling interferes with not only the lamellar bone, but with the DBBM particles also.

The reason for orthodontic induced bone remodeling was described in the literature a long time ago. Once an orthodontic- induced mechanical force presses the root into one direction, the PDL space on the pressure side starts to narrow. This causes a change and decrease in the vascular blood supply locally together with an anemic situation. Under this circumstances a lot of cytokines and molecules are released from the cells, which leads to the degradation of the local cells and matrix. This process is called hyalinization, which is manifested at the neighboring bone with an osteoclastic cell activity [Reitan 1967]. It is confirmed by our histologic samples, that this catabolic cell activity causes a similar interference with the inorganic particles also, which is in agreement with Araújo's finding, that there are many common features between the OTM induced bone resorption and the degradation of DBBM. However the detailed mechanism behind the remodeling and elimination of these particles is still not completely understood.

There are two findings which might need further explanation. Firstly, two samples in the apical region reveal also local bone pieces. This can be unfolded by the fact that during reentry the depth of the drilling was too deep, which caused the biopsy core to contain also local bone. If we assume that the depth was strictly controlled by calculating the change of the intrabony components, then the fact of this mistake can be rejected. Secondly, there is a tendency to observe fewer graft remnants at the coronal portion in T1 samples. Both observations can be also explained by the type of tooth movement. Most study teeth in this group underwent uprighting movement, due to the fact that these teeth were previously tilted toward the periodontal defect. An uprighting

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is a complex displacement, where simultaneous movements are happening. It is a combination of distalization and extrusion from the defect point of view (*Figure 43.*). Considering this, it can be calculated that the greatest tensile strength is located at the coronal aspect of the defect, which can cause the lower graft ratio in the histologic samples. On the other hand, it can explain the local bone at the apical portion, because the extrusion component can transposition original tissues in a more coronal manner. These observations need further investigations but this tendency could be beneficial in the long term, because a former defect healed with a larger amount of newly formed bone might be more stable and resistant to later periodontal bacterial attacks.

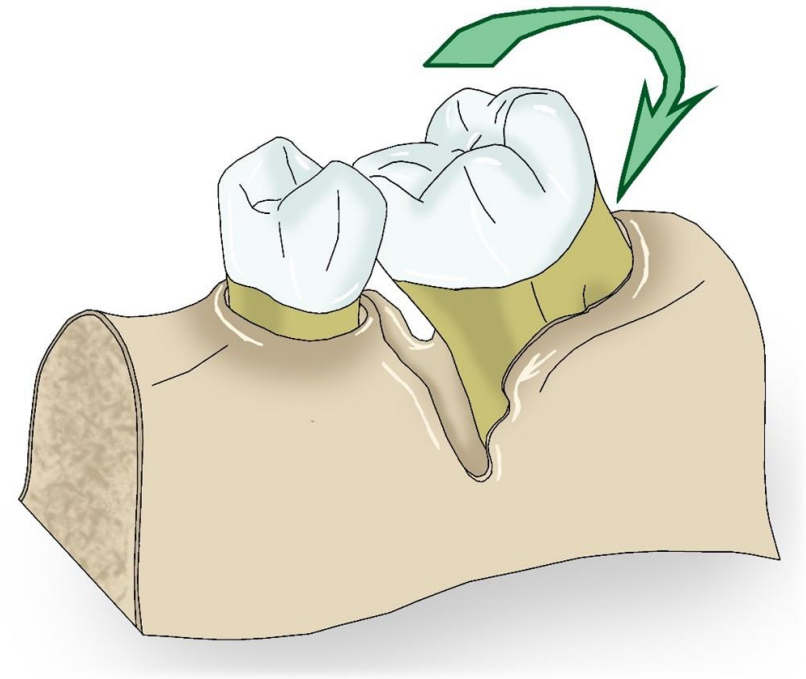

***Figure 43.*** *Schematic drawing demonstrates the complex type of tooth movement during uprighting. Periodontal defect is considered as a tension site (T1).*

Besides, there is a tendency of change in the amount of graft particles and the newly formed bone compared with the control sample's histomorphometry. The T1 subgroup revealed approximately 37,1% of de novo bone, 19,7% of graft and 43,2% of soft tissue elements. The amount of newly formed bone in this group is comparable with the ratio of graft particles found in the control group, while the distribution of DBBM in T1 is similar to the control's percentage of new bone. It is assumed that the tension caused by orthodontics induce bone remodeling and apposition, which interferes with the DBBM particles ending up with the previous results.

The highly increased new bone together with the decreased graft ratio is also present in the T2 subgroup. These amounts' of tissues compared to those ones of the control group are statistically significant. In view of the small number of samples representing histomorphometry data and the relatively high values of standard deviations, the revealed significant differences between the groups are striking. The proportion of the tissues in the test group is considered to be closer to an original bone's morphotype than those of the control group.

One sample in this subgroup disclosed in the coronal portion also a fibrous tissue displacement into the defect, for which the reason remains unknown, because the patient had an uneventful wound healing. The apical portion of this core showed nice bony encapsulation, which further strengthens the mixed healing potential of human periodontal defects (*Figure 32.*).

*5.2.3. Discussion of the Test 2 group's histology (pressure side)*

The core samples of this subgroup demonstrate a further decreased graft ratio while de novo bone formation was comparable with that of the T1 subjects. The overall histomorphometry results are as follows: 31,4% de novo bone, 13,7% of graft remnants and 54,9% of soft tissue. While these sites are under pressure by orthodontic forces, the induced resorption capacity of the osteoclasts affects the DBBM particles as well. Ongoing remodeling of graft material is evident by the presence of cutting cones and the development of giant cells in close contact with the bone substitute. The replacing bone ingrowth into the DBBM surface proves perfectly the remodeling of the originally non-resorbable graft material. The greater amount of connective tissue might be due to the fact that the bony samples were harvested in some cases at a still active phase of the OTM. It can be supposed that after removal of the appliance, ongoing bone formation with trabecularization would continue, which could possibly further increase the percentage of de novo bone [Ramaglia et al 2018].

Three patients' biopsies expressed some amount of local bone in the apical region of the core. This is not surprising in light of the fact that the tooth movement was utilized toward the defect in these cases, which caused a narrowing of the former dimension of the intraosseous lesions (*Figure 44.*). Due to the standardized place of the

trephine orientation, the biopsy site also moved a little bit mesially in accordance with the tooth. The calculated depth of the harvesting resulted in some apically contained local bone in the samples. However, the local bone presents different staining than the newly formed bone, therefore it was not involved into the quantitative analyses. One core discloses embedding of the DBBM into connective tissue, which can be explained by the possible wound failure described with the control biopsies.

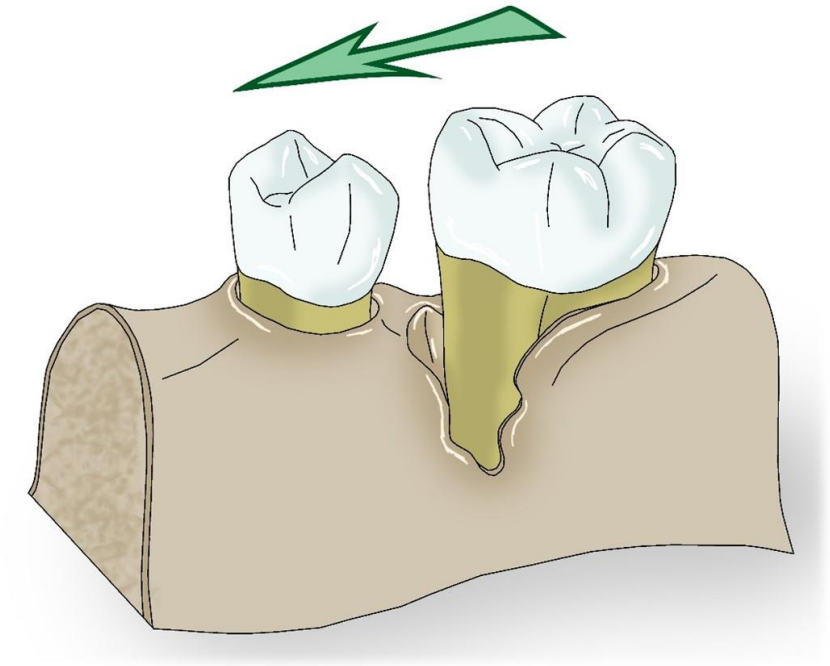

***Figure 44.*** *When the tooth is moved bodily toward the intrabony defect in some cases, the place of the biopsy might suffer some displacement. Periodontal defect is considered as a pressure site (T2).*

As demonstrated by different authors, active remodeling of DBBM can be explained by the upregulating effect of the recombinant human platelet-derived growth factor in bone metabolism, which created bone remodeling units around graft particles [Simion et al 2006, Nevins et al 2009]. The novelty of the present study is the histologic evidence of a similar resorption of the xenogeneic graft material in humans caused by a different stimulus, which is the OTM. The author would like to note here that this phenomenon is useful in other types of reconstructive surgeries combined with orthodontics (during the comprehensive treatment of the periodontally accelerated osteogenic orthodontics), where non resorbable graft materials are utilized [Nagy and Pörzse 2020].

According to the animal study of Nemcovsky et al, it is proved that OTM toward an iatrogenically created bony defect, where a simultaneous attachment loss is present; the orthodontic treatment without regeneration cannot prevent long junctional epithelium development, but can restrain the extent of it and can decrease the pocket depth [Nemcovsky et al 2007]. Another animal investigation by the same research group, with similar iatrogenic bony defects, demonstrated that OTM associated with intact PDL on root surface has a better outcome on bone healing than those without direct contact with existing PDL [Nemcovsky et al 2004]. The authors' conclusion was, that PDL originated cells may be involved in the mechanisms of both periodontal hard and soft tissue healing. A review also stated, that orthodontic-induced bone remodeling is strictly related to periodontal ligaments [Reichert et al 2009]. Therefore, it seems that early-initiated OTM does not compromise the periodontal wound healing phases, nor the establishment of a possible new attachment; otherwise, the later-developing ligaments would not cause the remodeling effect. According to the human histologic findings on periodontal regeneration, bone formation around DBBM particles is linked to preferable new cementum and PDL formation [Sculean et al 2003]. On the other hand, if a new periodontal attachment could not be verified, new bone development around DBBM was not reported. This can be in agreement with our histological results of those subjects where an early wound failure occurred and resulted in fibrous tissue encapsulation of the grafting agent. Although there is no full periodontal assessment in our trial, a conjecture in accordance with the previously- mentioned studies can be established, that in the case of a nice bone regeneration a simultaneous new attachment formation might also be present. Therefore, the presented minimally- invasive histologic harvesting and evaluation technique can be considered appropriate to fulfill the study aims demonstrating bone substitute remodeling, and to deduce simultaneous formation of a new attachment.

Least but not last it is observed, that there is a correlation between the results of the arbitrary bone quality examinations and the micromorphology image of the samples representing fibrous encapsulation at least on the coronal one third of the core. In case of 11 patients (7 in the control and 4 in the test) the crestal part of the bone showed easily scrapeable particles, which verifies the histologic pictures of these samples. In an interesting but not a surprising way, more control subject demonstrated the previous

finding. This is in agreement with the histologic results, that OTM might help to reduce also that type of graft amount, which is encapsulated coronally in connective tissue. The further biologic benefit of this is already described in the thesis. It is emphasized that easily scrapable biomaterials at the crestal part tend to occur more frequently than just the incidence of early wound dehiscence (11 compared to 5). This might be due to the inevitable fate of the mixed healing potential of a periodontal defect. Ultimately, it is advisable that once a reentry is indicated due to any reason after a periodontal regenerative surgery, then the coronally located xenogeneic bone substitute material not embedding in bone should be removed.

### *7.3. DISCUSSION OF THE RESULTS OBTAINED BY RAMAN, SEM AND EDS*

Raman spectra have distinct features related to bone specimens' concentration that are specific for each patient in relation to bone quality, phenotype and also correspond with the history of a periodontal disease. Representative and very strong peaks (955-960 $cm^{-1}$) corresponding to immature bone (morphologically associated with an amorphous phase) are present in all reference calcium phosphate compounds except crystalline HAP and graft material. Regarding initial bone samples in case of both patients (#1 and #2) the presence of amorphous phase (representing immature bone) is noticed. After the healing, there is no evidence of amorphous phase of calcium phosphates (immature bone). It is emphasized that there is no sign of phase transition (after healing), while a true mature bone formation occurs as the result of the testified HAP crystalline phase (quantification of crystallinity degree) [Nathanael et al 2011]. The proof for a mature type of bone, where the calcium phosphate compound has a more ordered and higher crystalline nature, is also marked by a representative Raman shift (960- 965 $cm^{-1}$) in both patients after treatment. The same Raman band (963 $cm^{-1}$) is also present in the spectra of the reference crystalline HAP (Sigma- Aldrich) and the DBBM material.

After patients' investigation the following conclusions can be drawn: the initial bone of a periodontally healthy patient consists both phases (balanced amorphous and crystalline) of HAP compound, while in a patient with a history of a periodontal disease only the amorphous phase corresponding to immature bone is confirmed.

OCP is a natural precursor of HAP in the process of natural bone mineralization, therefore it is believed that, compared with other calcium phosphates, it has the most pronounced bioactivity properties [Suzuki 2010]. However, this phase transition (OCP → amorphous HAP → crystalline HAP) and biomechanical process takes place under ideal circumstances (energy from adenosine triphosphate and optimal PPi concentration). Hence, there is a high interest with the Raman shift (1023 $cm^{-1}$) representing the presence of inorganic pyrophosphate (PPi). It is known that for a certain value of PPi concentration the phase transition process occurs [Foster et al 2012]. PPi might serve as a marker of the process dynamics, therefore its quantification can be important for clinical observations. Both patients' bone samples demonstrated higher peak intensity values before treatment: 73.04% (patient #1) and 81.22% (patient #2). The highest value was obtained for patient #2, who was registered with previous periodontal problems. PPi is known to act as a potent inhibitor of HAP crystals precipitation (biological mineralization). A decreasing trend was observed for both patients: 48.76% (patient #1) and 38.39% (patient #2) after the healing period. Thus, the transition phase process is confirmed of calcium phosphates to HAP crystalline phase (mature bone). A better result of healing process is noticed for patient #1, where bone tissue developed on a larger scale than for patient #2 (PPi obtained value for patient #1 is higher than that for #2, but both of them had decreasing values compared to those before treatment). An important observation might be that the level of Raman PPi peak intensity for reference amorphous HAP is sensitively close to that of patient #1's before treatment. It might be considered that the level of about 70% of peak intensity could be a reference level marker for a good balance of the transition process from amorphous to crystalline phase (immature bone→ mature bone).

Finally the collagen (CO) content of the bony samples can be an important aspect of the Raman spectra. The spectra are more or less intense curved depending on the CO quantity. The Raman shift corresponding to collagen proteins belongs to 800 – 900 $cm^{-1}$ interval (the most relevant is the fluorescence in the spectra). In light of this, more fibrous tissue and large amount of CO are detected in patient #2 at initial state (the spectra sector 800 – 900$cm^{-1}$ is well above that for patient #1) (*Diagram VI. (a) part*). After healing, the same spectra interval for patient #1 is slightly above compared to patient #2. That observation is consistent with the amount of collagen detected in the

samples (*Diagram VI. (b) part*). The explanation for this is a better CO matrix formation around the bone substitute after the healing. It emphasized here that an encapsulation of a graft particle into newly formed bone also presupposes the organic matrix formation, which is the collagen fiber network development around it.

EDS evaluation with a trend of Ca/ P fraction value indicates that the mixed amorphous and crystalline phases turns into a crystalline phase corresponding to the DBBM used as a grafting agent. The EDS is backing the Raman results and highlights that the two phases (amorphous/ crystalline) must coexist in a more or less balanced ratio according to bone type (augmented or not) or health status (any genetically determined disease, which affects the bone metabolism).

The SEM results seems to confirm those one acquired from Raman and EDS. The initial sample structure of patients without periodontal problem mainly contains mature bone (with a corresponding crystalline phase). In contrast, the initial bone encompasses rather immature components with amorphous phases in case of patients with susceptibility to periodontitis. After the augmentation both group of patients represent crystalline phases due to the bone substitute material, but crystallites seems to be in larger number for patient #1 with better CO fibrils organization around it.

In summary, Raman spectra obtained from patients with or without history of periodontitis are quite similar, but there are differences in peak intensities or with the presence of some specific shifts. This means that the bone composition is different, mainly the rate of immature/mature bone. The initial bone of those patients who have history of periodontitis shows a lover level of mineral phase with an increased level of collagen content. This trend is changing after an augmentative surgical intervention. This is not a surprise due to the fact that bone substitute increases the mineral content with a subsequent decrease in the amount of collagen. On the contrary, the original bone of a patient with healthy periodontium contains balanced mature/immature bone, while the augmented bone confirms a better organized collagen structure around graft particles. In this study Raman spectroscopy is considered to be a promising approach for bone quality evaluation and for periodontal disease associated analyses of the bone's chemical composition [Gatin et al 2019]. However, the findings obtained by the spectroscopic method supplemented with the EDS and SEM evaluations are based on small sample sizes, therefore the results and the drawn conclusions should be used

cautiously. On the other hand, it seems worthy to continue with these kind of investigations and to initiate the in vivo use of the portable Raman spectrometer with a sterile head to utilize it under intraoperative bone measures (*Figure 45.*). It is still challenging for the future to produce the device with as narrow diameter of the head as possible in order to perform minimal invasive flaps during the examination process. It means that reentry surgery cannot be neglected with Raman technique, but bone harvesting can be avoided. Overall, neither Raman spectroscopy nor histology is a diagnostic tool used in the daily practice due to the necessity of a special technique background and a second surgery in both procedures.

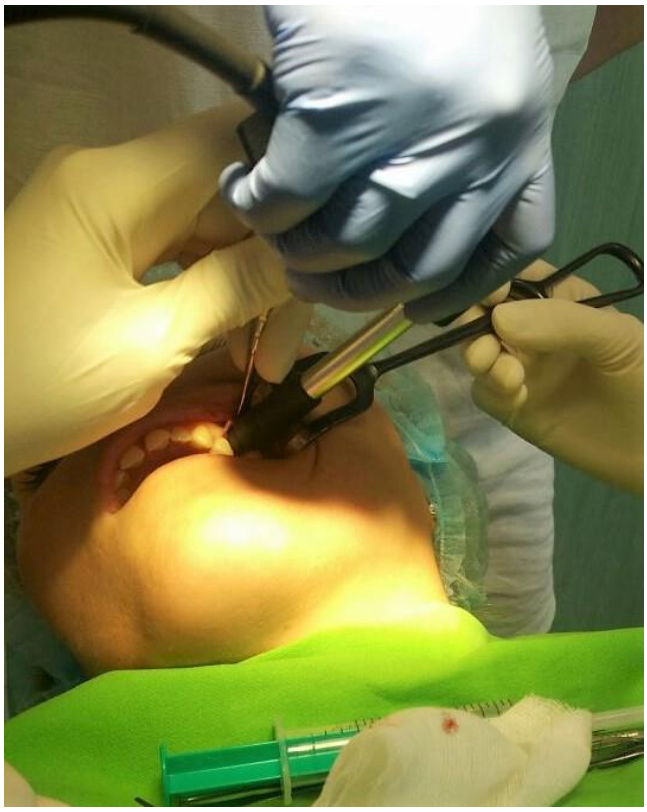

***Figure 45.*** *The in vivo use of the Raman device during an intraoperative measure with a sterilized head attached on the handpiece.*

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## 8. CONCLUSIONS

After having been the literature data analyzed through the thesis supplemented by the findings gathered from our histologic and clinical parametric results, the following conclusions can be drawn. The conclusion are divided into two parts. First part contains those statement, which corroborate the findings of the evidence based literature. Second part of the chapter comprises the candidate's new findings.

Corroborating findings:

1. When pathologic tooth migration or other malalignments have to be corrected in the presence of periodontal intraosseous lesions, a regenerative periodontal therapy bears fundamental relevance to orthodontics too.
2. It would seem essential to recommend the optimal and predictable time point of starting orthodontic tooth movement after a regenerative intervention.
3. The extent of periodontal regeneration is limited in humans. In cases of unfavorable defect morphology, full periodontal regeneration can hardly be achieved. The test and control groups testified to approximately a 2/3 intrabony fill of the former defects.
4. Coronally advanced flap design adapted to intrabony lesions is a good surgical option in cases of unfavorable defect morphology and during the use of a guided tissue regeneration technique. With the help of the mesio- distal extension and the coronal advancement of the flap, we are able not only to treat non- containing and multiple type of defects in adjacent interdental areas, but also to enlarge the space for a better coagulation. The enlarged coagulum is similarly and essentially supported by the flap design and by the GTR intervention, which is responsible for the favorable bony fill of the intrabony lesions.
5. We can conclude in the light of the data gathered from the literature, which is endorsed by our findings that the interaction of orthodontic treatment and periodontal regeneration may lead to attachment gain, new bone formation and to the correction of the malalignment. The present study's results of pronounced graft resorption in test patients, compared to control's, occurred presumably due to the effect of the orthodontic-induced bone remodeling. Hence, from a clinical point of view, it would be ideal to decrease the amount of graft ratio and consequently increase the amount of

newly formed bone and bone marrow. This is in agreement with Araújo's finding [Araújo et al 2001], who consider the biomaterial as an inactive filler, which distract the space from the bone marrow. However, the necessity of the graft material in non-containing defects cannot be neglected, therefore the advantage of OTM induced graft resorption can be another tool in order to establish a more likely original bony morphotype.

6. An early gingiva dehiscence with a concomitant secondary wound healing jeopardizes mainly the histologically proved new bone formation, resulting in a connective tissue down growth into the defect site.

7. It would be essential in the future to develop new less invasive techniques in order to gather relevant information regarding the healing of the periodontal tissues. Raman spectroscopy, as a highly sensitive method to characterize bone, can be a future alternative. However, we still lack enough clinical data about the reliability of the tool and how it can be used for in vivo studies. In addition, it is further a challenge optically how to make access to periodontal tissues from a less invasive exploration. Until that time histology and histomorphometry remain the gold standard for assessment and quantitative description of the structures in the tissues.

New findings:

1. There has not been previously published any human histological evaluation of the combined OTM + GTR techniques. The candidate and his coworkers presented the first human histological data on this comprehensive treatment.

2. It was one of the first human histology conducted with a less invasive trephine biopsy technique to evaluate the microscopic process of wound healing and bone regeneration after orthodontic tooth movement around periodontal defects.

3. It proved that both the pressure and tension sides showed marked new bone formation and remodeling.

4. It appears that an early (1 week postoperatively) initiation of tooth movement is safe, without influencing negatively periodontal wound healing.

5. The extent of periodontal regeneration of unfavorable defects were comparable both in the control and test groups.

6. Periodontal regeneration is successfully applicable combined with orthodontic tooth movement, resulting similar clinical endpoint parameters, than used alone without OTM.

7. It was one of the first attempt to investigate the feasibility of the RAMAN technique for the evaluation of the bone’s composition and quality.

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## 9. SUMMARY

The author's principal investigation was based on a RCT, where unfavorable periodontal intrabony defects were treated with the use of extended, coronally advanced flaps according to the GTR techniques with the utilization of DBBM particles. Patients after surgical intervention were randomly allocated either to test group with an early initiation of OTM or to control group in which the teeth healed splinted. After the observation period of 9 months, reentry procedures were scheduled.

Primary outcome variable was the qualitative and quantitative micromorphological analysis of the harvested bony specimens in order to reveal the biologic and cellular response of tooth movement on the hard tissue healing promoted by the DBBM. Secondary outcome variables were the changes of the clinical parameters within groups and the differences of the changes between the test and control groups.

The histologic results demonstrated an increased active resorption of the graft material along with new bone formation around the particles and between the bony lamellas. Histomorphometry showed in both test subgroups a noteworthy reduced amount of graft particles together with an increased percentage of de novo bone formation compared to control samples. There was a correlation between the reparative healing type of a connective tissue encapsulation of the bone substitute material and the early wound failures. The improvement of clinical parameters from baseline to endpoint was desirable in both groups. However, there were no statistically significant difference in the change of the values between the two groups, which retained our null hypothesis. Nevertheless, orthodontic- induced DBBM remodeling is a unique phenomenon and may be beneficial for patients treated with a combined periodontal- orthodontic approach, as it might result better stability of the grafted sites in the long-term.

In the second case series study the Raman spectroscope was introduced as a new device in order to establish differences between the quality of the bone of patients with or without the history of periodontitis and between the initial and augmented bone of these patients. The results of the Raman spectra supplemented by SEM and EDS in vitro measures revealed in increased amount of immature type of initial bone and a higher level of collagen content with periodontitis patients. It seems that there is a better organic component (collagen) organization around the graft particles in the augmented bone of healthy patients based on small sample sizes.

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## 10. ÖSSZEFOGLALÁS

Az elsődleges RCT vizsgálatunkban kedvezőtlen morfológiájú parodontális intraosszer defektusokat kezeltünk kiterjesztett, koronálisan elcsúsztatott lebenyek segítségével az GTR technika szabályai szerint DBBM graft anyag használatával. A pácienseket a sebészi beavatkozást követően random módom teszt vagy kontroll csoportba soroltuk. Az előbbi csoport a GTR műtétet követően korai inicializálású fogszabályozásban részesült, míg utóbbi csoportban fogmozgatás nélkül, tartós ideiglenes sínnel gyógyultak a vizsgált fogak. A 9 hónapos megfigyelési periódus leteltével egy második feltárást végeztünk biopsziás mintavételhez.

Az elsődleges eredmény változó a csontminta kvalitatív és kvantitatív mikromorfológiai analízise, míg a másodlagosak a klinikai paraméterek változása a csoportokon belül illetve e változások különbözősége a két csoport között voltak.

A hisztológiai leletek a teszt csoportban a DBBM aktív reszorpcióját igazolták újonnan képződött csont kíséretében a partikulumok körül és a csont lamellák között. A kontroll csoport mintáinak koronális harmadában a bioanyag inkább kötőszövetes beágyazódását tapasztaltunk. A hisztomorfometria mindkét teszt alcsoport esetén szignifikánsan redukált graft szemcse mennyiséget mutatott egy százalékosan megnövekedett új csontképződéssel egyetemben a kontroll csoporthoz viszonyítva. A csontpótló anyag kötőszöveti tokban való rögzülése és korai sebgyógyulási zavarok közötti kapcsolat jól kimutatható volt. A klinikai paraméterek javulása a kezdetitől a végállapotig mindkét csoport esetén jelentős volt. Azonban az értékek változását illetően nem volt statisztikailag szignifikáns különbség a két csoport között. Mindazonáltal a fogszabályozás által kiváltott DBBM remodelláció egy egyedülálló jelenség. A kombinált ortodonciai- parodontális kezelésben részesülők esetében hosszútávon egy stabilabb, ellenállóbb csontpótolt terület kialakulását eredményezheti.

A második esetsorozat vizsgálat során a Raman spektroszkópot, mint egy új eljárást teszteltük, hogy különbözőségeket tárjunk fel a csont minőségét illetően parodontitiszes és egészséges páciensek, illetve ezen egyének eredeti és augmentált csontmintái között. A Raman spektrumok eredményei, melyek kiegészültek SEM és EDS in vitro mérésekkel is, megnövelt éretlen típusú csont és kollagén tartalmat mutatottak a parodontitiszes páciensek eredeti csontjában. Az egészséges páciensek augmentált csontjában a graft szemcsék körül strukturáltabb szerves állomány figyelhető meg.

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## 12. THE BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

### *12.1. PUBLICATIONS RELATED TO THE THEME OF THE PHD THESIS*

Published in peer reviewed journals:

**Nagy P**, Molnar B, Nemes B, Schupbach P, Windisch P. (2019) Histologic evaluation of human intrabony periodontal defects treated with deproteinized bovine bone mineral in combination with orthodontic tooth movement: a case series. Int J Periodontics Restorative Dent, 40: 321–330.

**Nagy P,** Pörzse V. (2020) Preortodonciai kortikocízió és szimultán csont augmentáció a bukkális alveoláris dehiszcencia prevenciójában: Esetismertetés. Fog Sz, 113: 74-81.

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Martin A, Papp Z, Nemes B, **Nagy P**, Windisch P, Molnár B. (2018) Complex periodontal, orthodontic and implant-prosthodontic rehabilitation of a patient with generalized aggressive periodontitis: a case report. J Clin Periodontol, 45(S19): Paper PC404. Euro Perio 9. Amsterdam, Netherlands

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## *12.2. OTHER PUBLICATIONS BY THE AUTHOR*

Published in peer reviewed journals:

Fazekas R, Molnar E, **Nagy P**, Mikecs B, Windisch P, Vag J. (2018) A proposed method for assessing the appropriate timing of early implant placements: a case report. J Oral Implantol, 44: 378-383.

**Nagy P**, Kövér K, Gera I, Horváth A. (2016) Elektromos és kézi fogkefék hatékonyságának összehasonlítása az orális prevencióban: Irodalmi áttekintés, systematic review, meta-analízis. Fog Sz, 109: 3-22.

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