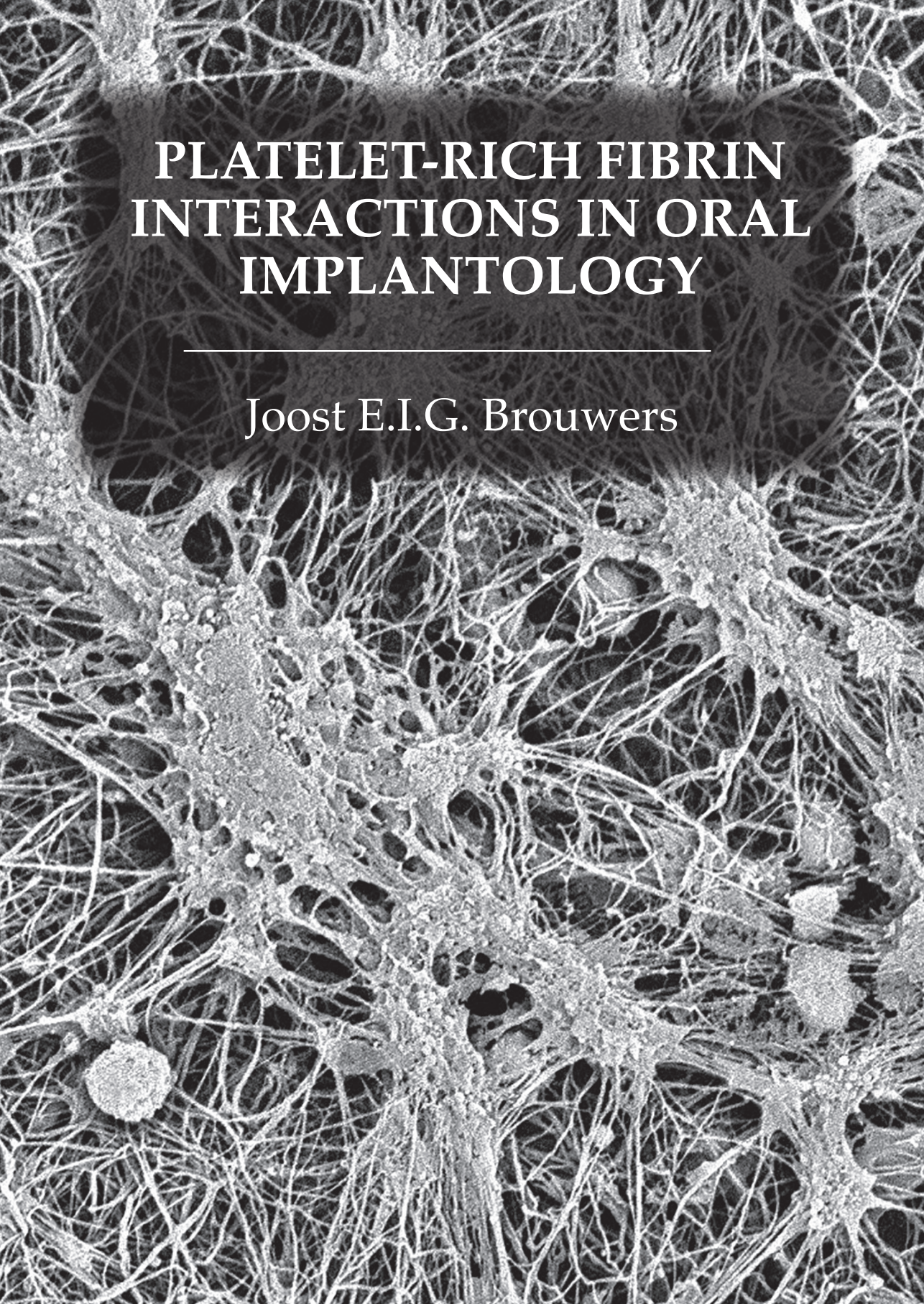
Platelet-rich fibrin interactions in oral implantology



PLATELET-RICH FIBRIN INTERACTIONS IN ORAL IMPLANTOLOGY

Joost E.I.G. Brouwers

Stellingen

1. Fibrin is a remarkable biological object with continual new suprises (Weisel & Litvinow, 2018).

2. Blutkonzentrate sind kein Produkt (Shahram Ghanaati, 2021).

3. Implant stability is associated with several hematological parameters (this thesis – Chapter 5).

4. Performing invasive oral procedures in the morning may be beneficial for patients with bleeding tendency (this thesis – Chapter 7).

5. The selection of proper collection tubes for preparing Platelet-Rich Fibrin is of great importance (this thesis – Chapter 8).

6. The influence of oral anticoagulation on PRF treatment outcome is still an unanswered question.

7. If all research projects would work, then we are doing the wrong research.

8. Le meilleur médecin est la nature: elle guérit les trois quarts des maladies et ne dit jamais de mal de ses confrères (Louis Pasteur 1822-1895).

9. Als je in je vijf en zestigste levensjaar promoveert, betekent dat onherroepelijk dat je géén gevaar vormt voor de jonge generatie wetenschappelijke onderzoekers. En dat is maar goed ook!

10. Het is totale nonsens dat een wijnglas voor witte wijn kleiner moet zijn dan een wijnglas voor rode wijn.

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PLATELET-RICH FIBRIN INTERACTIONS IN ORAL IMPLANTOLOGY

De invloed van bloedplaatjes fibrine concentraat in de orale implantologie

(Met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van Rector Magnificus, prof. dr. Pamela Habibović
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 23 juni 2022 om 10.00 uur

door

Joseph Everard Ida Gerardus Brouwers

Geboren 16 november 1956, te Heerlen

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“De wetenschap heeft geen andere vijand dan de onwetende”
Clément Marot (1496 – 1544)

Aan mijn moeder
Voor Flos, Titou en Zsa-Zsa

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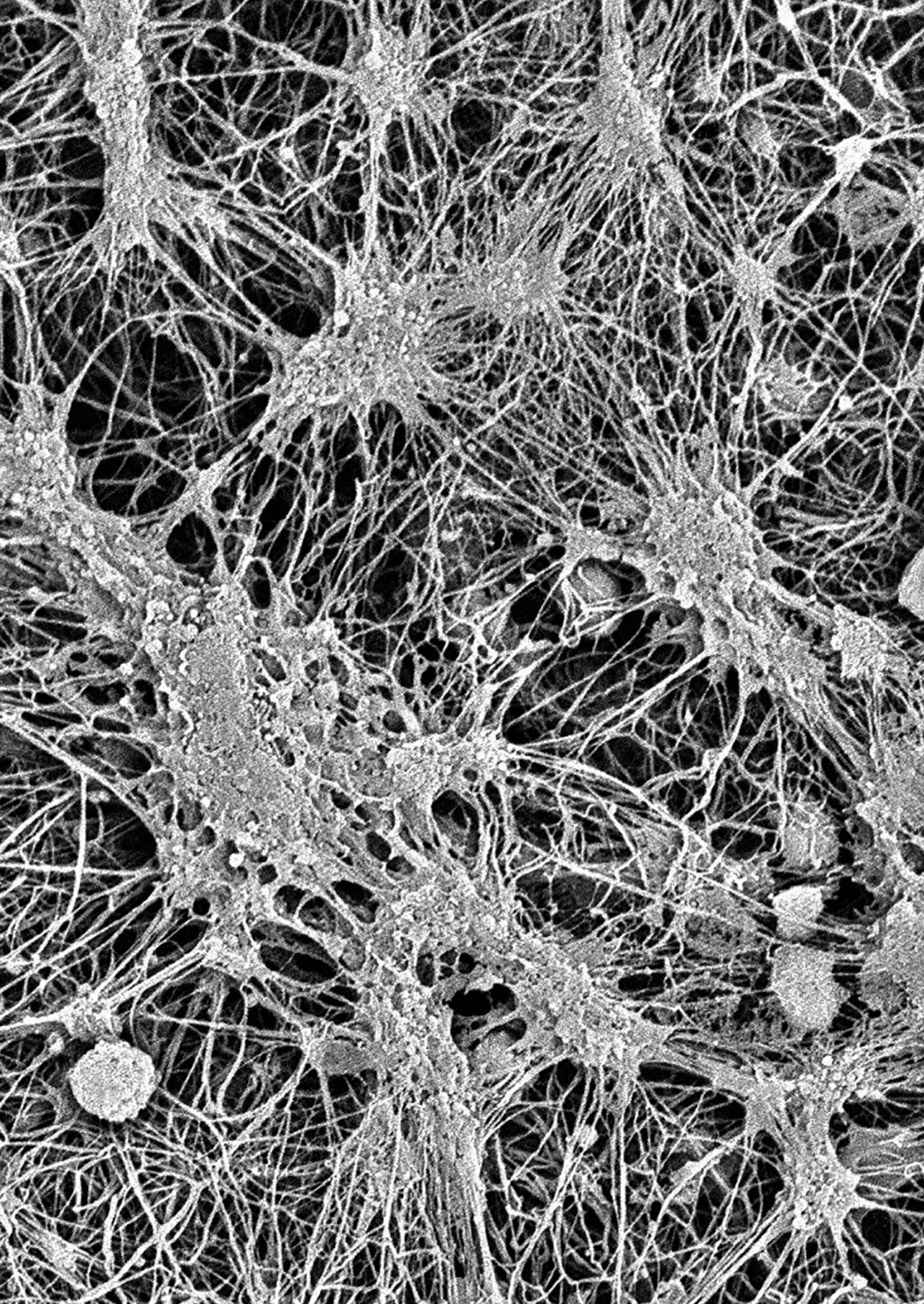


Chapter 1

General introduction

History of oral implantology

Since the introduction of oral implants by Prof. Dr. Per-Ingvar Brånemark[1] in the early sixties, clinicians have been searching for surgical protocols and techniques to enhance the predictability and success of oral implant procedures. The fusion of titanium with bone was already described in 1940 by Bothe et al.[2] In 1951 a similar mechanism was found and described by Leventhal et al. [3] The term osseointegration was introduced by Prof P-I Brånemark in 1969.[1] He defined osseointegration as "the formation of a direct interface between an implant and bone, without intervening soft tissue." In 1985 Lekholm and Zarb modified this definition of osseointegration into: "A process whereby a clinical asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading".[4] Osseointegration is a dynamic process. The roughness of the surface for instance and the geometry of the implant design, play an important role on a microscopic level. Cellular and molecular remodeling can take place. These findings have led to the development of different geometries and surface textures, different implant/abutment interfaces, prosthetic platforms and this all to enhance and speed up the osseointegration and maintain bone level.[5,6] Major advancements have been made in developing novel surfaces of dental implants (Figure 1). These innovations set the stage for rehabilitating patients with high success and predictable survival rates of these implants even in challenging conditions,[7] such as immediate or early loading of implants in patients with quantitatively as well as qualitatively compromised bone.

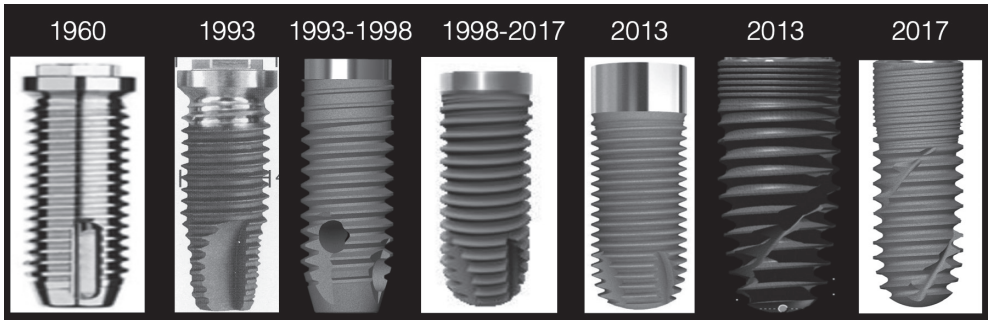


Figure 1. Development over time of implant surface and implant design.

Some decades ago, osseointegration periods of six months in the maxilla and three to four months in the mandibula were well accepted.[8] Nowadays treatment options exist for early loading (between 1 week and two months) or immediate loading (within a week after implant placement).[9,10] But nevertheless, there is an ongoing search for more predictable and faster methods for the osseointegration of oral implants with better bone conditions and better outcomes on the long term. Because oral implants become a more important treatment option in the treatment array of the dentist, cases become more challenging.

Age of the patients, with more underlying medical problems, as well as the group of young people having congenitally missing teeth and patients having trauma in the anterior esthetic zone, are both increasing. In addition, the increase in prosperity resulted in much higher esthetic demands and with that the complexity of cases.

Implant stability in oral implantology

Measurement of oral implant stability is an important parameter that predicts the outcome of treatment strategies in oral implantology. Primary implant stability has been identified as a pre-requisite for achieving full osseointegration.[11-14] Achieving and maintaining implant stability is of paramount importance for the success of oral implants over time. Primary stability is achieved through the mechanical engagement between oral implant and surrounding bone. Primary stability is of major importance for a good secondary stability and is depending on bone quantity and bone quality, surgical technique, implant geometry and implant surface conditions (Figure 3). Secondary stability represents the biological stability through the bone regeneration and remodeling. It is non-existent immediately after oral implant placement. It becomes apparent only as new bone cells are formed at the implant site, and increase in time (Figure 4).

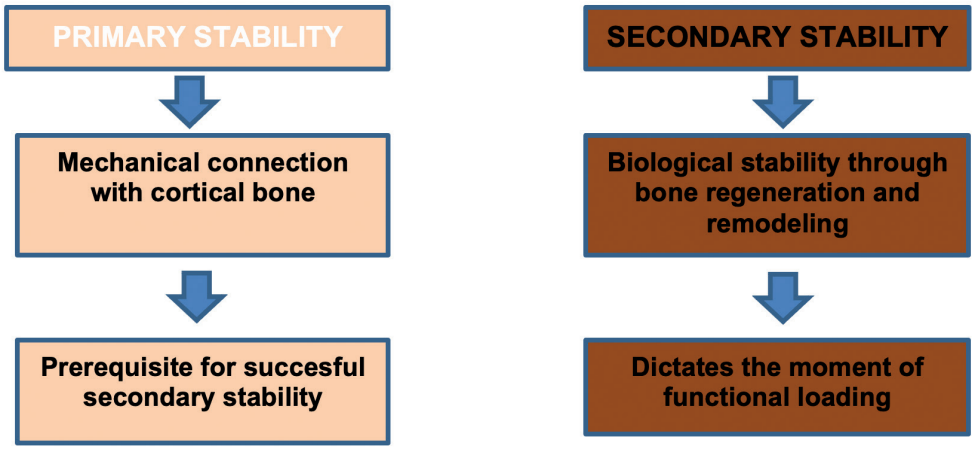


Figure 3. Differences between primary and secondary stability.

There are different ways of interpreting the stability of an oral implant in time:

- Radiographic evaluation of the implant stability is a non-invasive technique but is associated with many problems. There will always be some crestal bone loss over time. Evaluation of different radiographs in time is difficult because of lack of standardized reproducibility.
- Cutting torque resistance analysis can be done, but only at implant placement.[14]
- Removal torque values can be measured, but are unethically.[15]

- Percussion test is a simple method of evaluating implant stability, but are neither standardized, nor reproducible and not quantifiable.
- Periotest is a non-invasive method of measuring oral implant stability anytime during and after implant treatment, but has poor reliability.[16,17]
- Resonance frequency analysis (RFA) has been introduced more than 20 years ago by Meredith and coworkers for measuring implant stability based on vibration engineering and resonance frequency analysis.[13,18] RFA is nowadays the golden standard for non-invasive stability measurements.

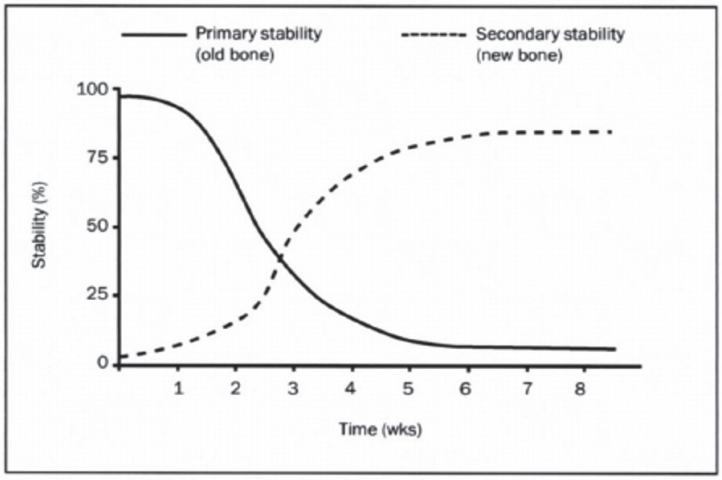


Figure 4. Implant stability development in time.

Resonance Frequency Analysis

Since 1996 the introduction of RFA, the Osstell device was the only instrument on the market to measure ISQ (Implant Stability Quotient) values.[19] Over time the Osstell Company developed different RFA measuring devices. It all started with an L-shaped transducer (Figure 5) which was connected with a cable and was screwed on the implant. Later on, this transducer was changed into an aluminum peg (Smartpeg™) which could be screwed in the implant.

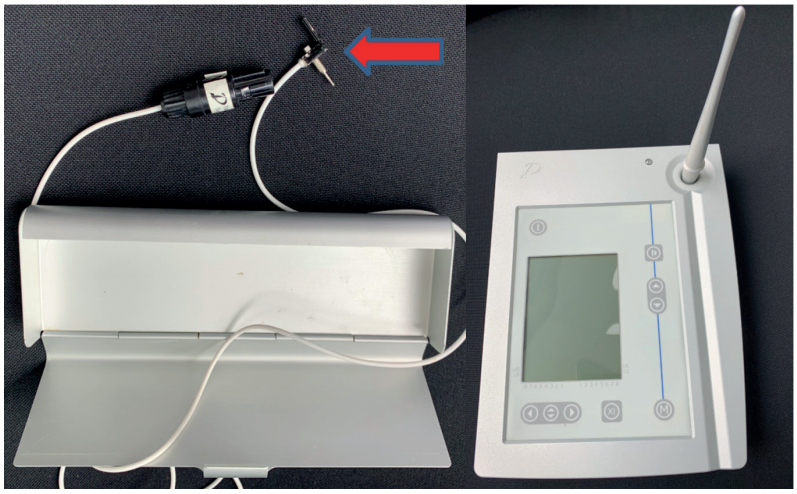


Figure 5. L-shaped transducer and the first commercial available Osstell measuring device.

One of the latest models was the Osstell ISQ; this was at the moment of this undergoing research the most compact machine of the Osstell Company. In 2015 PenguinRFA (Integration Diagnostics Sweden AB, Gothenburg) was introduced in 2015 by Lars Sennerby and Anders Petersson. They created a new transducer called MulTipeg™ and a cordless measuring device the Penguin. The MulTipeg™ is made of autoclavable titanium, while the (Smartpeg ™, Osstell) is made of aluminum. The striking difference between the two devices is reusable versus one-way transducers, and cable free versus cabled.

Biomaterials in oral implantology

For optimal osseointegration there is a need of bone regeneration and/or soft tissue regeneration procedures before implants can be placed.[20] There is an enormous range of different materials which can be used for patients with a need of bone-and/or soft tissue regeneration. We can divide them into different groups; autologous, allogenous, xenogenous and alloplastic materials. The most commonly used materials are: autologous, xenogenous or alloplastic materials. According to the literature the gold standard still is autologous material.[21,22] One of the disadvantages of autologous material is the limited amount of material available that can be harvested in the oral cavity.[23,24] As a result of these findings a combination of autologous material and xenogenous material could be of great benefit in bigger size defects. If the defects are of major category than a graft from the iliac crest is momentarily the only viable option. A major disadvantage of this procedure is the necessity of hospitalizing the patient.

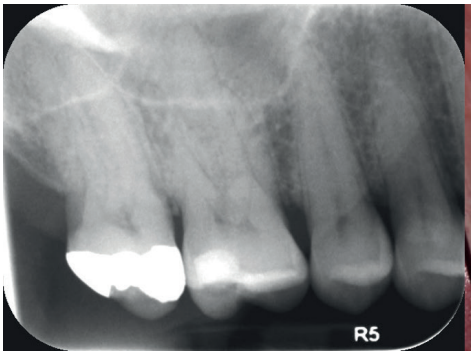
Xenogenous materials are materials harvested from different species. The most common sources used are equine, porcine, or bovine sources. The bone is deproteinized, sterilized, and it is both physically and chemically the same as the mineral phase of human bone. All

organic material is removed to mitigate immune reactivity or pathogen transmission. The remaining minerals act as a scaffold for native bone growth. The autogenous bone might be stimulated through the use of growth factors in combination with the xenogenous graft or allograft. Bone formation occurs mostly via osteoconduction. The resulting crystal structure is described as being rather similar to human cancellous bone.

Alloplastic grafting material is synthetically derived or produced from natural materials. This type of grafting material has major advantages like zero risk of disease transmission and low antigenicity. The most prevalent types of alloplasts are hydroxyapatite, dicalcium phosphates, and bioactive ceramics. These alloplasts can be used in combination with autogenous bone to provide an osteoconductive framework for bone. When transplanted, osteoid is produced directly onto the ceramic surface by native bone and later undergoes remodeling. Particle size and porosity influence the resorption rates of alloplasts along with other physical properties. Larger particles have a greater expectancy to remain at the augmentation site.

Greater porosity of alloplasts has faster resorption rates than osteoclasts and penetrates the graft more readily than dense materials.

After tooth extraction, the healing phase is initiated. Dimensional loss of bone height and bone width is a natural consequence. In the first eight weeks after extraction the biggest amount of resorption will take place through osteoclastic activity. This resorption is occurring buccally as well as lingually. The resorption is more pronounced on the buccal site in comparison with the lingual site.[25] These findings are of major importance in the anterior esthetic zone, but the literature is not conclusive about protocols to follow in the anterior esthetic zone.



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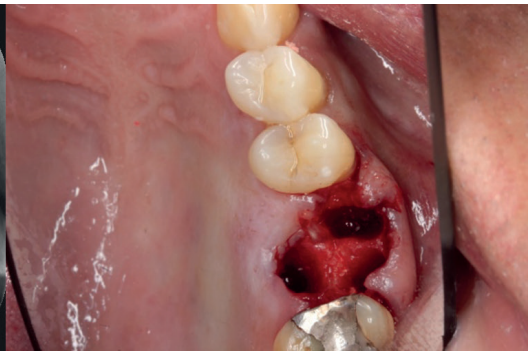

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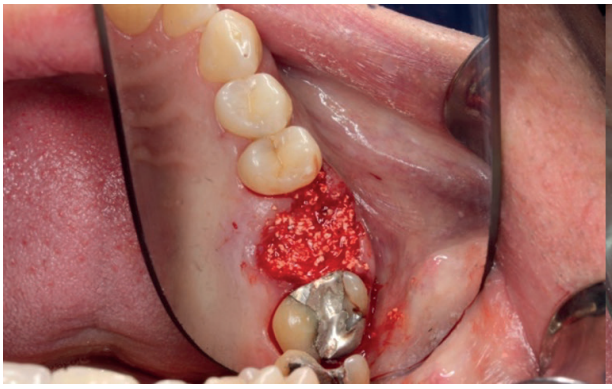

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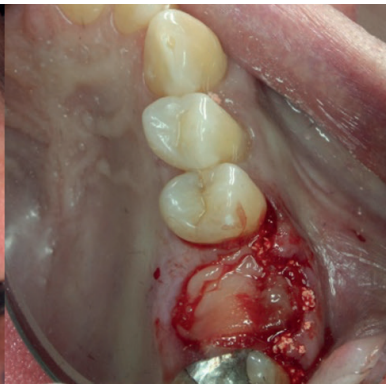

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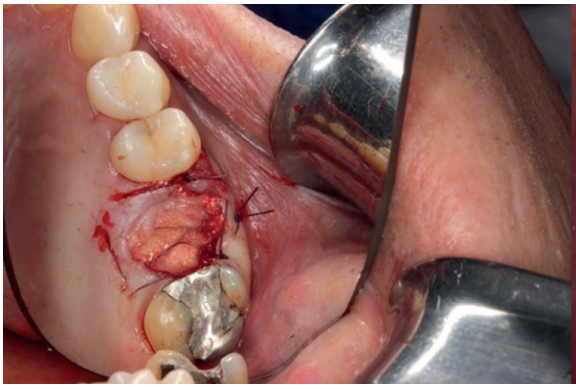

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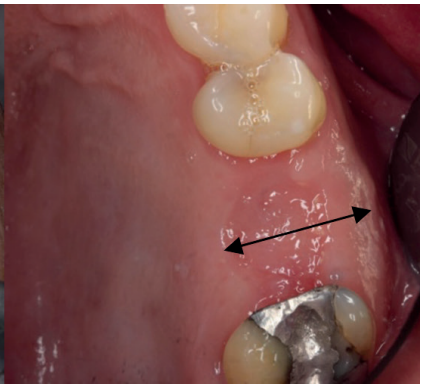

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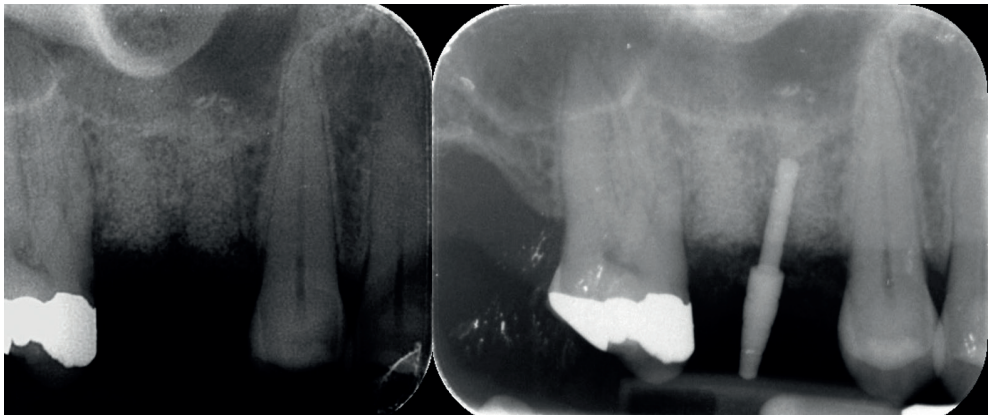

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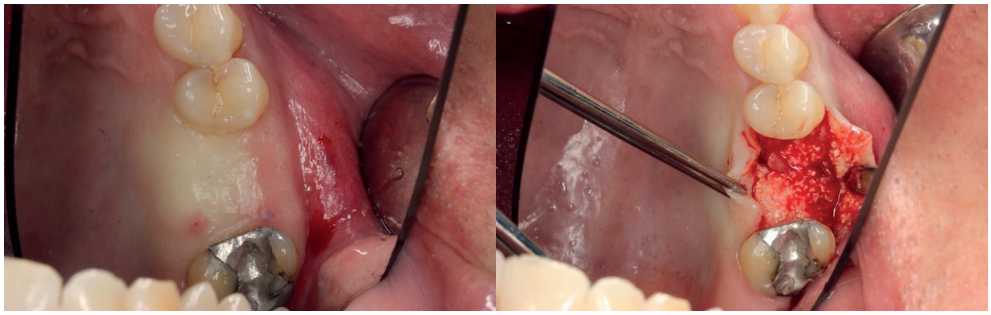

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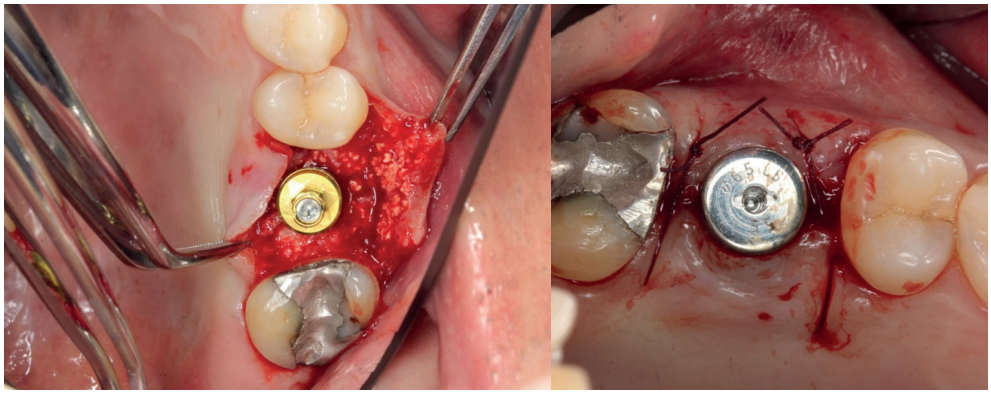


K L

Figures 2. A-L shows the protocol of extraction and site preparation.
(A)x -ray picture before extraction. (B) Occlusal view of the extraction site. (C) ridge preservation with DMBB and PRF parts incorporated in the mixture. (D) closing of the site with a PRF membrane. (E) PRF membrane sutured in place. (F) preservation of the width of the bone. (G) x-ray of the ridge preservation. (H) direction indicator in place. (I) after 10 weeks amount of preserved ridge. (J) opening of the site after 10 weeks, shows the amount of bone and preserved width. (K) implant installed in the site. (L) healing abutment after implant insertion, note the precise positioning of the implant in regenerated bone.

Immediate replacement of a lost tooth in the esthetic zone is a viable option! We need different approaches for a predictable result over time? Chen and Darby reported that eight weeks after flapless extraction, a reduction in orofacial dimensions of the ridge takes place due to resorption of the facial bone. The biggest facial alterations were found in sites were upfront dehiscence's and fenestrations were present. Furthermore, they found that the phenotype of the soft tissue plays an important role in the outcome of the treatment.[26,27] Another conclusion was that the soft tissue will thicken after extraction in the presence of a thin buccal wall (times 7). Narrow buccal walls are more complicated and less predictable than thicker buccal bone walls. A good understanding of the biological process which takes place after flapless extraction of anterior teeth is of paramount importance for the esthetic outcome of an immediate replaced tooth by an oral implant. In the understanding of the biological process a lot of research has been conducted the last two decades. Especially the introduction of autologous platelet-rich fibrin concentrates offers promising results in the immediate replacement treatment of natural teeth with dental implants.

Platelets in hemostasis and wound healing

Wound healing is a dynamic physiological process that restores the normal architecture and functionality of damaged tissue. Wound healing consists of three sequential phases of (1) acute inflammation (minutes, hours, days after injury); (2) proliferation and new tissue formation (days, weeks); and (3) remodeling (weeks, months, years).[28,29]

The different phases of wound healing are displayed in Figure 6. Immediately after tissue damage the first phase of wound repair is initiated. This phase starts with the formation of a platelet plug, followed by the activation of the coagulation cascade which results in thrombin formation and the strengthening of the platelet plug by a fibrin matrix. The fibrin matrix will serve as a scaffold for infiltrating cells. The simultaneously activated inflammatory and immune pathways are crucial to remove cellular debris and devitalized tissues and to prevent infection.

The second phase occurs between one and three weeks after injury. This phase mainly involves the migration and differentiation of different cell types. An important process in this phase is angiogenesis, growth of new blood vessels from preexisting ones, involving the action of endothelial cells.

The third phase of wound repair is remodeling which starts within 2-3 weeks of injury and can last for years depending on the wound characteristics.

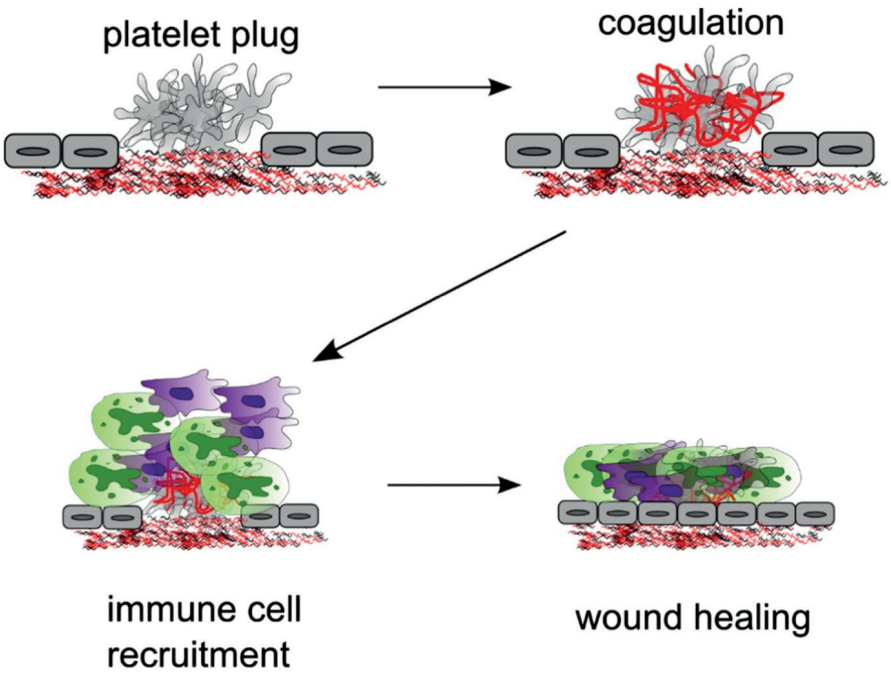


Figure 6. Simplified illustration of events leading to vessel injury repair.[30]

Platelets are widely recognized as having a critical role in primary hemostasis and thrombosis, increasing experimental and clinical evidence identifies these enucleated cells as relevant modulators of other physio pathological processes including inflammation and tissue regeneration. The growth factors present in the alpha granules of the platelets and released during the activation are mediators to promote tissue regeneration.[31] Due to their primary autologous origin, there are no concerns about immunogenetic reactions or disease transmission. Platelet enriched materials have become highly relevant in the last decade and constitute a growing object of experimental and clinical study in the context of wound healing and bone regeneration. Platelet concentrates are therefore often used to stimulate wound healing and bone regeneration. Two different methods are mostly used, PRP (platelet rich plasma) and PRF (platelet rich fibrin). Their differences are predominantly found in the collecting of the patient's own blood. PRP is collected in plastic tubes containing an anticoagulant; PRF is collected in glass tubes without any anticoagulant.

Platelet Rich Fibrin in oral implantology

Recently the focus in bone- and soft tissue grafting shifted to the use of platelet concentrates as a regenerative treatment. In the late nineties Whitman[32] and Marx[33] introduced PRP, the first-generation platelet concentrate. It is widely applied in different clinical scenarios. There are various methods described for the preparation of the PRP. The classic method of

preparing PRP involves two sequential centrifugation steps (Figure 7); namely, separation and concentration spins.

PRP can be activated by various compounds, such as autologous thrombin or calcium chloride, which induces the formation of a PRP gel.[34]

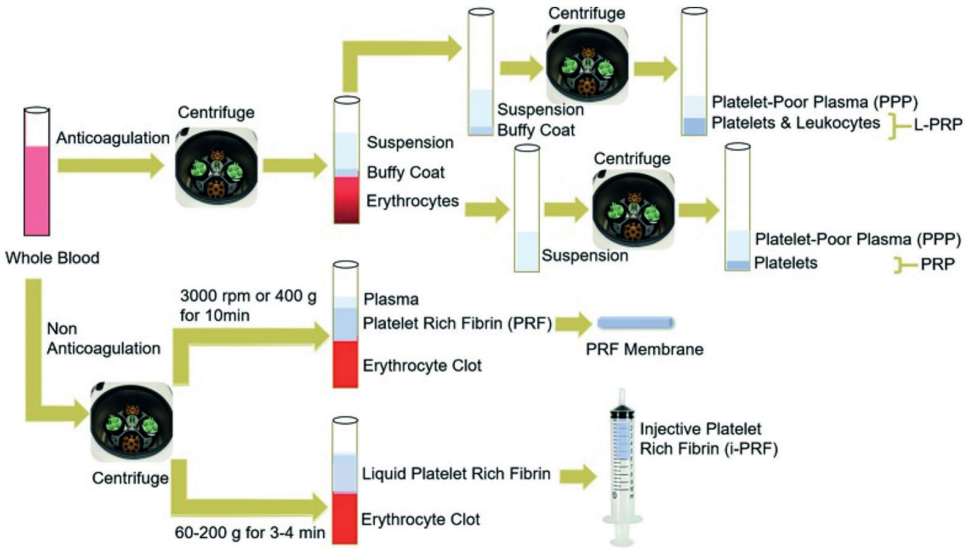


Figure 7. Flowchart of platelet-derivate preparations.

Whole blood is collected with an anticoagulant (top). Two sequential centrifugation steps occur under different force, speed and duration conditions. The first step separates erythrocytes from the buffy coat layer and the upper suspension, containing plasma and platelets. After a second spin, the buffy coat und upper suspension are separated into platelet-poor plasma (PPP) and leucocyte-rich plasma (L-PRP). A second centrifugation step of the upper suspension layer produces PPP and PRP. To generate PRF, whole blood is processed by a single centrifugation step without any anticoagulant (bottom). By means of different force, speed and duration conditions, a PRF membrane and platelet-rich fibrin (i-PRF) are generated separately.

Studies into the clinical efficiency of PRP are not conclusive and one of the main reasons is the difference in preparation techniques. It is a very operator sensible system, with high costs for the patients.

The disadvantages of PRP comprise the complex preparation in which Calcium Chloride ($CaCl_2$) and bovine thrombin have to be pipetted into the concentrate to achieve clotting,

depending on operator efficacy. So, it is not a 100% autologous concentrate. Soon it was adapted by the dental profession, but in time the limitations of this treatment became visible, probably because of lack of a standardized protocol. Given the paucity of RCT's (randomized controlled trials) relating to this topic, the scientific evidence regarding efficiency and efficacy is still controversial. The majority of these RCT's has been conducted using different graft materials and applying different protocols.[35,36]

Albanese et al. reported in their review article on PRP that the use of PRP in extraction sockets enables the improvement of soft tissue healing and influences positively bone regeneration but the effects disappear in a few days after the treatment. They found promising results of the use of PRP in implant surgery and sinus lift procedures. Some positive effects in the treatment of necrotic bone curettage were found as well.

In 1999 Anitua introduced the PRGF(Platelet rich in Growths Factors) procedure, a mix between the PRP method and the PRF.[37] The disadvantage of this system is that $CaCl_2$ is still needed to accelerate the clotting. This method appeared to be time consuming and has high costs.

Subsequently Nelson Pinto presented L-PRF (Leucocyte- Platelet-rich Fibrin), a second-generation platelet concentrate, at the 4th Congress of the World Union of Wound Healing Societies in the year 2000. Meanwhile, Choukroun introduced his Platelet-rich Fibrin also a second generation platelet concentrate.[38]

Choukroun, as well as Pinto claim to be the inventors of the PRF system; Choukroun introduced it as PRF and Pinto chose for L-PRF.

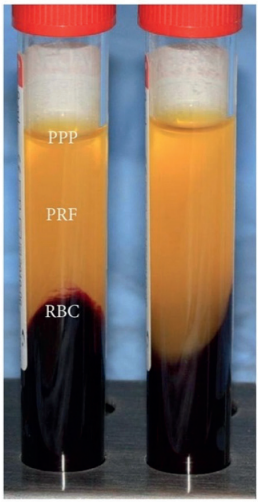
Platelet concentrates have been used for several decades in regenerative medicine, sports medicine, oral and maxilla facial surgery,[39] ear, nose and throat surgery,[40] neurosurgery[41] and plastic surgery[42] to improve the outcome of wound healing. Studies have shown reduction of pain, swelling, and infection and increase of soft tissue healing and bone density.[43,44] PRP differs from PRF because PRP does not contain a fibrin network; it is a platelet solution.

Research is inconclusive about the benefits of the treatment with platelet products. Protocols are not standardized and the product is often not well-characterized.[45]

The importance of platelets is illustrated by the role in vascular healing, hemostasis and thrombosis. Hypothetically, increasing the number of platelets will increase their effects on wound healing. Platelets release growth factors that are crucial in wound healing.[46,47] Growth factors that are secreted by the platelets include platelet-derived growth factor(PDGF), vascular endothelial growth factor(VEGF), epidermal growth factors (EGF), platelet-derived

endothelial growth factor (PDEGF), insulin like growth factor (IGF), fibrinogen, vitronectin, fibronectin, and thrombospondin-1, transforming growth factor-β (TGF-β).[43,46,48] Growth factors are involved in cell replication, bone resorption, attraction of undifferentiated cells and trigger cell division which are essential in wound healing.[46,47] In addition, other factors such as clotting factors, chemokines, cytokines and adhesive proteins are also secreted by platelets which might be important in wound healing.[49]

Nevertheless, multiple studies report no significant differences in wound healing between the group with a platelet product and the control group. In addition, a long-term follow-up study of 5 years did not show significant differences between the PRP group and the control group.[50] One of the explanations of the discrepancy between the results is the lack of standardization of protocols and characterization of the platelet-product.[51] There are many different protocols for the production of PRP or PRF with different centrifugal forces, times and types of centrifuges. Often the platelet product is not thoroughly characterized and methods are incompletely described. Information about blood panels, membrane histology and morphology are hardly presented. It is impossible to compare results due to incomplete information about the production and characterization of the product. Many parameters can influence the platelet yield in platelet products and a slight change of the protocol can result in different composition of the platelet product.[36,52,53] It was Ehrenfest et al. who showed us that terminology is confusing and that both standardization and consensus are lacking.[51] Miron et al. concluded later that the use of trade names like L-PRF and A-PRF (Figure 8)and the different centrifugal forces led to a lot of confusion in the field.[54]



The upper fraction containing the Platelet Poor Plasma (PPP)

The middle Fraction containing the Fibrin Clot (PRF)

The lower fraction containing The Red Blood Cells

Figure 8. 10 cc of whole blood processed for 8 minutes at 200G in a Duo Quattro centrifuge (Process for PRF, Nice, France), these settings were used during the whole study.

Many researchers claim the ability of platelet-rich fibrin to reduce the alveolar resorption after tooth extraction, the reduction of post extraction discomfort and therefore the use as a ridge preservation material.[55,56] Khiste and coworkers called it a "biofuel for tissue regeneration".[57] And exactly these differences led to a lot of confusion in the field of Platelet-rich Fibrin.

Aim and outline of the present thesis

The objective of the studies described in this thesis is to investigate the interactions between platelet-rich fibrin and the surrounding tissues of an oral implant, e.g. bone and soft tissue response.

In Chapter 2 we show the importance of the use of platelet-rich fibrin in a well-documented case report with a follow-up period of more than two and a half years. In Chapter 3 we describe the reliability and the validity of instrumental implant stability assessment (resonance frequency analysis) with the early Osstell™ apparatus. Chapter 4 investigates if there are differences in outcome of the RFA (Resonance Frequency Analysis) measurements (ISQ values) with two different devices in the study population. This because it has been suggested in recent literature that the use of platelet-rich fibrin concentrate could increase the implant stability.[58] Since there was inconclusive research on the differences between the two devices in combination with the use of platelet-rich fibrin, we incorporated this into our investigations. Chapter 5 describes the effect of peripheral blood cell composition and coagulation factors on the implant stability in patients treated with platelet-rich fibrin. Chapter 6 shows histological evidence for osteoconductive effects of autologous platelet-rich fibrin in oral implantology. As early as in the 1920s and 1930s Hunter and Glazko studied the role of blood clothing factors in human saliva. Since saliva contains coagulation factors, Chapter 7 describes the role and diurnal rhythm of salivary tissue factor. We noticed that the duration of post-extraction bleeding is highly variable between patients. Therefore, we hypothesized that this could be caused by variation in saliva derived TF-induced clotting activity. We aimed to assess the variability of saliva-induced thrombin generation (TG) in healthy individuals.

In Chapter 8 the findings of the studies described in Chapters 2-7 are integrated and discussed in a broader context and future projects are presented.

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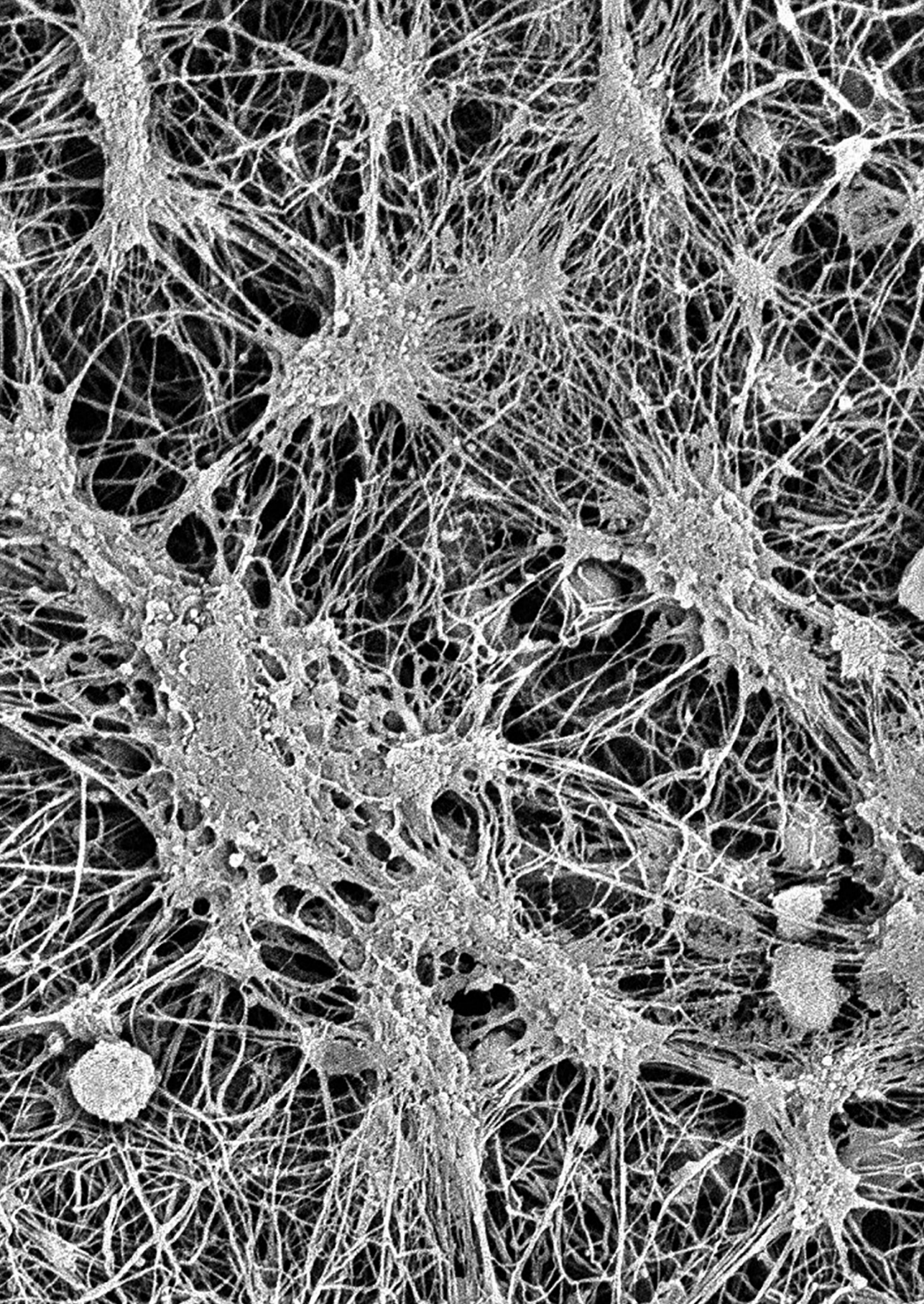

Chapter 2

Successful soft and hard tissue augmentation with platelet-rich fibrin in combination with bovine bone space maintainer in a delayed implant placement protocol in the esthetic zone

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Abstract

Background:
Placement of dental implants in the anterior maxilla region is challenging due to anatomically difficulties and pathologies such as gingival recession and bone loss. The anterior maxilla region is esthetically a precarious region.

Purpose:
A patient presented after a field hockey injury with an enormous gingival deficiency and a fractured central incisor (tooth number 11) with buccal bone loss. The affected site was not suitable for immediate replacement since there were large soft and hard tissue deficiencies.

Material and Methods:
The buccal bone deficiency was restored by a combination of Platelet Rich Fibrin (PRF) and a bovine bone space maintainer. After four months, the BEGO Semados® RS implant was placed in the augmented site. Implant stability was achieved in the augmented bone (30 Ncm, ISQ 65). In order to improve soft tissue thickness, PRF was used to restore gingival tissue.

Results:
Sufficient bone augmentation and soft tissue growth was achieved using PRF in combination with bovine bone. The augmented bone provided enough implant stability for successful implantation. The gingival deficiency was restored and the patient was pleased with the esthetic outcome.

Conclusion:
In the present case report we show that introduction of PRF in combination with bovine bone in the treatment of extreme buccal bone and soft tissue deficiencies, results in successful augmentation of buccal bone loss as well as increased amount of soft tissue in large defects.

Introduction

The anterior maxilla region is an anatomically difficult region for dental implantation. Soft and hard tissue augmentations are often needed to restore the affected site. A sufficient bone density and volume is needed for stable placement of dental implants. [1-3] In addition, esthetic outcome is an important parameter for the patient. The main esthetic objective for patients is to maintain a harmonious gingival contour with intact papillae and without abrupt changes.[1,4]

Placement of dental implants in the anterior maxillary region can be achieved by different methods.[4] The optimal method is dependent on anatomical parameters such as bone volume, bone density, alveolar crest position, adjacent teeth and gingival morphology. Moreover, esthetic outcomes are important for successful dental implantation which are determined by the smile and lip line.[1,3]

In order to increase the chance of successful dental implantation sufficient bone volume and quality are needed. Bone augmentation can be achieved by several different methods such as autologous bone grafting, xenogenous and alloplastic bone grafting, guided bone regeneration (GBR) and distraction osteogenesis.[1,3] Insufficient bone, including bone height, thickness, volume and quality, increases the risk of implant failure due to inadequate implant stability.[1,3] The bone is also a scaffold for soft tissue; when the implant is placed into bone with inadequate bone height and thickness, harmonious gingival contour is difficult to achieve.[1] The type of dental implant is especially a challenge in the anterior maxillary region. The choice of the dental implant is dependent on the bone volume and quality. An implant is often successfully placed in bone that has a width of 5 mm and a height of 7 mm.[5] Implants that are frequently used in the anterior maxilla are tapered implants, because of their ability to achieve a higher initial stability in spongeous bone[6]

Gingiva is often affected and soft tissue augmentation is needed to restore the harmonious gingiva line.[1] The type of gingiva influences the treatment method used for the repair of gingival recession.[7] Common approaches are free gingival grafts, coronally positioned flap, subepithelial connective tissue grafts, acellular dermal grafts, and enamel matrix proteins.[7] The subepithelial connective tissue grafts are generally considered as the 'gold standard' in gingival augmentation.[7]

Bone height and thickness are important for long-term stability of harmonious gingival margins around implants and adjacent teeth. [1] Loss of buccal bone around the implant frequently results in soft tissue recession potentially exposing implant collars and leading to loss of the harmonious gingival margin and horizontal or vertical bone loss of the alveolar

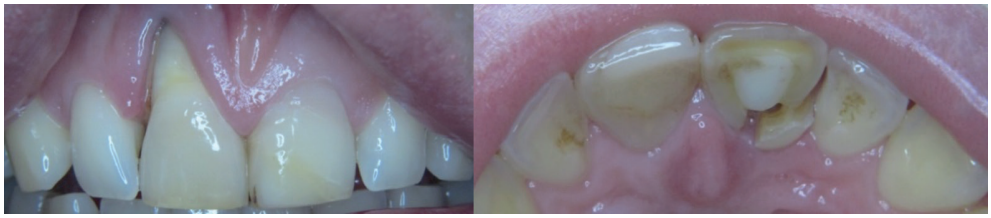
crest[1] The gingival morphology is important and can be divided into a thin highly scalloped gingiva or thick with shallow scalloped gingiva.[1]

We have used platelet-rich fibrin (PRF) successfully in our clinic for different applications such as bone and soft tissue augmentation, periodontal pocket reduction surgery, soft tissue dehiscence coverage and in combination with surgical removal of wisdom teeth. Autologous PRF is prepared from the patient's blood using a dedicated centrifugation protocol.[8] PRF consists of a polymerized fibrin network containing platelets and sometimes white blood cells (depending on the used protocol).[8,9] The membrane releases growth factors that influence the wound healing process.[10-14] PRF can be applied in both hard and soft tissue augmentation. The benefit of PRF compared to standard procedures is reduction of bone augmentation time.[15] For soft tissue augmentation and remodeling of the gingiva the PRF membrane is especially is placed in which strong fibrin architecture could be used as a matrix for wound repair.[10] In this case report we describe application of PRF for multiple procedures in a challenging case of hard and soft tissue deficiency in the anterior maxilla region.

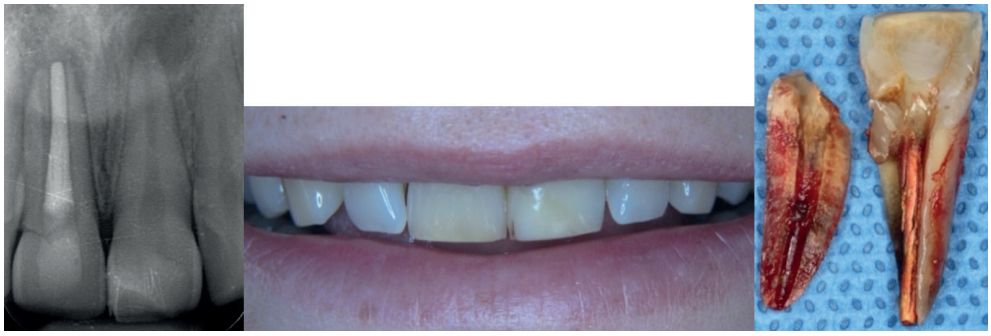
The case

A healthy woman (ASA-score I), 27 years of age, presented with a failing right upper central incisor (tooth number 11). The incisor was fractured in a vertical direction due to a field hockey injury 10 years ago. The patient had an endodontic treatment at the time of the injury on tooth number 11. Today, an enormous gingival deficiency on the buccal site was present and at the palatal side the fracture was clearly present (Figures 1A, 1B). Radiographic inspection confirmed the endodontic treatment and demonstrated the fracture line (Figure 1C). Furthermore, the buccal bone was completely destroyed. The patient had a normal smile line, a thick gingival biotype, and probing depths of the adjacent teeth were within normal limits (Figure 1D).

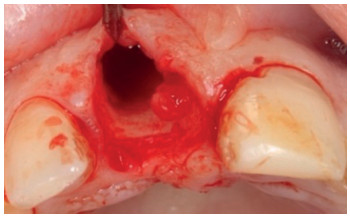
The tooth could not be salvaged; therefore, the treatment was to replace the central incisor with an implant. The tooth was extracted (Figure 1E) and the severe loss of buccal bone was clearly visible after extraction (Figure 1F). The alveolus was carefully curetted to eliminate any residual infective tissue to prevent compromised osseointegration.



1A 1B



1C 1D 1E



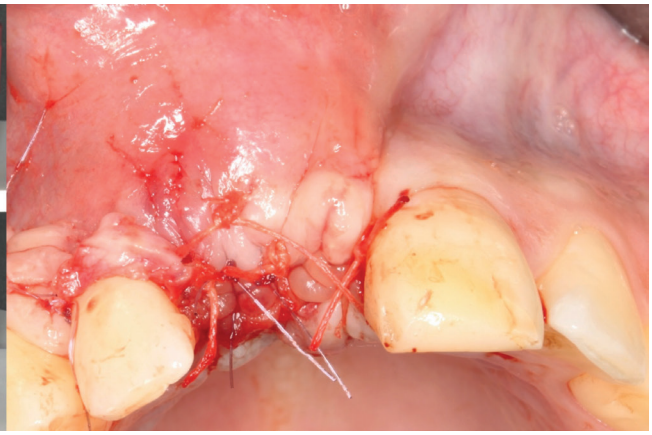
1F

Figures 1 A-E. Esthetic and radiographic overview of the failing right upper central incisor.
Fig 1A and B clearly show the hard and soft tissue deficiency and the fracture line, respectively. Fig 1C shows the radiological assessment of failing tooth number 11 and fracture line. The patient's smile line was not visibly affected by the tissue deficiency, Fig 1D shows the patient's smile line. Fig 1E shows the extracted tooth and Fig 1F shows both the hard and soft tissue deficiency after tooth extraction.

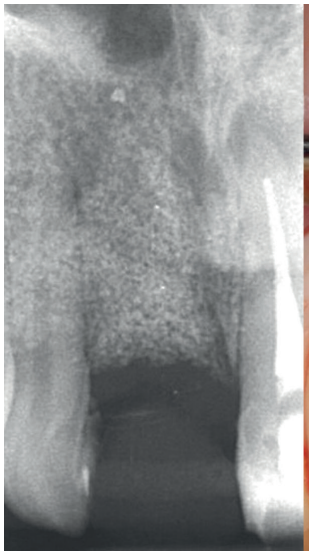
Due to the severe bone loss, bone augmentation was needed to reconstruct the buccal bone and increase bone volume to improve chances for successful implantation. Bone augmentation is usually achieved by intra-oral autogenous bone augmentation. However, PRF has showed promise above the conventional methods of bone augmentation.[17,18] The PRF method is thoroughly described;[8] Briefly, whole blood is taken in a PRF tube and immediately centrifuged (Process for PRF, Nice, France) at 200 g for 8 min.[16] The PRF tube is left in an upright position for 10 min at room temperature (Figure 2A). The PRF is separated from the red blood clot and pressed in the PRF box for 1 min. The liquid fraction, which was collected after compression, is mixed with the bovine bone space maintainer (BEGO OSS, BEGO Implant Systems, Bremen, Germany). The space maintainer is put into the cavity combined with the PRF liquid fraction and covered with the PRF membrane (Figure 2B).

The patient was closely monitored for the next 18 days. The patient showed little swelling, but had no pain. The 3-month follow-up showed stable bone augmentation which was radiographically examined by X-ray (Figure 2C). Endodontic treatment was recommended and executed by the referral dentist on central incisor 21 because of an apical translucency on the central incisor 21 and no vital reaction of the pulp.

2A



2B



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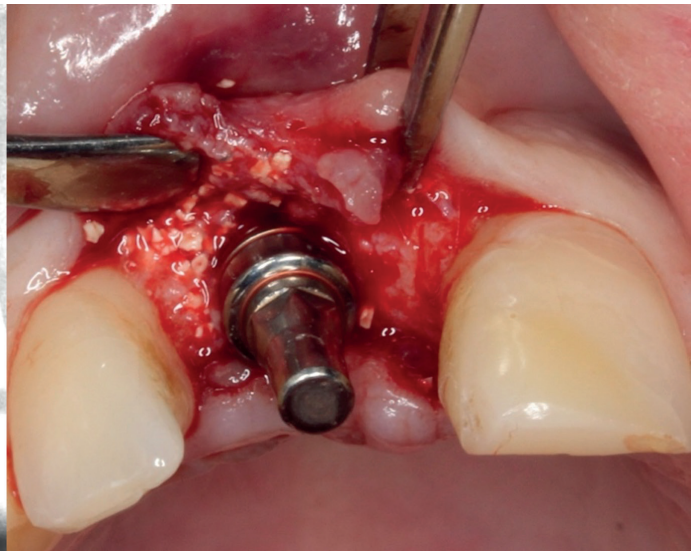
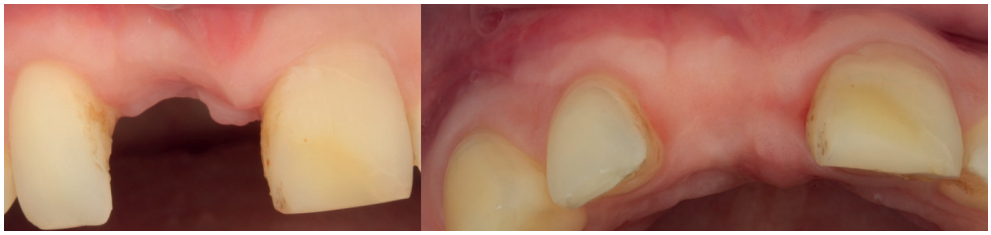
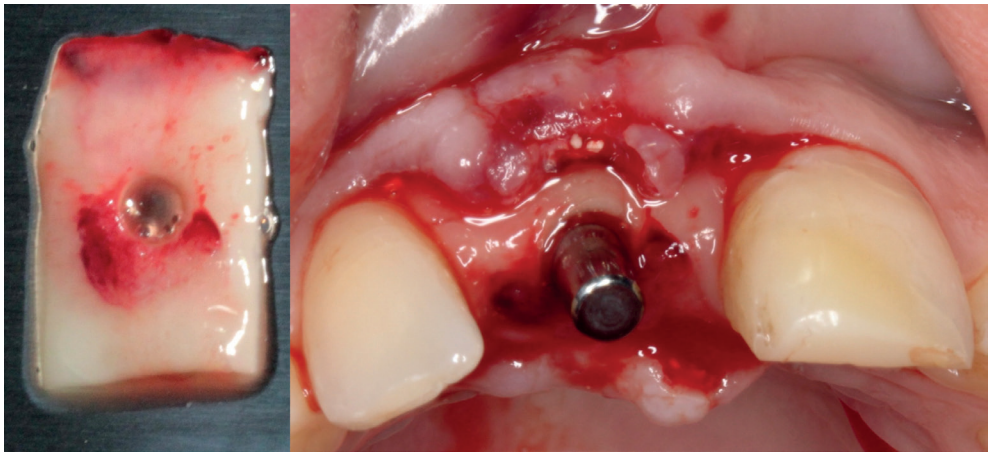


2D



2E 2F



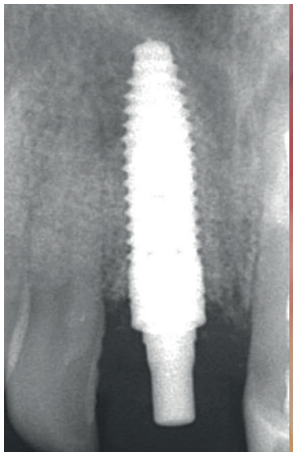
2G 2H

Figures 2 A-E. Preparation and the use of the PRF membrane.
Fig 2A the PRF clot obtained after centrifugation. Fig 2B PRF membrane inserted into the cavity. Fig 2C radiologic assessment of deficiency at 3 months after augmentation. Fig2D implant placement with Immediate Temporary Abutment. Fig 2E and F buccal deficiency after build up and occlusal appearance after build up, respectively. Fig 2G shows the punched PRF membrane. Fig 2H PRF membrane placed in the buccal/palatal envelope.

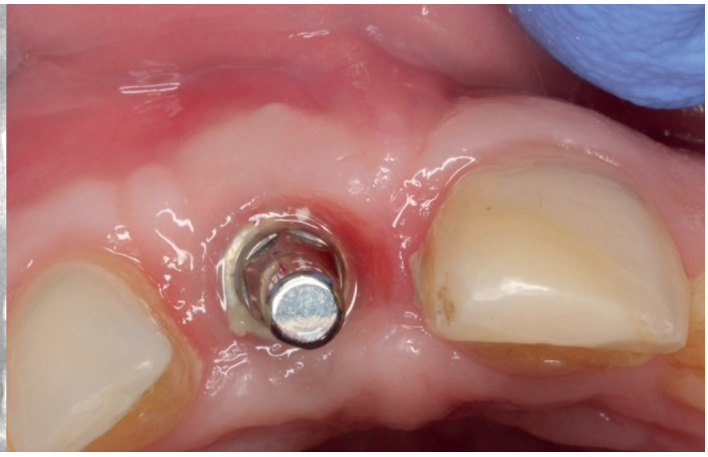
After four months, bone augmentation was sufficient for a BEGO Semados® RS implant (BEGO OSS, BEGO Implant Systems, Bremen, Germany), length of 15 mm and 4.1 mm diameter, placement (Figure 2D). After placement, a primary stability of 30 Ncm was reached which was within the advised range for immediate loading.

A BEGO Immediate Temporary Abutment (PS ITA) (BEGO OSS, BEGO Implant Systems, Bremen, Germany) was placed on the implant and was restored with a provisional crown. However, there was still insufficient tissue in the anterior maxillary region. From the buccal perspective there was a deficiency in the hard and soft tissue contour (Figure 2E) but the frontal aspect of the soft tissue was in normal range (Figure 2F). The gingival zenith was initially very apical and became almost symmetrically with the neighboring teeth. PRF was used for thickening of the soft tissue (Figure 2G, 2H). Four weeks after implant placement the proper dimensions in buccal and palatal direction were obtained (Figures 3A, 3B). Fourteen months after the augmentation of soft and hard tissue, the final crowns were placed on the

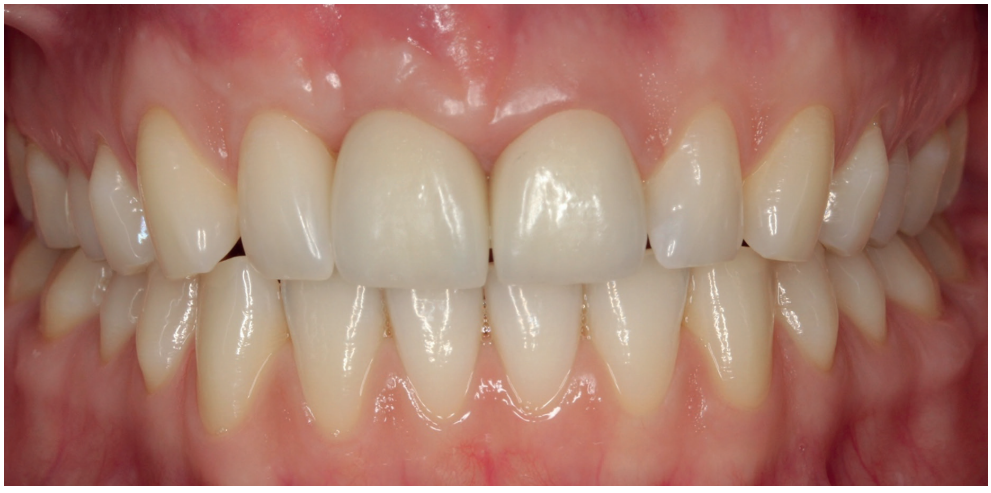
implant and the central incisor 21(Figure 3C). Follow up after one (Figures 4A, 4B, 4C) and two years demonstrates a very stable hard and soft tissue volume (Figures 5A, 5B, 5C).



3A

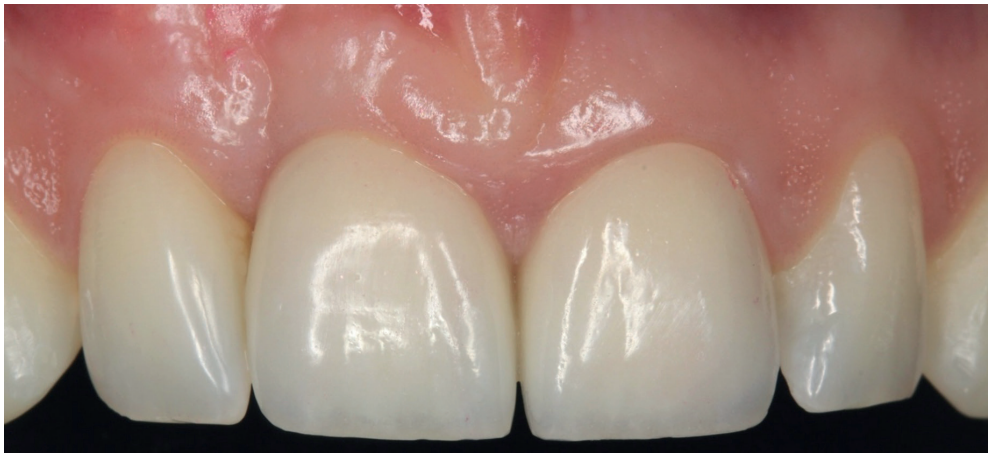


3B



3C

Figures 3 A-C. Implant placement.
Fig 3A radiographic assessment of implant placement 4 weeks after implant insertion. Fig 3B thickening of the soft tissue 4 weeks after membrane placement. Fig 3C final restorations in place. Notice the position of the attached gingiva and the zenith.



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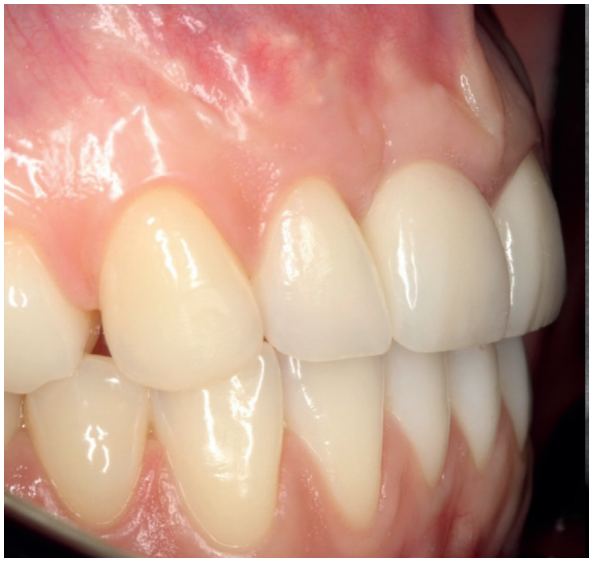

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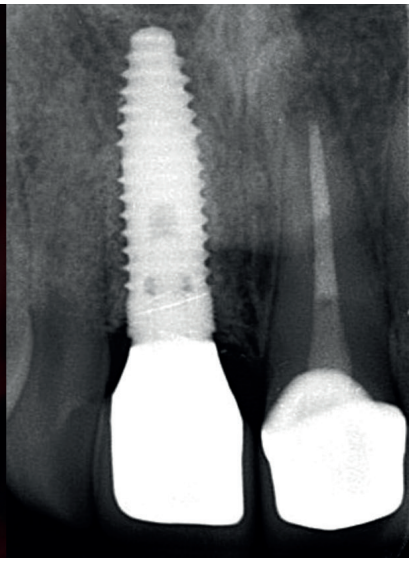
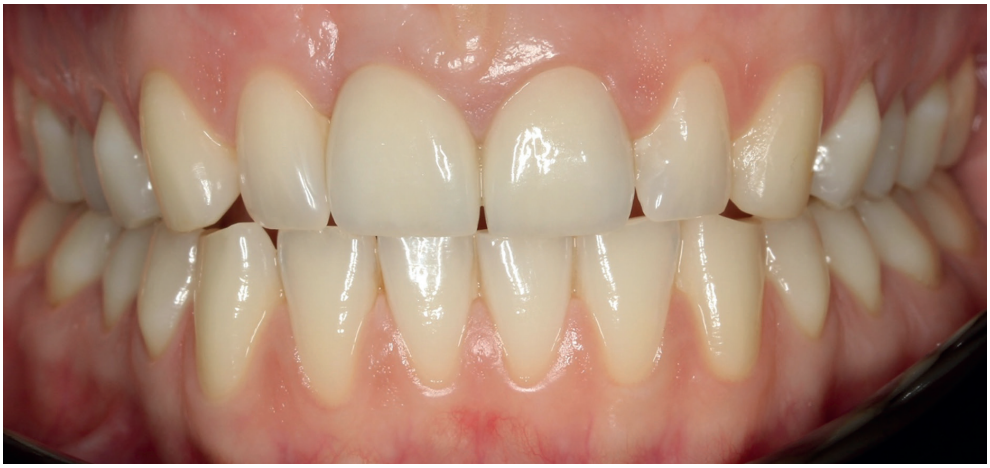


4C

Figures 4 A-B Postoperative assessment of the implant placement after one year.
Fig 4A and 4B one-year post-op. Fig 4C shows radiographic assessment one-year post-op. No change in position of attached gingiva and zenith.



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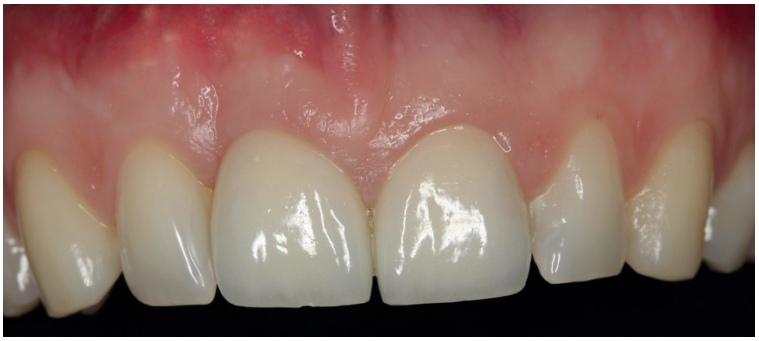

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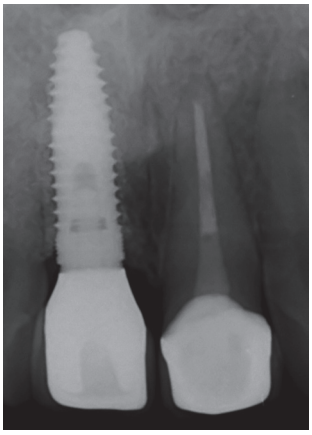

5C

Figures 5 A-C. Postoperative assessment of the implant placement after two and a half years.
Fig 5A and 5B two and a half years post-op. Fig 5C shows radiographic assessment two and a half years post-op, note there is no change in soft and hard tissue position.

Discussion

Soft tissue augmentation is especially important in the esthetic zone. The facial soft tissue parts will resorb quite quickly which should be prevented. The interdental papillae will disappear fast after extraction and in addition the enormous bone loss will influence the gingiva.[18] The initial quality of the gingival tissue is important.[19-23] A thin biotype is less predictable then a thick biotype. The wound should be closed primarily without any tension. If the gingival tissue is weak or damaged, sloughing of the soft tissue is likely to occur and will lead to a compromised healing site due to contamination.

Long term clinical studies have shown that functional osseointegration is a predictable outcome. However, the success of dental implant therapy is no longer based only on functional osseointegration, but also on positive patient outcomes as esthetic harmony with the remaining dentition. In the present case report, we describe the use of PRF in combination with bone substitute for hard and soft tissue augmentation. We have applied this approach in our clinic for several years, because it may have a stimulating effect on the healing and maturation of soft and osseous tissues.

After extraction of the central incisor the extent of bone loss was clearly visible. Therefore, immediate loading of the implant was not possible due to the resorption of the alveolar ridge. One of the prerequisites for immediate loading is primary stability, which only can be achieved if there is enough surrounding bone.[24,25] Without immediate grafting after extraction the alveolar ridge would resorb immediately, resulting in inadequate bone volume.[26] In order to increase the bone volume, PRF and bovine bone substitute was used. In previous studies, it was shown that PRF is a suitable technique for bone augmentation.[9] One of the benefits of PRF is that PRF increases the bone-to-implant contact compared to other bone augmentation techniques.[9] However, some bone augmentation techniques have the potential of angiogenesis therefore it cannot be excluded that PRF solely is a superior technique for bone augmentation.[28] The role of PRF in wound healing has been demonstrated by Agrawal et al. by prolonged release of platelet-derived growth factors at the wound site, proliferation of fibroblasts and osteoblasts, promoted angiogenesis, induced collagen synthesis, guided wound coverage, mechanical adhesion by fibrin, trapped circulating stem cells and regulation of immunity.[27]In addition, the membrane acts as a bio-barrier and an engineering scaffold.[15]

Conclusion

In this case report we have showed that PRF in combination with bovine bone space maintainer is a promising method for buccal bone augmentation as well as soft tissue restoration. This approach resulted in natural healing and maturation of the peri-implant

bone and soft tissues. Bone augmentation was achieved with enough bone and bone density in order to achieve sufficient implant stability. The patient had a good functional and esthetic outcome. In this case the application of PRF was a simple, affordable and accessible method. The approach of PRF in combination with bovine bone substitute may be a promising development in oral implantology, although more knowledge about the molecular properties of PRF is needed for optimal implementation.

Ethical approval

A written informed consent was obtained according to the ethical guidelines of the 1975 Declaration of Helsinki (2013).

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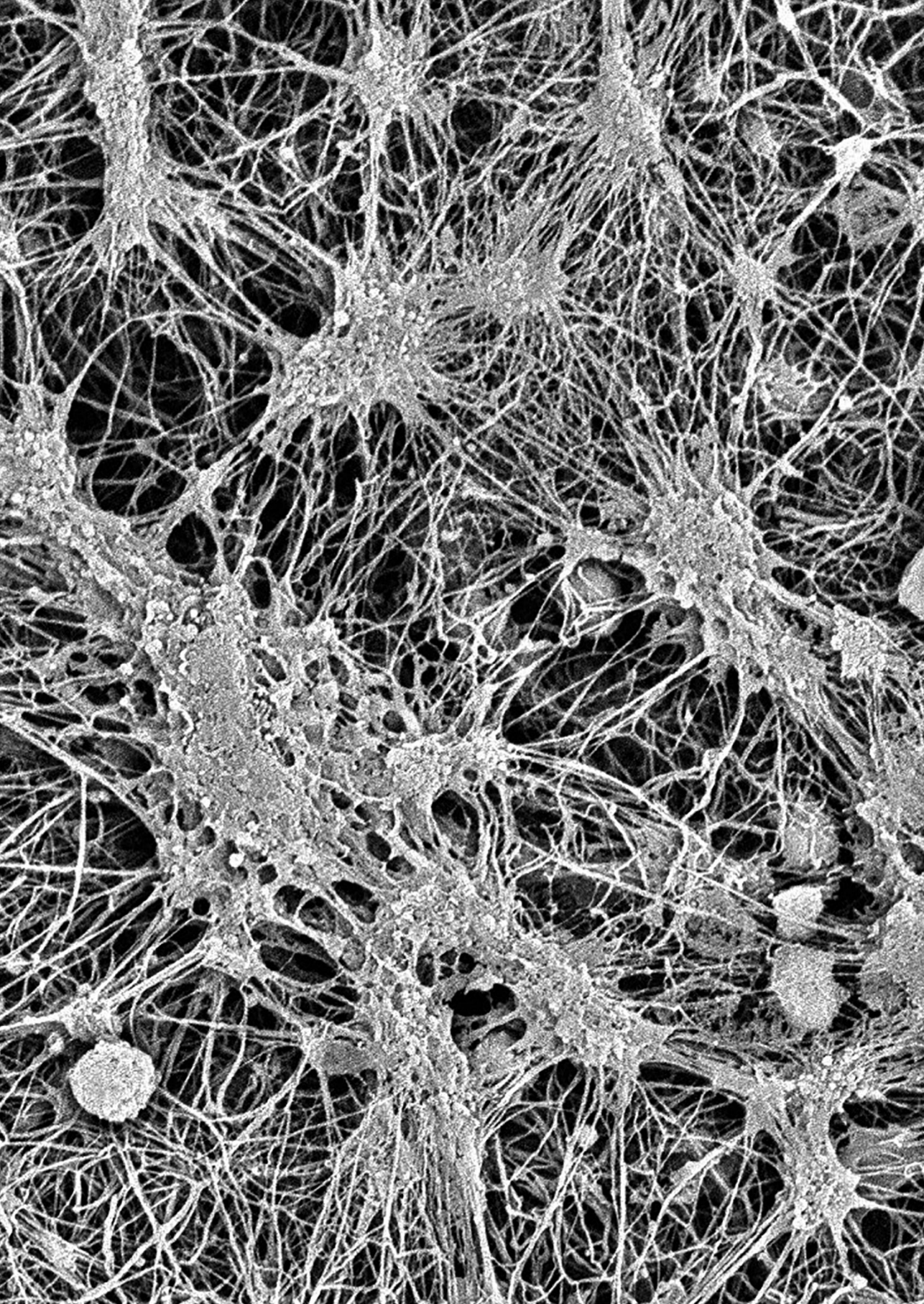

Chapter 3

Reliability and validity of the instrumental assessment of implant stability in dry human mandibles

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Abstract

The aim of this study was to determine the intra- and interobserver reliability and validity of the instrumental assessment of primary dental implant stability, using resonance frequency analysis (RFA). Sixteen tapered implants and 16 cylindrical implants were installed in eight unfixed dry human mandibles (Cawood classification IV/V). Implant stability quotients (ISQ; the outcome variable of RFA) and peak removal torque were determined. Both the intra-observer reliability and the interobserver reliability of the RFA measurements were fair-to-good, while no significant correlations between the ISQ values and removal torque were found. The removal torque of the cylindrical implants was higher than that of the tapered implants. The smallest detectable difference was almost nine ISQ units. Within the limitations of the present dry cadaver study, it was concluded that (i) primary dental implant stability can be assessed reliably with RFA measurements, (ii) the concurrent validity between RFA measurements and removal torque is poor, (iii) cylindrical implants may be more stable than tapered ones and (iv) two subsequent readings of RFA measurements need to differ at least nine ISQ units before the difference between the two measurements can be considered statistically significant. More research is needed to see whether these conclusions can be extrapolated to the clinical situation, including the assessment of implants during function (secondary stability).

Keywords:
dental implants, resonance frequency analysis, reliability, validity, smallest detectable difference

Introduction

Implants have become increasingly important for a durable dental rehabilitation, with reported treatment success rates for the mandible of up to 100%.[1] Primary implant stability has been identified as a predictor for, and even a pre-requisite to, achieving full osseointegration,[2-6] not only as part of the traditional two-stage approach but also following one- stage surgery and subsequent immediate loading of the implants.[7-10]

In the past, several methods were proposed to measure primary implant stability, such as the Periotest (Gulden, Bensheim, Germany) and insertion torque measurements.[4] The Periotest is a non-invasive technique that can be performed anytime during and after the implant treatment, but its reliability is poor.[11,12] Insertion torque measurements, on the other hand, can only be made per operandum.[13,14] As an alternative, resonance frequency analysis (RFA) has been introduced to provide, amongst others, an objective, non-invasive and easy-to-perform technique for measuring primary implant stability.[4,15-18] However, until now, there is only little information available about the reliability and validity of RFA measurements. Most of the literature concerning the Osstell™ RFA device (Osstell, Gothenburg, Sweden) is published by the inventors of the device. These studies have been carried out on cadavers or under clinical conditions. A variety of variables can influence the reliability and validity of these measurements, like fenestrations of the bone that are often unrecognizable even in cadavers. Importantly, before recommending the RFA technique for use in daily practice, acceptable levels of the reliability and validity of this technique need to be established.

Hence, the purpose of this study was to evaluate the intra- and interobserver reliability as well as the validity of the resonance frequency analysis of two types of dental implants that were placed in dry human cadaver mandibles. Furthermore, the smallest detectable difference of the RFA technique was assessed.

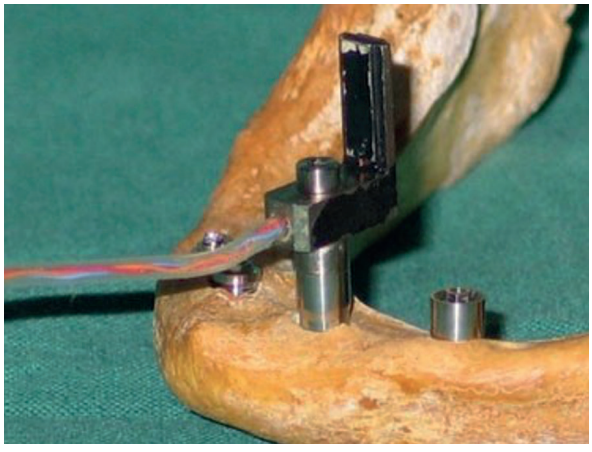

Figure 1. The L-shaped Osstell™ transducer, designed for the 3i® system, in situ on an implant in one of the experimental dry mandibles.

Materials and methods

Study sample

Eight Caucasian dry human mandibles with a Cawood classification IV–V (graveyard abolition, Department of Anatomy, University of Utrecht) were provided by the department of Dental Radiology of ACTA. Seven of the eight mandibles were totally edentulous. In one mandible, there was a retained root in position 33.

Procedure

Thirty-two implants (3i®, Implant Innovations Inc., Palm Beach Gardens, FL, USA) were placed by an experienced clinician. In all eight mandibles, the implants could be successfully installed with a 45 N cm insertion torque. All implants were positioned with the shoulder at the marginal bone level, i.e. marginal bone height was the same for all implants. Four implants per mandible were placed in the interforaminal region in position 44, 42, 32 and 34. In four mandibles, cylindrical implants (3i®, Osseotite™, type I) were used; in the other four, tapered implants (3i®, Osseotite NT™, type II). All implants had a length of 10 mm and a diameter of 4 mm. The abutment height used in all implants was 4 mm.

Measurements

To obtain the RFA measurements, the Osstell™ device was used, with L-shaped transducers fabricated for the 3i® system (Figure 1). The Osstell™ transducer is designed as an offset cantilever beam with an attached piezoceramic element. This transducer quantifies the implant stability as an implant stability quotient (ISQ), which is a function of bone-implant stiffness (N $cm^{)1}$) and marginal bone height. The ISQ value varies on a scale from 0 to 100 and is a dimensionless quantity. Higher ISQ values indicate increasing levels of interfacial bone- implant stiffness and thereby higher integration stability. To perform the RFA measurements, the transducer was mounted on the implant orthoradially and perpendicular to the jawbone, with the upright part on the oral side. The fixation screw was tightened with 10 N $cm^{)1}$. Measurements were performed by two experienced clinicians in separate sessions. To gain experience with the Osstell™ device, the observers received training before doing the measurements. Observer 1 did two sessions of measurements on all the implants to assess the intra-observer reliability, whereas observer 2 only performed one session of measurements to assess the interobserver reliability.

Observer 1 and observer 2 performed their measurements at separate time points. The time between the measurement sessions was at least 1 week.

In animal studies, removal torque has been used to determine implant stability (20–22). In the present study, a removal torque measurement was performed to obtain a reference

standard for implant stability. This measurement was executed once per implant by observer 1. The necessary force to unscrew the implants in the mandible was measured with a torque control device. Before using the device, it was calibrated by the manufacturer (NobelBiocare™, Gothenburg, Sweden). The unscrewing forces were divided in five groups: 1 = 0–10 N $cm^{)1}$; 2 = >10–20N $cm^{)1}$; 3 = >20–32 N $cm^{)1}$; 4 = >32–45 N $cm^{)1}$ and 5 = >45 N $cm^{)1}$.

Data analysis

For each implant stability assessment, the intra-observer and interobserver reliability were estimated by the intra- class correlation coefficient (ICC). Following the recommendations of Schuck (23), the two-way mixed model with measures of absolute agreement was used. The ICCs were qualified as follows (24): poor reliability when ICC < 0.4; fair-to-good reliability when ICC = 0.4–0.7; and excellent reliability when ICC > 0.7. Spearman's correlations (q) were calculated between the ISQ and the removal torque data. Using the latter data, the Mann–Whitney U-test was used to determine to most stable implant type. The four implants in every mandible were thereby considered as independent cases. For the ISQ, the smallest detectable difference (SDD) was determined according to Kropmans et al. (25). All statistical tests were performed with SPSS 11.0 software package (SPSS Inc., Chicago, IL, USA). Probability levels of P < 0.05 were considered statistically significant.

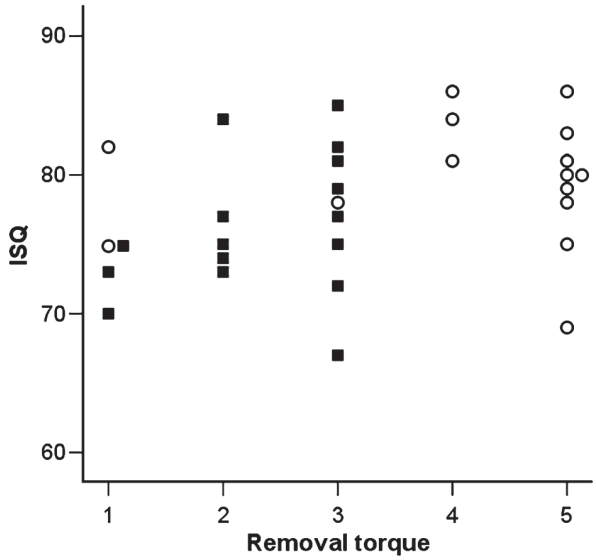

Figure 2. Implant stability quotients (ISQ) values for the various removal torque classes (1–5; see Materials and Methods, Measurements) for the implant types I (open circles) and II (closed squares).

Results

All data of one implant were discarded from the analyses because it was inserted too close to the retained root.

Intra- and interobserver reliability

The ICC value for the intra-observer reliability was 0.46, which can be qualified as fair-to-good. The ICC value for the inter-observer reliability was 0.70, which is on the edge between fair-to-good and excellent.

Validity

For both implant types, the relationship between ISQ and removal torque is shown in Figure 2. No significant correlations between both variables were found either for type I implants or for type II implants (p=0.11 and p=0.34, respectively).

A highly significant difference was found between the removal torques of the two types of implants. The mean rank of type I implants was 21.80; that of type II implants, 10.56 (Mann–Whitney U-test; p=0.00).

Variability of measurements

The SDD of the ISQ was 8.8 for both types of implants combined. When calculated for both types separately, the SDD of the ISQ was 8.6 for type I implants and 9.2 for type II implants.

Discussion

Studies on the intra-observer and inter-observer reliability of the instrumental assessment of dental implant stability by means of resonance frequency analysis (RFA) measurements are scarce. Nedir et al.[19] reported a relative variation between consecutive measurements of the Osstell™ device of 1.14% when implant stability was being assessed clinically. However, unlike reliability measures like the ICC that yielded fair-to-good reliabilities in the present dry cadaver study this percentage has not been adjusted for the contribution of chance to agreement. More animal or clinical studies are thus needed on this topic, using chance-adjusted reliability measures.

In a recent review of the clinical literature, Aparicio et al.[20] found no randomized clinical trials or prospective cohort studies for testing the validity of the RFA method. In addition, Schliephake et al.[21] concluded on the basis of a histomorphometric study of bone anchorage of implants in foxhound mandibles that the validity of individual implant stability measurements using RFA should be considered with caution. Furthermore, Rabel et al.[22] found no correlations between ISQ and insertion torque measurements. They therefore

concluded that RFA does not appear to be suitable for the evaluation of implant stability when used as a single method. The present study, in which no correlations between removal torque and RFA measurements on implants in dry cadaver mandibles were found, corroborates the outcomes of these previous studies. Hence, more research is needed using fresh cadavers or animals to assess the validity of this clinical tool for measuring implant stability. In a published study protocol, Jung et al.[23] described the intention to collect ISQ data in relation to the quantity of direct implant-bone interface of retrieved palatal implants that were placed for orthodontic reasons. The outcome of that study may con- tribute to our insight into the validity of the RFA measurements. A striking finding was the highly significant difference in removal torque between the two types of implants. The cylindrical implants were better anchored in the bone than the tapered ones. In the literature, on the contrary, tapered implants are found more stable than the cylindrical ones.[24] Amongst others, this difference may be because in the present study, dry mandibles were used, whereas O'Sullivan et al.[24] used the femur and tibia of living rabbits, which were later sacrificed. The literature indeed suggests that dehydrated bone has different properties from those of fresh bone. For example, Rho and Pharr[25] found an increase in the elastic modulus by approximately 10% for dry bovine bone compared with fresh bone. This stresses the need to study further the reliability and validity of implant stability measurements in fresh bone specimens. Furthermore, as we analysed our data non- parametrically, the four implants per mandible were assumed to be independent cases. A split-mouth design, with both implant types in every mandible, might be a better design option for future studies on this subject. Therefore, the conclusions of this study must for the present be drawn with caution.

In their manual, the Osstell™ Company suggests that if the transducer is reattached, the difference between two subsequent measurements should be <2 ISQ units. However, we found an SDD of about nine. This would mean that the precision of the RFA measurements is considerably worse than the company suggests. It should again be noted, however, that this number was obtained from dry cadavers and may thus not be fully applicable to fresh cadavers or living patients.

Conclusions

Within the limitations of the present dry cadaver study, it was concluded that (i) primary dental implant stability can be assessed reliably with RFA measurements, (ii) the concurrent validity between RFA measurements and removal torque is poor, (iii) cylindrical implants may be more stable than tapered ones and (iv) two subsequent readings of RFA measurements need to differ at least nine ISQ units before the difference between the two measurements can be considered statistically significant.

More research is needed to see whether these conclusions can be extrapolated to the clinical situation, including the assessment of implants during function (secondary stability).

Acknowledgments

The authors would like to thank Implant Innovations Inc., Florida, USA, for supporting the study, Dr Gerard Sanderink of the Department of Oral Radiology, ACTA, for providing us with the cadaver mandibles, Dr Herman Hosman for operating on the cadaver mandibles and Dr Maurits van Selms for his help with some of the statistical analyses.

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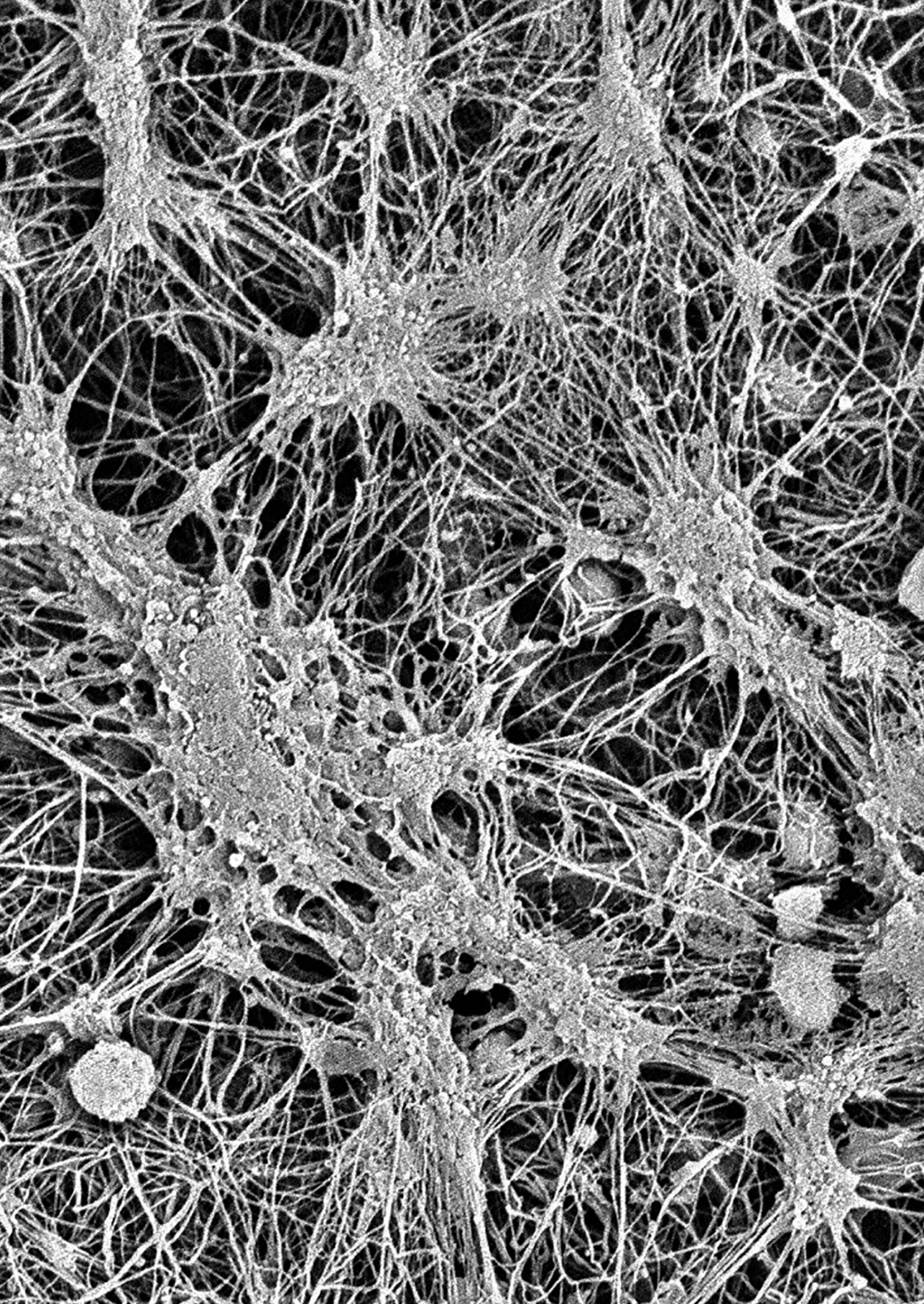

Chapter 4

Resonance Frequency Analysis with two different devices after conventional implant placement with ridge preservation

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Abstract

Background:
Primary and secondary implant stability is of high importance for survival and success of dental implants in the short and long term. Measurements of implant stability during healing provide the opportunity to monitor the course of the osseointegration process.

Purpose:
To compare implant stability quotient (ISQ) by resonance frequency analysis (RFA), recorded with two different devices after implant placement.

Materials and Methods:
Patients with the need of single tooth extraction in posterior sites of the maxilla and the mandible were treated in a surgical centre. All patients received additional augmentation with a bovine bone substitute and platelet rich fibrin (PRF) after atraumatic tooth extraction. After a healing period of 10 weeks, 28 self-tapping titanium-implants were placed. Implant stability was recorded with two different devices (Osstell and Penguin) at the time of implant insertion (T0), 10 days later (T1), and after 7 (T2), or 17 weeks (T3).

Results:
No implant was lost, and no post-operative complication occurred during follow up. Patient cohort comprised 9 female (32.1 %) and 19 male patients (67.9 %), with a mean age of 52.8 years, 64.3 years respectively. Mean overall insertion torque was 43.6 Ncm at implant placement with no significant difference between implant location, age, or gender. No patient dropped out. During observation period, a significant increase in mean ISQ was recorded with both devices. Significant positive correlations between insertion torque and ISQ were recorded with both devices at T0, T2 and T3. No significant differences were observed in ISQ-values between both devices, and measuring directions at any point of measurement.

Conclusions:
Within the limitations of this cohort study, both devices were suitable for RFA-measurement and revealed comparable results. Due to the cordless design, handling of the Penquin device was more comfortable. Reusability of the Penguin™ MultiPeg-transducers may offer an additional benefit with regard on ecological aspects.

Keywords:
Resonance frequency analysis, insertion torque, implant stability quotient, platelet rich fibrin, ridge preservation, osseointegration.

Introduction

To date, a whole range of different tools and concepts for measurement of implant stability has been introduced in order to enable clinicians predicting a successful osseointegration of implants. Invasive methods for implant stability assessment are histological or histomorphometric analysis, tensional tests, push-out/pull-out tests, and removal torque analysis.[1] Insertion torque measurement, percussion tests, Periotest (damping capacity analysis), or Resonance Frequency Analysis (RFA) are examples for non-invasive techniques to determine implant stability.[1,2] As a non-invasive diagnostic method, RFA is a commonly used approach to asses implant stability in oral implantology.[3,4] RFA was developed by Meredith and co-workers nearly 30 years ago, and has become one of the main tools for implant stability assessment.[3,5] This technique uses a transducer peg, which is attached to the implant and is excited over a range of frequencies by electro-magnetic waves, in order to measure resonance frequency (RF) of the implant.[3] RF values are computed in Hertz (Hz) by a simple recalculation method. Hereinafter they are converted into the Implant Stability Quotient (ISQ), usually on a scale of 1 (lowest stability) to 100 ISQ units (highest stability).[3,6] ISQ value as determined by RFA, is a measure of interface stiffness between bone and implant surface.[3] RFA represents a bending test of the implant-bone complex, measuring the implant´s displacement by lateral forces, applied by the transducer peg.[7] This non-invasive and repeatable technique enables reflection of multidirectional fixation strength.[7-9] In the field of dentistry, the ISQ value was reported to correlate with the percentage of the implant surface, being in close contact with bone tissue (i.e. bone-to-implant contact ratio).[10,11] This feature enables measurement of the ISQ values and the temporarily changes in implant stability during osseointegration process after implant placement.[7,9] The Osstell ISQ (Osstell, Gothenburg, Sweden) is a medical device which measures RF by vibration analysis of the dental implant with magnetic pulses (Figure 1). In order to measure implant stability, a peg with threads (SmartPeg™) is attached to the implant. The Osstell aluminium pegs are single use items. Their threads are weak in order not to damage the inner threads of the implant. Further, attempts to clean and sterilize the peg will result in corrosion and the reuse of the peg probably will lead to questionable measurement results.

Eversince the introduction, the Osstell ISQ has been the only instrument on the market to measure ISQ values. Penguin™ (Integration Diagnostics Sweden AB, Gothenburg, Sweden) was introduced on the dental market in 2015 (Figure 2). The Penguin device utilizes a reusable transducer (MulTipeg™) for ISQ-measurement.[12,13] MulTipeg™ is made of durable and tissue friendly titanium and can consequently be autoclaved up to 20 times, according to manufacturer´s specifications. When mounted on an implant, the MulTipeg™ is excited with magnetic pulses. RF is measured without any contact between the attached MulTipeg™ and the Penguin™ measuring device. Frequency of the vibration is picked up by the device and translated into an ISQ value between 1 and 99. The higher the ISQ value, the better the implant stability.

Since comparative human clinical studies on measurement validity between Osstell and Penguin[RFA] are scarce, the purpose of our clinical investigation was to analyse RF with both measurement devices after conventional implant placement with ridge preservation measures after tooth extraction, and to compare RF-values after certain time intervals. Ridge preservation was performed with a mixture of a xenogenous bone substitute and platelet rich fibrin (PRF).

In this cohort study, RFA-measurements were performed with two different measurement devices. Main objectives of the study were as follows:

1. To explore the influence of different independent variables, like age, gender, implant location, or implant dimensions on insertion torque-value (IT) and ISQ, primary and secondary stability respectively.
2. To compare the two devices in order to analyse differences, correlations respectively of the recorded RFA values.
3. To explore the course of implant stability over a mean follow up-period of 17 weeks after conventional insertion of self-tapping implants in healed extraction sites with preservation procedures.

Materials and methods

Patients

Twenty-eight patients of the Institute for Dental Implantology Amersfoort were treated with augmentation procedures after tooth-extraction in the posterior parts of the maxilla or the mandible, and received single implant treatment in a conventional insertion protocol after a healing period of 10 weeks. The medical ethical review board of the Gelre Hospitals approved the study protocol (TCO nr 15.38; 28-07-2015) and written informed consent was obtained from all study participants according to the ethical guidelines of the Declaration of Helsinki (Version 2013).

All patients had an ASA score I (healthy individual) or II (mild systemic disease) according to the American Society of Anaesthesiologists.[14]

Surgical intervention

Surgical intervention was performed under local anaesthesia. Teeth were extracted carefully with periotomes and small luxators in order to preserve as much bone as possible and to avoid fracture of the buccal bone plate. The extraction sockets were thoroughly scraped after extraction and rinsed with a sterile 0.9 % sodium chloride solution. Ridge preservation was performed for each extraction socket with a mixture of growth-factor rich liquid and an amount of 0.5-1.0 ml Demineralized Bovine Bone Mineral (BEGO-Oss, BEGO Implant

Systems). After augmentation, the socket was covered with an A-PRF membrane. Platelet rich fibrin was prepared according to the Choukroun method (A-PRF DUO, Process for PRF, Nice, France).[15] No antibiotics or pain medication were prescribed before extraction and postoperatively in the test group.

Implant placement

After a mean healing period of 10 weeks, 28 bone level implants (BEGO Implant Systems) with diameters between 3.75 and 5.5 mm and different lengths (BEGO Semados RS implants in the maxilla and BEGO Semados SC implants in the mandible, BEGO Implant Systems) were placed according to the manufacturer´s guidelines (BEGO Work instructions with BEGO Tray, instruments and drills) in a conventional placement protocol, according to the ITI consensus conference.[16,17] One hour before surgery, prophylactic antibiotic medication was performed either with 2.0 g Amoxycillin or 600 mg Clindamycin. An envelope flap was created on the buccal and oral side, and the implant site was prepared first by using a trephine drill (Hager & Meisinger GmbH, Neuss, Germany) with a diameter of 2.0 mm (for histological evaluation in a later study) followed by the drilling protocol according to the manufacturer's specifications. Insertion torque (Ncm) was determined using the Oral Implantcenter (Acteon, Bordeaux, France). Implants were immediately provided with healing abutments (PS HP, BEGO Implant Systems) according to the manufacturer's specifications. Thereafter the flap was closed, and welded over the PRF membrane with mattress sutures.

RFA measurements

RFA measurements were performed at time of implant insertion (T0), after 10 days (T1), as well as at seven (T2), and 17 weeks (T3) after implant placement. ISQ values were registered in two directions: 1) sagittal (mesio-distal: md), and 2) transversal (bucco-oral: bo).

At every time point, ISQ values were measured with the Ostell SmartPeg and the Penguin MulTipeg device according to the manufacturer´s specifications. Healing abutments were removed for each measurement, and the transducers of each measurement device were screwed manually onto the implants and tightened firmly. In order to reduce potential errors, all recording procedures were performed by the same operator (JEIGB).

After recording the implant´s stability in two directions, the respective transducer-peg was unscrewed, and the healing abutments were reattached.

Statistical analysis

Statistical analysis was performed with the MS Excel AddIn Winstat version 2012.1.0.96 (Robert K. Fitch), Graphpad software version 3.1 (San Diego, USA), and BiAS for Windows (epsilon-Verlag) version 11.10. Test for normal distribution was performed with the Kolmogorov-Smirnov test statistics. In case of a non-parametric distribution of variable values, a Box-Cox transformation was utilized, in order to enable calculation with mean values and statistical testing with parametric tests. Person and Spearman correlation tests

were performed in order to detect correlations between IT at time of implant insertion, as well as ISQ at four different points of measurement, and other independent patient- and implant related variables. The level of statistical significance was set at $p<0.05$.

Results

Patients

Mean age of the 28 participants was 60.6 years. Patient cohort comprised 9 female (32.1 %) and 19 male patients (67.9 %) with a mean age of 52.8 years, 67.9 years respectively. There was a statistical significant difference in mean age between both genders (p=0.011).

Implants

BEGO Semados® RS/SC implants are fabricated from Grade 4-titanium. They comprise an internal taper abutment connection with a hexagonal anti-rotation protection. They have a conical shape and a self-tapping thread design, which shall reduce mechanical loading on the implant and avoid damage of surrounding peri-implant bone during implant insertion. Twenty-eight implants with five different lengths (8.5 mm, 10.0 mm, 11.5 mm, 13.0 mm, and 15.0 mm) and four different diameters (3.75 mm, 4.1 mm, 4.5 mm, and 5.5 mm) were inserted in the maxilla (n=18 implants, 64.3 %) and in the mandible (n=10 implants, 35.7 %). All implants were placed in the posterior sites of both jaws (Fig. 3). No implant was lost during the observation period. Overall mean IT was 43.6 Ncm. Mean IT in the mandible was 44.5 Ncm and in the maxilla 43.1 Ncm, revealing no significant statistical difference between both jaws (p=0.873). Likewise, statistical tests did not reveal any significant difference in mean IT-values between female and male patients (p=0.598). No significant correlation between age and IT-value could be identified as well (p=0.232). Pearson test revealed no significant correlation between implant length (p=0.444), or diameter (p=0.210) and IT.

RFA-measurements

Ascending mean RFA-values were recorded with both devices during the observation period in both measurement directions at every point of measurement, except for the Penguin^{RFA} device in mesio-distal direction between T1 and T2 (Tab. 1, 2). Any significant difference could be observed in RFA-values at any point of measurement between both measurement devices, and between both measurement directions (Tab. 3). A significant increase in mean implant stability was recorded with both devices in both directions between baseline (T0) and T2, T3, as well as between T1-T2, and T1-T3. No significant differences were observed between mean baseline values and measurements ten days after implant insertion (T0-T1) for both devices in both measurement directions (Tab. 4, 5). No statistical significant increase in implant stability was recorded with the Osstell device in bucco-oral direction between T2-T3, and between T0-T2, when measured in mesio-distal direction with the Penguin device (Tab. 5). Recorded ISQ-values were consistently higher in mesio-distal direction, compared

to those, measured in bucco-oral direction for both devices, although these differences never reached any statistical significance (Tab. 6). All RFA-records were higher in the mandible, reaching statistical significance when recorded bucco-orally at T1, T2, and T3, as well as mesio-distally at T3, when measured with the Ostell device. When measured with the Penguin device, significant differences were observed in bucco-oral direction at T1 and T3, and in mesio-distal direction at T3.

No statistical differences were observed in RFA-values between female and male participants at any time point of measurement, independently of the measurement direction or the measurement device. A significant positive correlation was observed between mean IT- and ISQ-values at T0, T2 and T3 for both measurement directions, when measured with the Osstell device, and at T0 and T2 for both directions, when recorded with the Penguin device (Tab. 7).

Whereas implant length didn´t reveal any influence on implant stability, irrespective of the used device or the measuring direction, a significant correlation was observed between implant diameter and ISQ-values at T0 in both directions with both devices, and at T3, when measured mesio-distally with the Osstell device, as well as when measured bucco-orally with the Penguin device.

Significant correlations between implant´s primary stability at T0, and RFA measurements at T1, T2, and T3 were observed for both measurement directions with the Osstell device and for T2 in both measurement directions with the Penguin device (Tab. 8). Significant correlations were observed between measurement values of both devices, when all records from all measurement points were pooled (bucco-oral: $r=0.7861$, $p<0.001$; mesio-distal: $r=0.8651$, $p<0.001$) (Fig. 4, 5).

Discussion

Osseointegration plays a fundamental role in oral implantology and is a prerequisite for implant success and implant survival. Primary and secondary implant stability are key functions for a direct and functional connection between peri-implant bone tissue and implant surface, during implant healing, as well as under functional loading conditions.[18] While primary stability is a result of mechanical stability at time of implant placement, secondary stability is a consequence of biological rigidity, evoked by the implant´s osseointegration.[19,20] Implant´s primary stability is highly dependent on bone density and bone quantity, surgical technique, and implant design, and will be achieved by compression of the surrounding bone during implant placement.[3] For a long term implant success, achievement of osseointegration and preservation of implant stability is the key to clinical success of implant treatment.[3,21] During osseointegration, an initial loss of implant stability

will be observed, followed by an increase of stability during remodelling of the surrounding bone tissue. The newly formed bone matures with time, which will result in an increased bone density, a close bone to implant contact, and an increased implant stability.[3]

Hence, assessment of implant stability at time of implant insertion and during healing period represents an important tool to monitor the course of this turning process in peri-implant bone tissue. Primary implant stability is considered to be the result of mechanical friction between implant surface and peri-implant bone.[22] Implant stability at early healing stages is influenced by a variety of factors, such as local bone quality and quantity, the implant´s size and geometry, the respective drilling method (e.g. undersized preparation of the implant bed, insertion of self-tapping implants), or loading conditions during healing.[23-29] Implants placed into grafted post-extraction sites obviously exhibit a similar clinical performance in terms of implant survival and marginal bone loss as implants, placed in non-grafted sites.[30-32] During implant placement, drilling of the implant bed and implant insertion will lead to hematoma and a surgically damaged peri-implant bone tissue. In the first two weeks during healing process after implant insertion, damaged bone tissue will gradually be replaced by immature woven bone, formed in close contact to the implant surface. This transformation of bone tissue is designated as remodelling process. Bone remodelling triggers a transitional loss of implant´s former primary stability. Clinical studies report a decrease of ISQ-values after implant placement, indicating the transformation of primary mechanical stability into a secondary biological stability, induced by the osseointegration process. A re-gain of implant rigidity occurs as a result of new peri-implant bone formation, determining the emerging of the implant´s "secondary stability" through the biological process of ongoing osseointegration.[33] Clinical data on duration of this transformation process show a high variability. Induced by the variety of bone healing patterns, a distinct spread of time intervals for a decrease in implant stability during healing period was observed in different investigations.[34-36] A number of studies reported a decrease in ISQ following implant installation, reaching its lowest values after three to four weeks.[37-39] Another significant decrease in ISQ is described in scientific literature, with lowest ISQ-values at day two and 21 after implant placement,[40] while other observations were showing the lowest values seven days,[41] two weeks,[34] or one month[36] after insertion respectively. Due to the obvious high variety of bone healing patterns between patients, the shift from primary stability to secondary stability remains unpredictable.[19] In contrast, results of other investigations displayed no decrease in implant stability, but an increase in mean ISQ six to twelve weeks after implant placement.[40,42-44]

Results in our investigation displayed increasing mean ISQ-values, measured with both devices at implant placement, thus being in accordance with measured values of a certain number of other clinical studies.[10,37,40,42-44] As for ISQ-values during implant healing, higher implant stability values were recorded in our study in relation to the findings in other, similar

investigations.[34,36] This observation may be due to the self-tapping thread design of the used implant system in our study, which is showing higher stability values related to non-self-tapping implants, as confirmed by the findings of Markovic and colleagues in 2013.[26]

Two present in vitro investigations reported a merely weak correlation between mean IT at time of implant placement and RFA values during healing process.[45,46] Since the insights of a recently published systematic review by Lages et al. exhibited no statistically significant correlations between ISQ and IT, the impact of IT on implant stability remains debatable.[47] Our results displayed a significant statistical correlation between IT and ISQ at T1, when recorded with both measuring devices in both measurement directions. This observation is comparable with the insights of a clinical study recently published by McCollough and colleagues.[39] In this study high RFA values were traced back to the macro-thread design of the investigated implant system. Since the implant system in our study revealed a comparable design, implant stability in the early post-operative healing period (at T1 after 10 days) might be due to the macro-threads of the applied implants.

PRF and bovine bone were implemented as standard measures, due to our good clinical experience in terms of a proper handling by combining both materials for bone augmentation, ridge preservation respectively, as well as for the supposed beneficial effects of PRF on implant primary stability. As assessed in a recent publication of our study group, increased implant primary stability might be due to a presumed stimulation of coagulation factors.[48] Our findings were confirmed by the results of a randomized controlled clinical trial, displaying similar beneficial effects of PRF on implant primary stability.[49] As recently shown by Tarnow and colleagues, bovine bone substitute is working well, when used as augmentation material for ridge preservation immediately after tooth extraction, as we performed in our study.[50]

As bone quality represents another important parameter for implant osseointegration,[51,52] primary stability is highly dependent on bone mineral density, showing lower values in low quality bone.[51] Obviously, different forms of implants require different insertion torques in order to obtain primary stability.[27] For instance, implants with a large thread design are recommended in low-density bone for a proper primary stability.[23,39] Furthermore, bone quality obviously seems to be an important factor to determine readings of RFA-values.[9,12] In our present study, bone density was not assessed before implant treatment. This may represent a potential source of bias related to effects on implant´s osseointegration. Anterior and posterior sites of the maxilla and mandible represent comparable bone quality in each jaw, with lower quality bone in posterior sites.[36] However, implants were placed all in posterior sites of the maxilla and the mandible of systemically healthy individuals. Due to the implant´s distribution in our study, potential source of bias could be reduced. On the other hand, RFA-values seem to be not suitable for the evaluation of bone quality, due to

the insights of a comparative clinical study, where no significant correlation could be found between implant stability and bone type.[53] For this reasons, and with a view to the used large-threaded implant system, assessment of bone density/quality might be a negligible feature in our investigation.

Nonetheless, we recorded higher RFA-values at nearly all time points in implants, placed in the mandible, reaching statistical significance of RFA values in particular when measured in both directions with both measurement devices at late healing stages at T3 after 17 weeks. A number of experimental clinical studies confirmed our observation, by presenting superior RFA-values in implants placed in the mandible compared to implants placed in the maxilla.[36,54-57] Relying on the insights of our investigation, implant location (mandible vs. maxilla) may have had a significant impact on implant stability during the late healing period.

Our observation, that gender had no significant influence on implant´s primary and secondary stability, as expressed by RFA-values recorded at different time-points, was contradictory to findings of other investigations, where female gender was associated with lower implant stability at implant placement.[36,58] One reason for this contradictory finding may be linked to the low number of female participants in relation to male subjects, to their significant lower mean age, or due to the assumed influence of the used implant´s thread design in our investigation.

The fact, that we used only one implant system in our study, may be of high importance for the comparability of RFA-values. Since various clinical investigations displayed a high influence of implant design on implant stability, the use of the same implant system may have had avoided potential bias by impeding any influence of implant design parameters.[59-61] Another advantage of the application of a single implant system was located in the same connection type between implants and transducer pegs of both measurement devices. Thus, repeated calibration procedures could be avoided. Since misfits in the implant-peg connection were identified as potential source of erroneous measurements, best possible fit between implants and measurement devices is prerequisite for precise and reproducible RFA-measurements.[6] Due to the same implant-peg connection type, this potential source of bias could be impeded for both devices during measurement. Additionally, all measurements were performed on implant platform-level. Since results of a cross-sectional study revealed a significant influence of measurement height on RFA-values, this potential source of bias could be expelled as well.[5]

Due to the experimental setup of our investigation, smartpegs were screwed manually by the investigator onto the implants, resulting in a missing torque control during tightening of the transducer. For this reason, tightening of smartpegs may have served as another potential source of bias concerning validity and reproducibility of RFA measurements.

Obviously there is still disagreement about the optimal torque for tightening smartpegs on implants in order to create valid and reliable ISQ records.[62] Transducer torques ranging between 10.0 and 17.0 Ncm (hand tightening),[63] 2.0 to 11.0 Ncm (hand tightening), and 2.0 to 6.0 Ncm (machine tightening),[62] were recently reported in two in vitro-studies as adequate for accurate implant stability measurement. Since only minor measurement differences were reported between hand- and machine tightening,[62] a potential distortion of measurements, being caused by improper hand tightening of the transducer pegs could also be excluded.

Implant diameter was described as an influential factor on implant´s primary and secondary stability in clinical studies, showing a high correlation between larger implant diameters and increasing stability values.[58] In contradiction, another clinical study revealed no relationship between implant diameter and increasing ISQ values.[64] Other investigations revealed a positive correlation between shorter implants (8.0 mm) and implant stability.[42] A recent in vitro investigation revealed no correlation between implant stability and diameter/length of the implants, as angular stiffness at the implant´s neck seemed to be a superior index for quantifying implant stability in relation to the implant´s design.[65] In our investigation, both devices displayed a significant correlation between implant diameter and RFA-values in both measurement directions at time of implant placement in bucco-oral direction. Our observation may serve primarily as a reference for a significant correlation between implant diameter and primary stability. No correlation was found between implant length and implant stability in our investigation.

RFA and ISQ were judged by several authors as predictors for implant success, indicating increased risks for implant failure, when records were falling below certain threshold levels.[58] Nonetheless, judging higher risks for implant loss, merely on the basis of ISQ-, and RFA-values, should be considered with caution, as shown in an animal study.[35] Furthermore, RFA readings with the Osstell and Penguin device were not able to predict implant failure, unless they were reliable in determining implant stability, as displayed in a recently published clinical human trial.[12] Results of another clinical investigation revealed no correlation between distribution of ISQ and successful implant osseointegration, when measured with the Osstell device.[66] Thus, RFA-measurements may be looked at as an additional feature to predict implant success and as a tool for subsequent follow up examinations during healing stages mainly in the short-term,[21] and not as an instrument with a reliable prognostic value in the long-term.[66] Therefore, RFA measurements should be considered as supplementary tools to radiographic assessments and clinical records.[44] Additionally, measurement values for implant stability seem to be more reliable, when implants were placed in stiff materials (e.g. bone with high density) indicating measurement errors in bone with lower quality.[67]

Nonetheless, RFA can provide relevant clinical information about the state of implant-bone interface at any time during treatment and at follow-up examinations.[7] Thus, RFA measurement is a very viable and repeatable diagnostic method to monitor osseointegration at any conceivable time point after implant placement,[1] obviously being the most frequently used method for clinical measurement of implant stability.[68]

The present study did not reveal any significant differences between RFA-values, when recorded in two different directions and with two different measurement devices. This observation is in agreement with the findings of other clinical investigations,[46,69,70] but it stands in contradiction to certain observations, revealing significant differences between measurements with the Osstell- and the Penguin-device.[68,71] The reasons for these controversial results remain inconclusive and should be subject of further investigations of our study group. Our findings support the application of both measurement devices as reliable and useful tools to determine implant stability, as shown in other clinical investigations.[12,45,68] Notwithstanding the lack of statistical significance, RFA-values were consistently higher, when measured with both devices in a mesio-distal direction. This observation is confirming the results of a study performed by Capek et al. in 2009, which demonstrated an increase in RFA-values, by turning the transducer peg from a perpendicular (bucco-oral) into a parallel (mesio-distal) position to the long axis of the alveolar crest.[8] The authors pointed out, that transducer pegs may not be rotated more than 30 degrees during recording in order to avoid distortion of RFA-values. Hence, measurement position was standardized for valid and reproducible measurements.[8,72]

Conclusion

From a clinical point of view, both devices reveal functional differences in terms of handling and sustainability. SmartPegs of the Osstell device are merely articles for single use, while MultiPegs of the Penguin device are reusable items, due to their sterilisability. Due to the cable connection of the Osstell, handling with the cordless Penguin was stated as much easier, based on the experience, we´ve made during the experimental part of our present investigation. With the Osstell Beacon, the Osstell Company introduced a cordless device as well to a larger audience in June 2018 during the EuroPerio 9 in Amsterdam. Due to the fact, that marketability was reached after the onset of our study, which was in 2015, this new device could not be taken into account in the present investigation. This should be part of further comparative studies. Finally there is an obvious difference in price between both measurement devices, with Penguin and reusable Multipegs being advantageous from an economical point of view. Reusability of MultiPegs may offer an additional benefit with regard on ecological aspects.

Tables and Legends

Table 1. Changes in mean RFA values between measuring points for the Ostell device

Osstell RFA bucco-orally (bo)				
Point of measurement	**Valid cases**	**Mean RFA (Hz)**	**CI**	**STD**
T0 (time of implant insertion)	28	76.8	±2.3	6.0
T1 (after 10 days)	23	78.3	±2.5	5.8
T2 (after 7 weeks)	26	79.6	±2.8	6.9
T3 (after 17 weeks)	24	81.6	±2.3	5.4
Osstell RFA mesio-distally (md)				
Point of measurement	**Valid cases**	**Mean RFA (Hz)**	**CI**	**STD**
T0 (time of implant insertion)	28	78.2	±2.1	5.5
T1 (after 10 days)	23	79.7	±2.0	4.7
T2 (after 7 weeks)	26	80.3	±2.6	6.4
T3 (after 17 weeks)	24	82.9	±1.9	4.6

Table 2. Changes in mean RFA values between measuring points for the Penguin^{RFA} device

Penguin^{RFA} RFA bucco-orally (bo)				
Point of measurement	**Valid cases**	**Mean RFA (Hz)**	**CI**	**STD**
T0 (time of implant insertion)	28	75.8	±2.1	5.5
T1 (after 10 days)	22	77.4	±2.7	6.0
T2 (after 7 weeks)	25	77.8	±3.2	7.7
T3 (after 17 weeks)	25	82.2	±2.0	4.9
Penguin^{RFA} RFA mesio-distally (md)				
Point of measurement	**Valid cases**	**Mean RFA (Hz)**	**CI**	**STD**
T0 (time of implant insertion)	28	77.5	±2.0	5.2
T1 (after 10 days)	22	79.2	±1.9	4.3
T2 (after 7 weeks)	25	78.9	±3.2	7.9
T3 (after 17 weeks)	25	83.0	±2.0	4.9

Table 3. RFA values of both measurement devices at different time points of measurement

1. RFA bucco-orally (bo)								
Measuring points	**T0**		**T1**		**T2**		**T3**	
Device	**Osstell**	**Penguin**	**Osstell**	**Penguin**	**Osstell**	**Penguin**	**Osstell**	**Penguin**
RFA values	76.9	75.8	78.3	77.4	79.6	77.8	81.6	82.2
Significance p	0.533		0.616		0.398		0.700	
1. RFA mesio-distally (md)								
Measuring points	**T0**		**T1**		**T2**		**T3**	
Device	**Osstell**	**Penguin**	**Osstell**	**Penguin**	**Osstell**	**Penguin**	**Osstell**	**Penguin**
RFA values	78.2	77.5	79.7	79.2	80.3	78.9	82.9	83.0
Significance p	0.652		0.726		0.490		0.904	

Table 4. Osstell: mean ISQ and differences between time points of measurement

1. ISQ bucco-orally (bo)						
Measuring points	**T0-T1**	**T0-T2**	**T0-T3**	**T1-T2**	**T1-T3**	**T2-T3**
Significance p	1.000	*0.001*	*<0.001*	*0.006*	*<0.001*	0.146
1. mesio-distally (md)						
Measuring points	**T0-T1**	**T0-T2**	**T0-T3**	**T1-T2**	**T1-T3**	**T2-T3**
Significance p	0.732	*0.003*	*<0.001*	*0.004*	*<0.001*	*0.022*

Table 5. Penguin: mean ISQ and differences between time points of measurement

1. RFA bucco-orally (bo)						
Measuring points	**T0-T1**	**T0-T2**	**T0-T3**	**T1-T2**	**T1-T3**	**T2-T3**
Significance p	0.758	*0.033*	*<0.001*	*0.028*	*<0.001*	*0.025*
1. RFA mesio-distally (md)						
Measuring points	**T0-T1**	**T0-T2**	**T0-T3**	**T1-T2**	**T1-T3**	**T2-T3**
Significance p	0.912	0.100	*<0.001*	*0.004*	*<0.001*	*0.031*

Table 6. Differences between RFA values in dependence on the measuring directions for both devices

	Osstell								**Penguin**							
Point of measurement	**T0**		**T1**		**T2**		**T3**		**T0**		**T1**		**T2**		**T3**	
Direction	bo	md	bo	md	bo	md	bo	md	bo	md	bo	md	bo	md	bo	md
ISQ	76.9	78.3	78.2	79.6	76.9	78.3	78.2	79.6	75.9	77.6	77.4	79.3	77.9	79.1	82.2	83.0
Significance p	0.332		0.363		0.677		0.364		0.209		0.222		0.575		0.510	

Table 7. Correlation between mean IT and RFA-values

Device	**Osstell**				**Penguin**			
Measuring direction	**bucco-orally (bo)**				**bucco-orally (bo)**			
Measuring points	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**
Correlation coefficient r	0.409	0.031	0.305	0.255	0.416	0.119	0.169	0.371
One-sided significance	*0.015*	0.443	0.064	0.114	*0.013*	0.297	0.208	*0.037*
Measuring direction	**RFA mesio-distally (md)**				**RFA mesio-distally (md)**			
Measuring points	**T0**	**T1**	**T2**	**T3**	**T0**	**T1**	**T2**	**T3**
Correlation coefficient r	0.483	0.108	0.250	0.406	0.496	0.091	0.149	0.340
One-sided significance p	*0.004*	0.311	0.108	*0.024*	*0.003*	0.342	0.237	*0.048*

Table 8. Correlation between primary stability at T0 and RFA-values at T1, T2, and T3

Device	**Osstell**			**Penguin**		
Measurement direction	**RFA bucco-orally (bo)**			**RFA bucco-orally (bo)**		
Measuring points	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Correlation coefficient r	0.113	0.693	0.724	0.225	0.744	0.186
One-sided significance p	0.302	*<0.001*	*<0.001*	0.156	*<0.001*	0.185
Measurement direction	**RFA mesio-distally (md)**			**RFA mesio-distally (md)**		
Measuring points	**T1**	**T2**	**T3**	**T1**	**T2**	**T3**
Correlation coefficient r	0.337	0.739	0.758	0.241	0.772	0.274
One-sided significance p	p0.057	*<0.001*	*<0.001*	0.139	*<0.001*	0.092

Figures

Figure 1. Osstell device

Figure 2. Penguin device

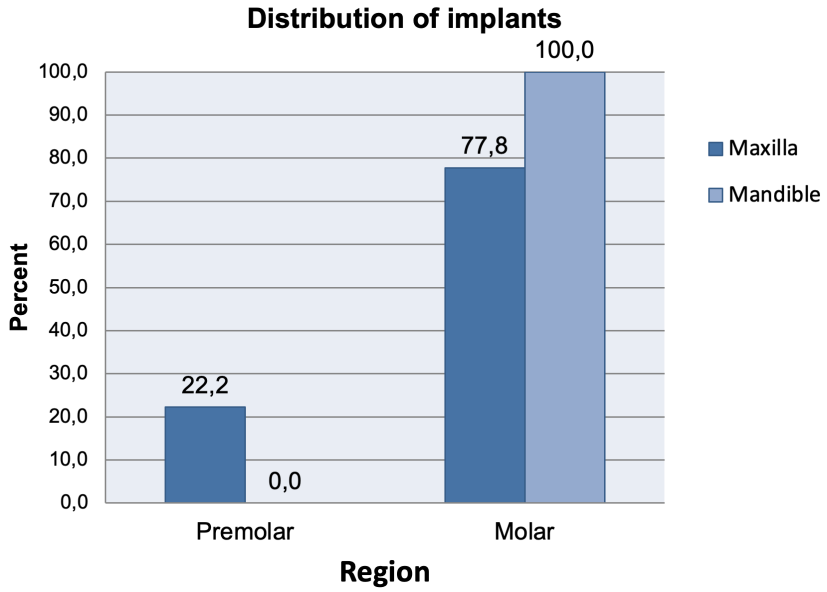

Figure 3. Distribution of implant locations (jaw and area)

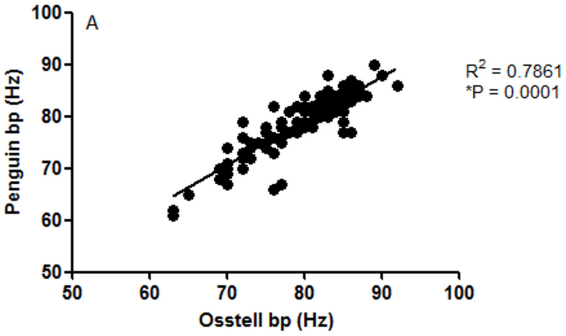

Figure 4. High correlation between both devices in bucco-oral (bo) direction

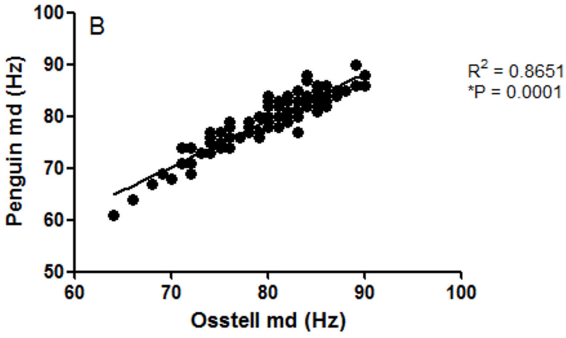

Figure 5. High correlation between both devices in mesio-distal (md) direction

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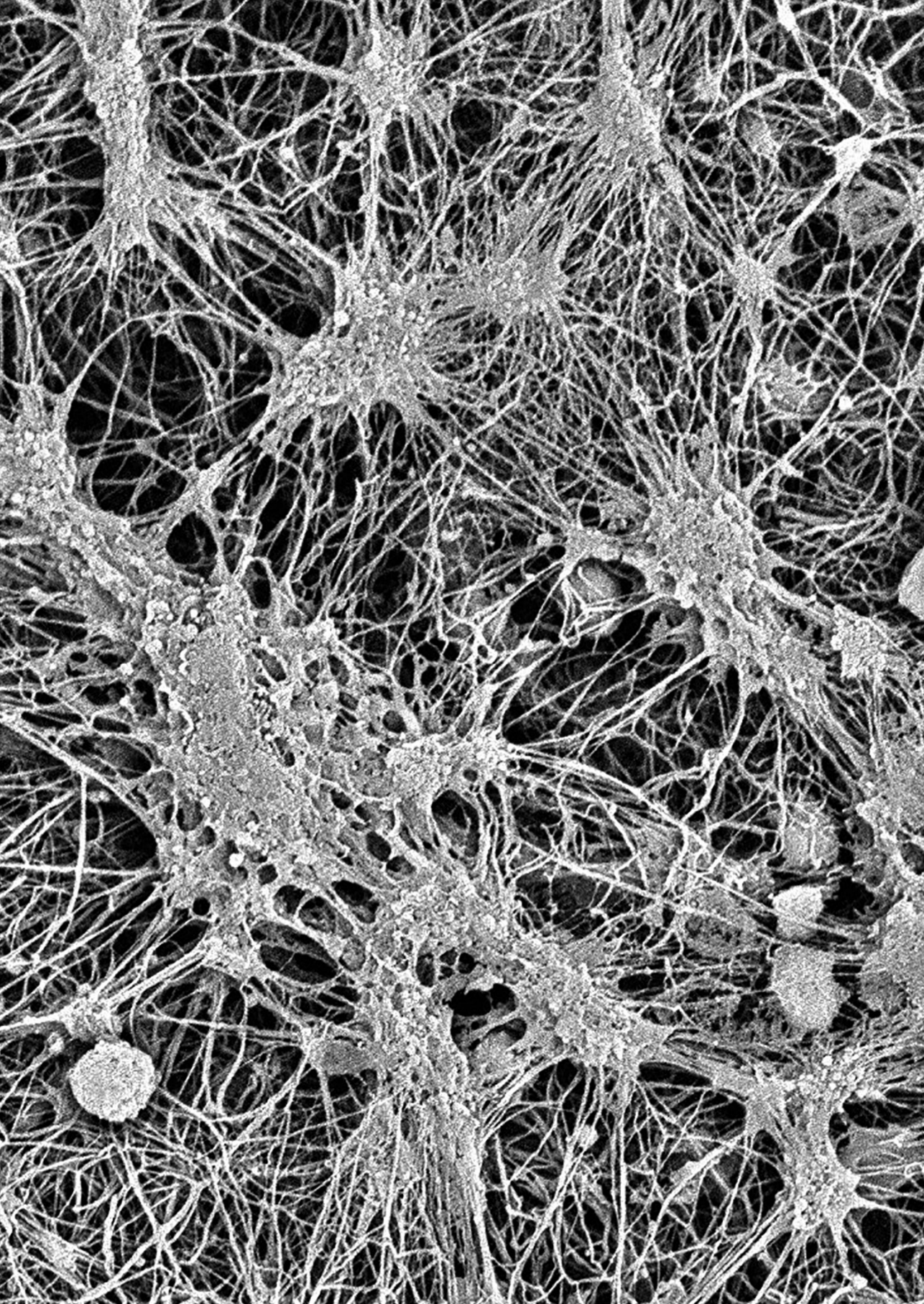

Chapter 5

Implant stability in patients treated with platelet-rich fibrin and bovine bone substitute for alveolar ridge preservation is associated with peripheral blood cells and coagulation factors

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Abstract

Aims:
The aim of the present study was to assess the association between dental implant stability and peripheral blood cell composition and levels of coagulation factors in patients treated with alveolar ridge preservation with platelet-rich fibrin (PRF) and bovine bone substitute.

Materials and Methods:
Fifty patients were included between 2015 and 2017. PRF was prepared from autologous blood, in which blood cells and coagulation factor levels were measured. PRF and bovine bone (DBBM) were placed in the socket, followed by closure with PRF membrane. Implants were placed 14 (± 2.5) weeks post-extraction. The implant stability quotient (ISQ) was measured at t=0, t=10 days, t=7 weeks and t=17 weeks by resonance frequency analysis.

Results:
Erythrocyte count was inversely associated with PRF membrane length, but not with implant stability. Conversely, platelet count did not correlate with membrane size, but inversely correlated with implant stability at 7 and 17 weeks. In addition, implant stability was directly correlated with levels FXIII (t=0, $p<0.01$), active von Willebrand Factor (t=0 and 7 weeks $p<0.05$) and total VWF (t=7 weeks, $p=0.012$).

Conclusion:
Implant stability following alveolar ridge preservation with PRF and bovine bone substitute is associated with circulating blood cells and coagulation factors. In particular, fibrin structure, von Willebrand Factor and FXIII may be important modulators of implant stability.

Key Words:
Platelet-rich fibrin, implant stability, alveolar ridge preservation, blood coagulation, blood cells

Introduction

Upon tooth extraction or tooth loss, progressive resorption of the alveolar bone occurs.[1,2] This can lead to insufficient height and width of the residual bone, which negatively influences the prognosis of implant-supported prostheses.[3,4] One of the available treatment options to prevent this is alveolar ridge preservation.[5,6]

Platelet-inspired biomaterials have been extensively studied for this purpose. In particular, second-generation platelet concentrates, such as leukocyte- and platelet-rich fibrin (L-PRF),[7,8] have important advantages: preparation of PRF is relatively fast, easy and no additives or anticoagulants are required, which makes PRF more economic than other platelet-dependent biomaterials used to fill the alveole.[9] PRF consists of a slowly polymerized fibrin network with enmeshed cytokines, glycanic chains, structural glycoproteins, platelets and leukocytes.[9,10] Platelets secrete fibrinogen, fibronectin, and vitronectin, which behave as a matrix for connective tissue and adhesion molecules, facilitating cell recruitment to the wound area.[11-15] The fibrin network has a key role in early wound healing and functions as a scaffold for ingrowing cells and as a reservoir of cytokines.[16,17] Importantly, the cells entrapped in the fibrin mesh release a variety of growth factors.[10,18,19]

The use of PRF (with or without additional bone grafting material, such as deproteinized bovine bone mineral, DBBM) for ridge preservation has been reported previously. However, its efficacy has been evaluated with heterogeneous approaches and results are inconsistent.[20-22] Interestingly, it was observed that some patients have smaller and/or shorter PRF membranes than others.[23] This may in part be due to differences in red blood cell content, as individuals with lower hematocrit have larger PRF membranes.[23,24] Indeed, the composition of the peripheral blood, including but not limited to red blood cell content, might influence the size and composition of PRF. Besides the cellular fraction, circulating coagulation factors may also influence the properties of the PRF and hence implant stability.

Therefore, in the current study we investigated the association between implant stability and the peripheral blood cell composition and levels of coagulation factors closely involved in fibrin network formation and platelet incorporation.

Materials and Methods

Ethics statement

The research complied with all the relevant national regulations, institutional policies and the tenets of the Helsinki Declaration (2013), and has been approved by the Medical Ethical Review Board of the Gelre Hospitals Apeldoorn (reference 15.38; 28-07-2015). All participants gave full written informed consent.

Study population

This study was designed as a prospective observational study. All study participants were enrolled from the Institute of Oral Implantology (Amersfoort, the Netherlands) between September 2015 and September 2017. We included patients with age ≥18 years, requiring an implant in posterior maxilla or mandible, and classified as American Society of Anaesthesiologists (ASA) I (healthy individual) or ASA II (mild systemic disease) patients. Six of our patients were current smokers, with an average smoking quantity of 6 cigarettes per day (SD=3). Three patients were suffering from high blood pressure, treated with an ACE inhibitor or angiotensin 2 receptor blocker. Five patients had a history of respiratory illness (i.e. asthma and bronchitis), and two of them were treated with either a sympathomimetic or inhalation steroids. One patient had a previous myocardial infarction (2007) and was therefore treated with aspirin and statins.

Preparation of PRF membranes

Three to four (depending on the implant site) 10 mL A-PRF tubes (Process for PRF, Nice, France) were obtained by venipuncture prior to the atraumatic extraction procedure. The A-PRF tubes were immediately centrifuged (PRF DUO, Process for PRF, Nice, France) at an RCF of 200 g (calculated using the formula RCF = 1.12 x Radius x (rpm/1000)2, based on rpm of 1,336 and rotor radius 100 mm), for 8 min. After centrifugation, the tubes were left in an upright position for 10 min. Three layers were obtained, namely red blood cells (RBC) at the bottom, platelet-poor plasma (PPP) at the top and PRF in between. PRF membranes were separated from the red thrombus and placed in the metal PRF box (Process for PRF, Nice, France). Subsequently, PRF exudate was obtained using the lid of the PRF Box as weight. PRF membrane length and width (both in cm) were measured. Subsequently, one of the membranes was cut into fragments and mixed with DBBM (Bego-Oss®, approximately 0.5-1 mL) and the growth-factor rich PRF exudate. The other PRF membranes were used to close the socket.

PRF treatment protocol

The study procedure consisted of two stages, namely ridge preservation with PRF and implant placement, as described below. All procedures were performed under local anesthesia by the same implantologist (J.B.). Atraumatic extraction was performed to preserve as much bone as possible and avoid fracture of the buccal plate. Periotomes and small luxators were used to remove the teeth. DBBM (on average 0.5-1 mL, Bego-Oss®, BEGO Implant Systems GmbH & Co., Bremen, Germany), on average 0.5-1 mL, mixed with PRF (see preparation paragraph 2.3) was placed into the socket until the desired vertical height was achieved. An envelope flap was created on the buccal and palatal side. A PRF membrane was placed in the envelope flap, followed by a mattress suture to fixate the membrane. No antibiotics or pain medications were prescribed before extraction and postoperatively. After a healing period of 14±2.5 weeks, the implant site was prepared using a trephine drill with a diameter of

2 mm . Bone level implants (Bego RS or SC implants, BEGO) with diameters between 4.1 and 5.5 mm were placed. Insertion torque (N/cm) was determined using the Oral Implantcenter (Acteon, Bordeaux, France). All patients were followed 17 weeks after implantation.

Analysis of blood cell and coagulation parameters

Peripheral blood samples were obtained by venipuncture (concurrently with blood for PRF preparation, prior to extraction) into two 2 mL EDTA tubes and two 2 mL 3.2% sodium citrate tubes (Vacuette, Greiner Bio-One, Kermsmunster, Austria). Blood cell counts (platelets, erythrocytes, hematocrit, leukocytes) were measured using the hematology Cell-Dyn Sapphire analyzer (Abbot Diagnostics, Wiesbaden, Germany). Clotting times (PT, APTT) and the coagulation factors fibrinogen, FVIII and FXIII were measured using the STA-Max analyzer (Stago, Asnières-sur-Seine, France). To measure VWF and active VWF, i.e. VWF in its unfolded state able to bind platelets via the GPIb platelet receptor, we used in-house sandwich enzyme-linked immunosorbent (ELISA) assays, as described earlier [25]. (Active) VWF levels are expressed as a percentage (%) of levels measured in normal pooled plasma (NPP).

Measurement of implant stability

The stability of the implants was evaluated with resonance frequency analysis (RFA). The measurements were carried out with the Osstell device (Osstell, Göteborg, Sweden). The implant stability quotient (ISQ) has a range from 1 to 100, with a higher number indicating a more stable implant. ISQs were measured buccal/palatinal (bp) and mesial/distal (md). Ideally, the same ISQ value is found from both directions, indicating that the bone-implant interface is the same around the implant. However, if the bone is inhomogeneous the implant can have different stability in different directions. Therefore, we provide both the bp and md ISQ values. ISQ measurements were performed immediately after surgery (t=0, n=38) and 10 days (n=36), 7 weeks (n=39) and week 17 (n=50) after implant placement.

Statistical analysis

The normality of continuous variables was assessed graphically in histograms and normality Q-Q plots and tested using the Shapiro-Wilk test. Continuous variables with a skewed distribution are presented as median (interquartile range, IQR). Values for continuous non-skewed variables are presented as means ± SD. Correlations between parameters are expressed as the Spearman's rank correlation coefficient r, with the corresponding p-value. Comparison of the clinical outcome parameter between different time points was performed by Kruskal Wallis test with pair-wise posthoc test (including Bonferroni correction for multiple comparisons). IBM Statistical Package for Social Sciences (SPSS) version 25 software was used for all statistical analyses (SPSS Incorporated, Chicago, USA). Figures were prepared using GraphPad Prism version 5.00 (GraphPad Software, San Diego, USA). A p-value of <0.05 was considered as statistically significant.

Results

Patient characteristics and implant stability measurements

Patient demographics are provided in Table 1. The study consisted of 50 patients (28 males (56%) and 22 females (44%)) with an age range between 35 and 82 years (mean 58.8 ± SD 11.2 years), treated with a composite graft of PRF and DBBM for alveolar ridge preservation prior to placement of a dental implant.

Implant stability, measured as the ISQ, was determined at several time points over the course of the study (Table 1, Figure 1a, b). Directly after placement of the implant (t=0), median ISQs were bp 73.5 (IQR 5.0) and md 74.5 (IQR 5.0). After 10 days ISQs were 78.5 (SD 5.4) bp and 79.6 (SD 4.3) md. The implant stability increased slightly more during the remaining follow-up, with median bp ISQs of 77.0 (IQR 7.0) and 79.0 (IQR 9.0) and md ISQs of 79.0 (IQR 5.0) and 81.5 (IQR 8.0) at 7 and 17 weeks after implantation, respectively. Together, these data confirm the good primary and secondary stability of the dental implants.

Effect of peripheral blood cell composition on PRF membrane

We hypothesized that peripheral blood cell composition and coagulation factor levels may influence PRF membrane characteristics (Table 2). A significant inverse correlation between erythrocyte count and PRF membrane length (n=41) was observed: PRF membranes prepared from patients with a higher erythrocyte count in peripheral blood (and higher hematocrit) resulted in shorter PRF membranes than patients with a lower erythrocyte count (Figure 2a, b). Conversely, there were no significant associations between platelets (Figure 2c) and PRF membrane size parameters. FVIII and active VWF levels correlated significantly (p=0.024 and p=0.033, respectively) with PRF membrane area (n=17) (Figure 2d, e), whereas fibrinogen levels did not correlate with PRF membrane area (Figure 2f) or length.

Table 1: Patient demographics and clinical data on implant stability.

Variable	Mean ± *SD* or medium (IQR)	N
Age, years	58.8 ± 11.2	50
Sex (% male)	56	50
Current smoker (%)	12	50
Membrane and implant		
Length (cm)	3.1 ± 0.4	41
Width (cm)	1.2 (0.3)	17
Area (cm^2)	3.9 ± 0.9	17
Insertion Torque (*N*/com)	50 (18)	44
Maxillary implant[a]	62%	31
Total nr. of extractions	62	50
Molars extracted (*n*)	37	50
ISQs		
t = 0 bp	73.5 (5)	38
t = 0 md	74.5 (5)	38
t = 10 days bp	78.5 ± 5.4	28
t = 10 days md	79.6 ± 4.3	28
t = 7 weeks bp	77.0 (7.0)	30
t = 7 weeks md	79.0 (5.0)	30
t = 17 weeks bp	79.0 (9.0)	50
t = 17 weeks md	81.5 (8.0)	50

Abbreviations: bp, buccal-palatal; ISQ, implant stability quotient; md, mesial-distal.
[a]Out of the 50 implants in total, 19 (38%) were placed in the mandibula and 31 (62%) in the maxilla. *N* in the last column indicates parameter were available.

Effect of peripheral blood cell composition on implant stability

Because we found that specific cellular- and coagulation parameters influence PRF characteristics, we were interested in the effect of the composition of peripheral blood on implant stability. Platelet count was significantly inversely correlated with bp (Figure 3a) and md (Figure 3b) ISQ scores at 7 and 17 weeks, while erythrocytes were not significantly correlated with implant stability. At 7 weeks post-implantation, higher implant stability was associated with lower leukocyte count (Figure 3c), but this was not observed at 17 weeks.

Significant correlations between implant stability and a number of coagulation parameters were observed, namely between ISQ at t=0 and FXIII (Figure 4a) and active VWF (only bp, Figure 4b), and between ISQ at t=7 weeks and active VWF (Figure 4b) and total VWF (only bp, Figure 4c).

Discussion

In the present study, we analyzed the stability of non-immediate implants placed following ridge preservation with a mixture of autologous PRF and DBBM xenograft. We found several interesting correlations between implant stability, peripheral blood cell counts and the coagulation factors (active) von Willebrand Factor and FXIII.

Blood composition may have dual effects on wound healing and tissue regeneration, namely a direct effect on the composition of the (PRF) clot and an indirect effect on the circulation of the wound area. Conversely, the underlying condition requiring tooth extraction (e.g. periodontitis) may influence peripheral blood cell composition and coagulation status. We found several interesting correlations between blood cell counts and coagulation factor levels on the one hand and membrane characteristics and implant stability on the other hand. Surprisingly, platelet counts were not associated with PRF membrane size. However, higher erythrocyte counts in peripheral blood correlated with shorter PRF membranes. This may be explained by the fractional volume of erythrocytes (hematocrit) and plasma: a lower erythrocyte count (and thus hematocrit) is likely accompanied by a larger plasma fraction after centrifugation, which results in a longer clot. Indeed, hematocrit showed a significant inverse correlation with membrane length ($r=-0.321$, $p<0.05$), in accordance with findings by others.[23]

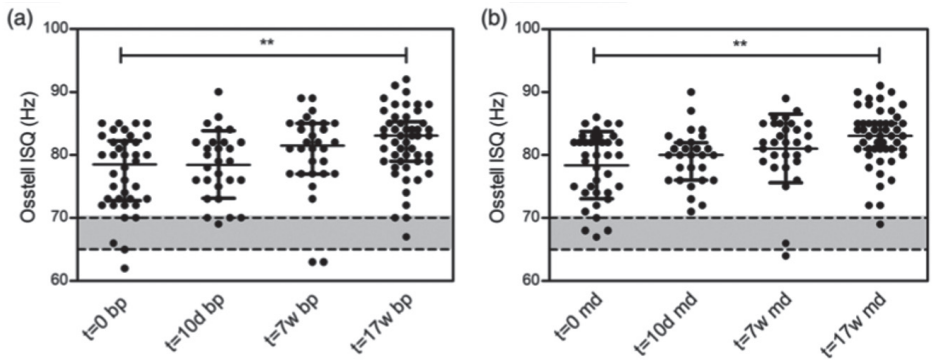

Figure 1. Changes in ISQ values during 17-week follow-up after implant placement. Whiskers represent mean and corresponding SD (t=0, t=7w, t=17w) or median and interquartile range (t=10d) for bp (panel a) and md (panel b) ISQs. Gray area indicates reference value for ISQ. **, $p<0.01$ in Kruskal-Wallis test and post hoc comparison with Bonferroni correction. Bp, buccal/palatal; d, days; md, mesial/distal; w, weeks.

Table 2. Blood cells and coagulation factors in PRF-treated patients prior to extraction.

Variable	N	Mean ± SD or medium (IQR)	Reference values
Blood cells			
Platelets (x10^9/L)	50	254.7 ± 55.3	150-400
Erythrocytes (x10^{12}/L)	50	4.7 ± 0.5 (Total)	
		4.9 ± 0.4 (Men)	4.4-5.8 (Men)
		4.4 ± 0.3 (Women)	4.0-5.3 (Women)
Leukocytes (x10^9/L)	50	6.9 (2.5)	4-10
Coagulation factors			
Fibrinogen (g/L)	41	2.9 ± 0.5	2.0-4.0
FXIII (%)	38	83.5 (18)	70-140
Active VWF (% of NPP)	38	126.1 (75.7)	91.6-154.8†
Total VWF (%)	38	99.6 (47.6)	50-150

Note. All reference values were derived from the Dutch Society for Clinical Chemistry ("Nederlandse Vereniging voor Klinische Chemie NVKC. Algemeen overzicht referentiewaarden," 2018) except those indicated by †, which were derived from (van der Vorm et al., 2019).
Abbreviations: NPP, normal pooled plasma; VWF, von Willebrand factor.

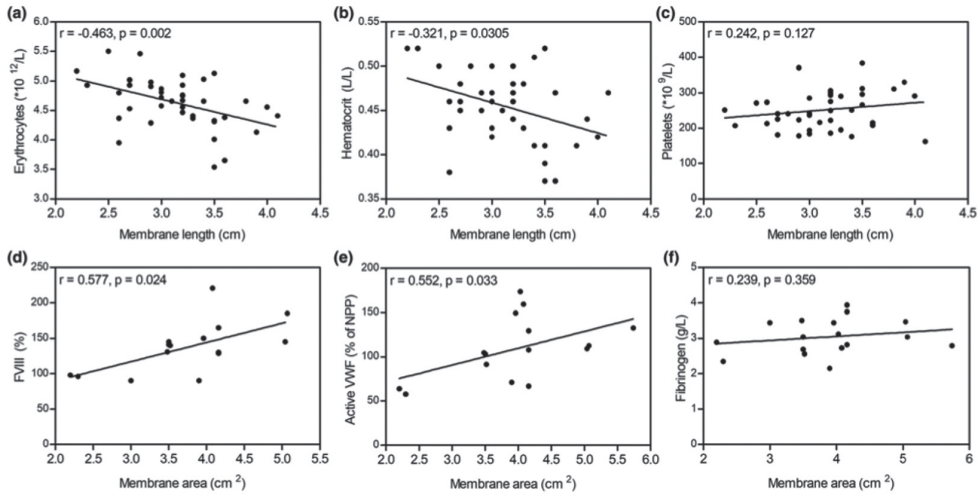

Figure 2. Effects of peripheral blood cells and coagulation factors on PRF membrane. PRF membrane size correlated significantly with (a) erythrocyte count and (b) hematocrit but not with (c) platelet count. Of the coagulation factors, PRF membrane area correlated with (d) FVIII and (e) active VWF levels but not with (f) fibrinogen levels. Spearman correlation coefficients and corresponding p values are indicated in the upper left corner. PRF, platelet-rich fibrin; VWF, von Willebrand factor

Furthermore, membrane area correlated significantly with FVIII and active VWF levels. FVIII and (active) VWF both play a central role in hemostasis, illustrated by the severe bleeding phenotype associated with hemophilia A and von Willebrand disease, respectively. Hemophilia A patients are known to have more irregular fibrin clot structure than healthy individuals, composed of thicker and shorter fibers, and this effect is mediated through reduced thrombin generation.[26] Although none of our patients suffered from hemophilia, it could be hypothesized that lower FVIII levels affect the fibrin structure in the PRF clot to produce a smaller membrane. Regarding active VWF, one of the key functions of VWF is to tether platelets to the exposed subendothelial collagen after vessel wall damage. Circulating VWF can only exert this function after conversion to its active conformation, which is induced amongst others by shear stress.[27] In healthy individuals, only a minute amount of VWF circulates in its active conformation, but nevertheless there is an inter-individual variation of around 15%.[25] During clot formation, functionally active VWF readily incorporates into the fibrin network and subsequently supports platelet adhesion.[28] Also, the presence of VWF results in the formation of a less dense fibrin network.[28] Thus, during PRF clot formation higher active VWF levels may also facilitate binding of more platelets and may result in a more loose fibrin network, and hence a larger membrane area. Of note, no significant correlation between membrane size and fibrinogen levels was observed, probably because fibrinogen circulates in excess amounts and hence does not limit the size of the PRF membrane.

More of clinical interest is the effect of these peripheral blood parameters on implant stability. Surprisingly, we found that peripheral platelet and leukocyte counts were inversely associated with secondary implant stability (at 7 weeks post-implantation). This finding is counterintuitive as both platelets and leukocytes are considered essential for tissue regeneration and osteogenesis by the release of growth factors and by supporting angiogenesis and lymphogenesis.[16] Our findings challenge this view on the cellular fraction of PRF as the key elements responsible for the clinical efficacy. However, future studies quantifying cell counts as well as growth factor- and inhibitor levels within the clot itself are required to support these findings. Similar to our findings on membrane size, active VWF was also associated with primary implant stability and both active VWF and total VWF levels correlated with secondary stability at 7 weeks. As mentioned, (active) VWF may contribute to both platelet incorporation in the fibrin mesh and to fibrin network structure.[28] Moreover, activated VWF may indirectly affect implant stability by supporting primary wound healing.[28] Levels of FXIII, the coagulation factor responsible for fibrin crosslinking, also correlated with primary implant stability. FXIII is known to be involved in wound healing, as demonstrated by impaired wound repair in FXIII deficient mice.[29] Thus, circulating FXIII levels may both directly (through fibrin crosslinking) and indirectly (through wound healing) affect implant stability. Altogether, our data suggest that the blood composition and the fibrin structure of PRF may be critical modulators of implant stability, and require more emphasis in future, more detailed studies.

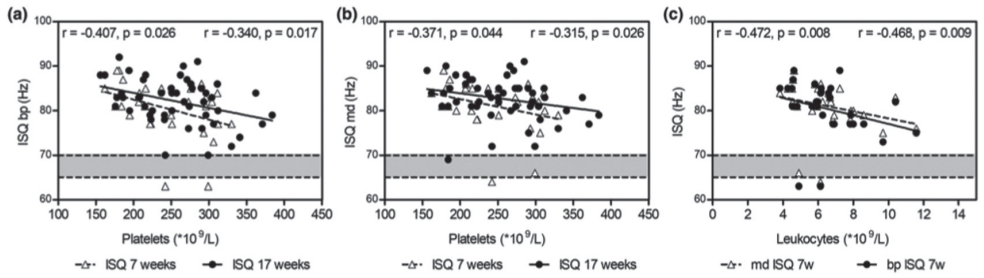

Figure 3. Effects of peripheral blood cells on implant stability. Platelet (a, b) and leukocyte (c) counts correlate with implant stability after 7 and 17 weeks. Spearman correlation coefficients and corresponding p values are indicated in the upper left corner for the parameter indicated with open triangles and in the upper right corner for parameters indicated with closed circles. Gray area indicates reference value for ISQ. bp, buccal/palatal; ISQ, implant stability quotient; md, mesial/distal

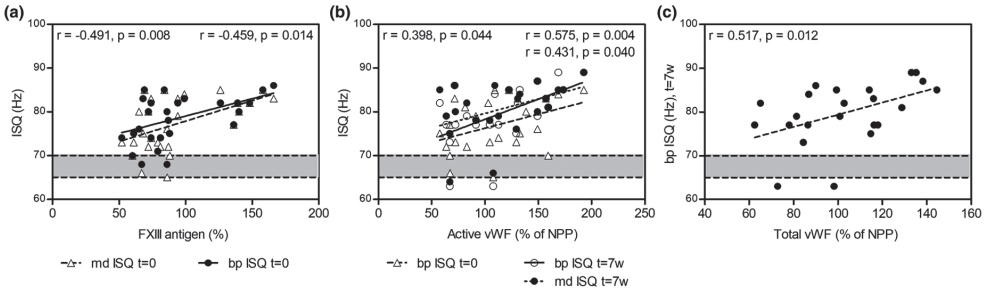

Figure 4. Effects of coagulation factor levels on implant stability. Levels of the coagulation factors FXIII (a), active VWF (b), and total VWF (c) correlate with implant stability after 0 and/or 7 weeks. Spearman correlation coefficients and corresponding p values are indicated in the upper left corner for the parameter indicated with open triangles and in the upper right corner for parameters indicated with closed circles (top for open circles and bottom for closed circles in Figure 4b). bp, buccal/palatal; md, mesial/distal; NPP, normal pooled plasma; VWF, von Willebrand factor; w, weeks

The most important limitation of our study is the lack of randomization to a PRF treatment group and a control group treated with only DBBM, or alternatively a split-mouth model with these two treatments. Given the clinical success of implants placed with PRF and DBBM as a grafting material (as observed by the implantologist involved in this study, J.B.), in particular in patients with an infamous prognosis, withholding this treatment from patients to serve as controls for the current study was deemed unethical. As an alternative, we considered including patients with a more favorable prognosis (such as the absence of inflammation and better residual bone at the time of extraction) as a control group treated with only bovine bone substitute. Although this would be a more ethically acceptable approach, it would confer considerable bias to the effect size of the PRF. A second limitation is that we could not determine blood cell count and coagulation factors in the PRF itself. Previously, methods for measurement or estimation of platelet counts in PRF have been proposed, such as the "subtraction method",[30-32] but unfortunately we did not perform these measurements in the liquid fractions after clot formation. The associations between peripheral blood composition and outcomes (membrane size and implant stability) should therefore be interpreted with caution, as the composition of the PRF may be altered relative to the peripheral blood composition. Thirdly, it was not always possible to schedule patients at each of the three time points, explaining that the number of observations is not 50 for all time points and parameters. A future study should take into account this larger than expected loss-to-follow-up rate. Finally, bone quantity around the implants could not be evaluated histologically and correlated with implant stability. Primary stability measurements were previously demonstrated to correlate significantly with bone density.[33] However, the lack of a control group in the current study already precludes drawing conclusions on the effect of the combination graft of PRF and DBBM on bone density and implant stability, hence in further studies both a control group and histological analysis should be included.

In addition, we are fully aware that association is not the same as causation, and that a large variety of patient-related factors can potentially confound the observed correlations between haematological parameters and implant stability. For instance, age is known to influence coagulation, and older age is associated with higher VWF levels.[34] Although our inclusion criterium was to include all patients >18 years, the majority (43/50, 86%) of our patients was older than 50 years. This may explain why we see no significant associations between age and implant stability itself nor with any of the haematological parameters associated with implant stability. Furthermore, implant stability was not significantly different between men and women or between smokers and non-smokers. Although the negative effects of smoking on implant stability have been described extensively in literature,[35] the number of smokers in the current study is likely too small to detect a significant difference. Likewise, subgroups with co-morbidities were too small to perform meaningful statistical analysis. Larger studies are required to assess possible confounding effects of these patient-related factors on implant stability.

In conclusion, implant stability, following alveolar ridge preservation with PRF and bovine bone substitute, is associated with several haematological parameters. Our results suggest that fibrin structure and levels of (active) VWF and FXIII, more than platelet and leukocyte count, may be determining factors for PRF membrane characteristics and implant stability.

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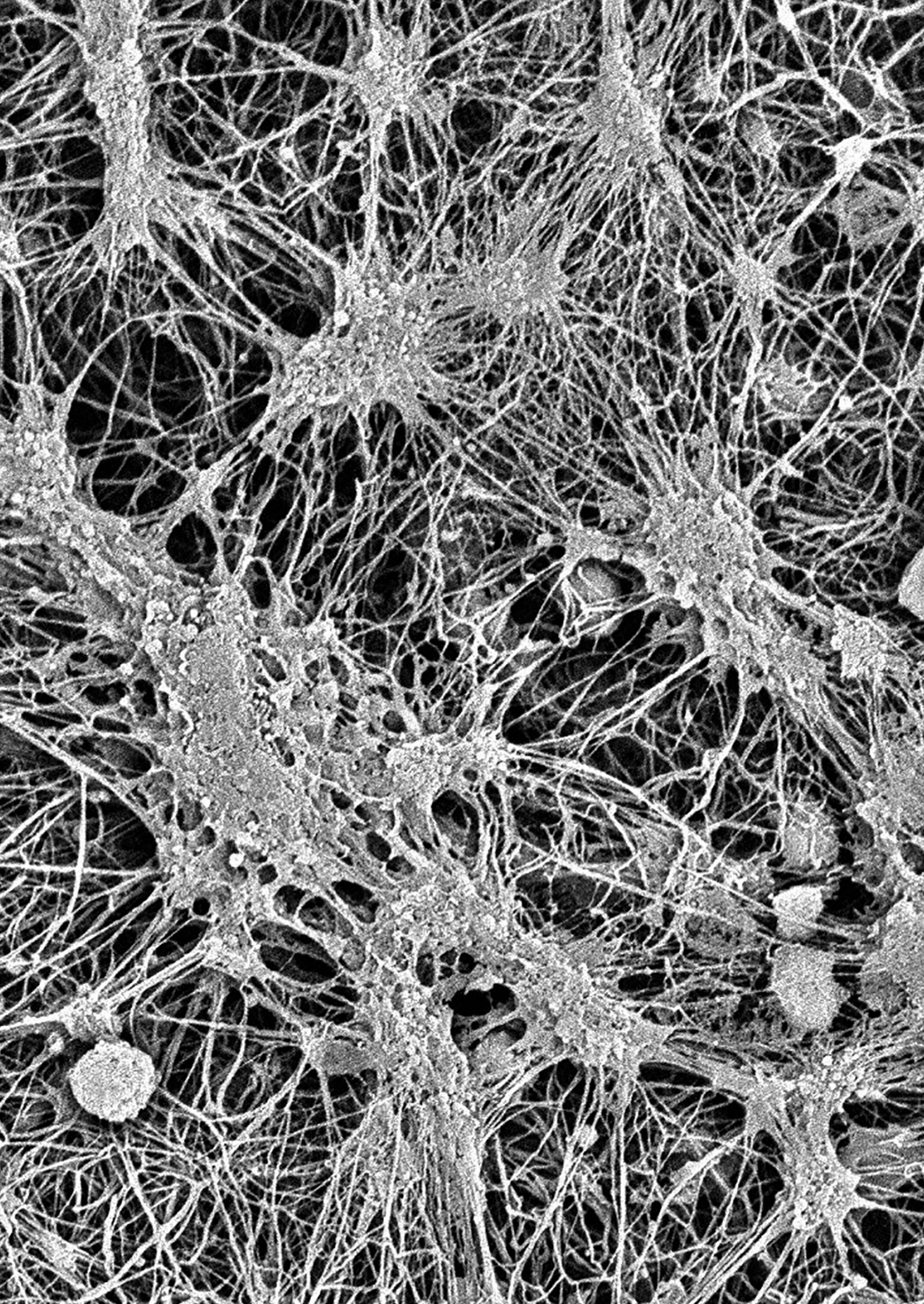

Chapter 6

Histological evidence for osteoconductive effects of autologous platelet-rich fibrin in oral implantology

Joost E.I.G. Brouwers[1], Lisa N. Van der Vorm[2,3,4], Sharon Buis[1,2], Rianne Haumann[2], Joke Konings[3,4], Philip G. de Groot[3,4], Bas de Laat[2,3,4], Jasper A. Remijn[2,3,4,5]

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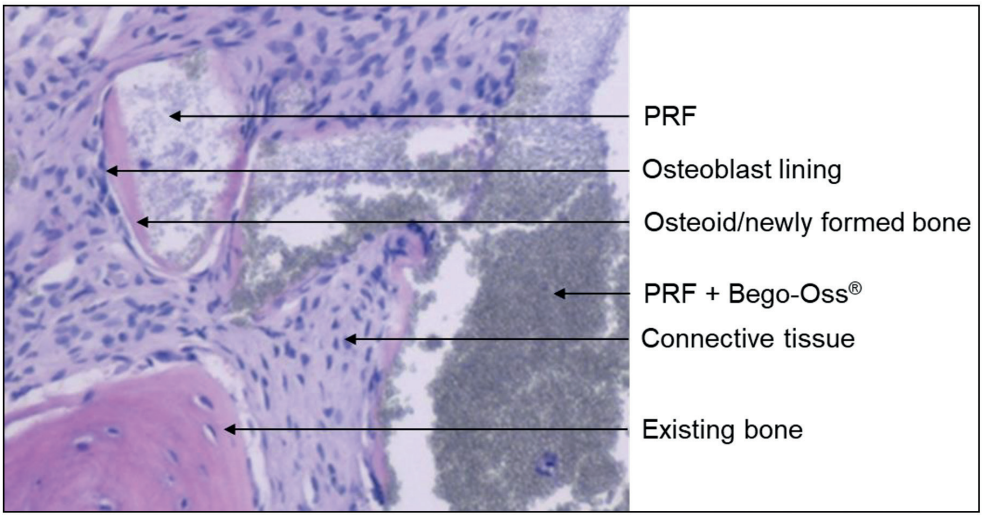
Adapted from:
Journal of Dentistry and Oral Biology 2020: 5(4): 1174: Clinical Image

Application of autologous platelet-rich fibrin (PRF) has emerged as a promising therapy to accelerate wound healing and bone regeneration. In oral implantology, PRF has been used to improve osseointegration of dental implants to increase implant stability, although cellular mechanisms are not fully understood. Therefore, we histologically assessed the effect of pre-implantation extraction socket grafting materials, PRF and bovine bone substitute, on bone regeneration in 19 cases. The extraction sockets contained deproteinized bovine bone mineral (Bego-Oss®) and autologous PRF membrane supplemented with autologous plasma growth factor concentrate.[1] The socket was closed using a PRF membrane, on top of the graft, in an envelope technique. At 14 ± 2.5 weeks post-extraction, the surgical area was reopened for implant insertion and a biopsy cone of transcortical bone was taken. Cones were embedded in paraffin, followed by de-calcification and haematoxylin-eosin staining. The presented Clinical Image shows a representative histological image of a biopsy revealing the presence of newly formed bone. This osteoid matrix is deposited at the borders of the remnants of PRF and bovine bone, and dense cellular osteoblasts lined in fronts residing in connective tissue. These findings suggest that PRF and bovine bone have osteoconductive effects as early as 14 weeks post-PRF treatment.



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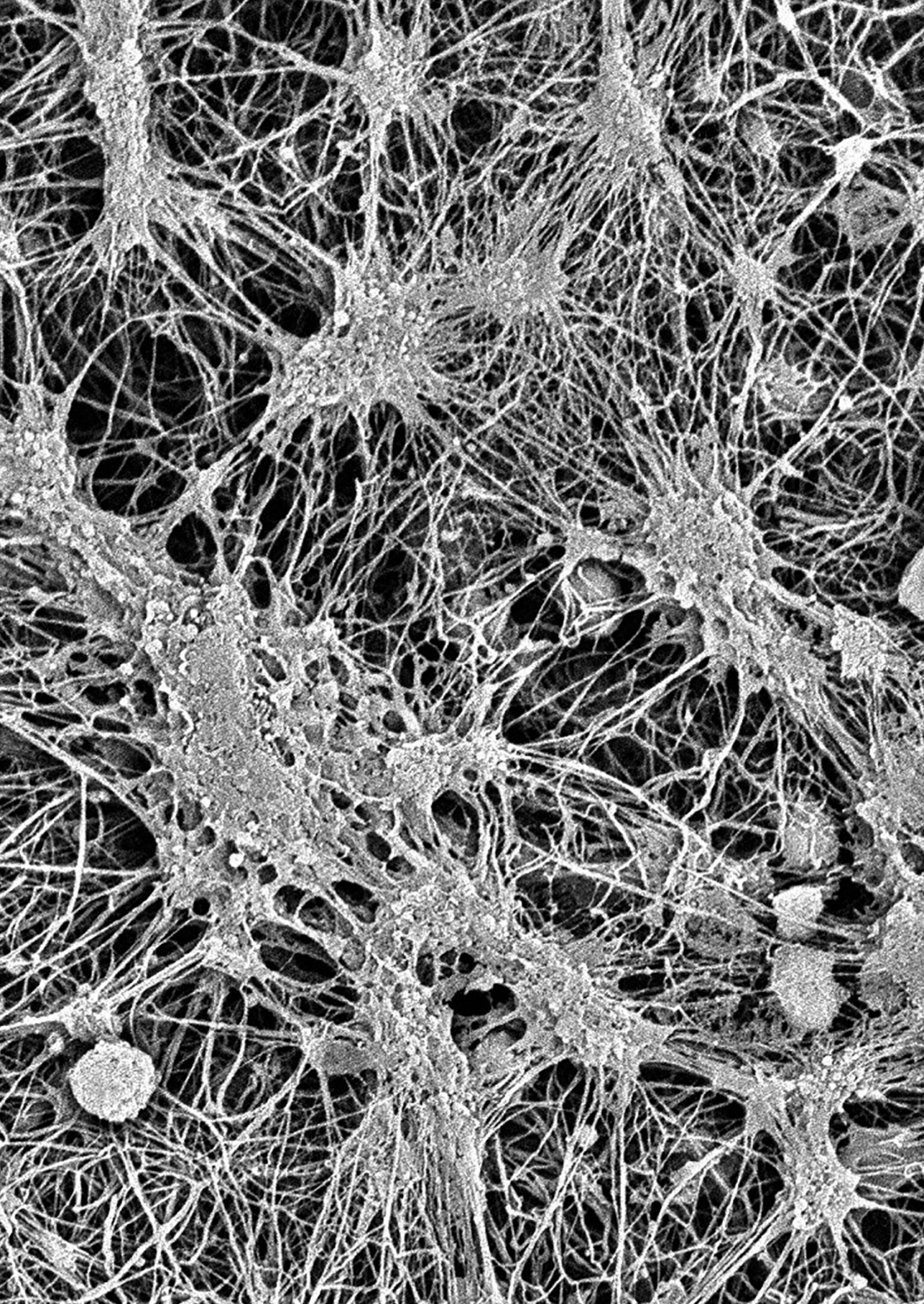

Chapter 7

Salivary tissue factor induces thrombin generation in a diurnal rhythm

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Adapted from:
Res Pract Thromb Haemost. 2018; 2:757-761

Abstract

Background:
Upon tooth extraction, extravascular tissue factor (TF) initiates coagu- lation to arrest bleeding. Additionally, saliva is in constant contact with the wound and contains extracellular vesicle-derived procoagulant TF. Since the duration of postextraction bleeding is highly variable between patients, we hypothesized this may be caused by variation in saliva-derived TF-induced clotting activity.

Objectives:
We aimed to assess the variability of saliva-induced thrombin generation (TG) in healthy individuals.

Methods:
TG was performed according to the calibrated automated thrombinography (CAT) method. Diluted saliva was added (instead of recombinant TF and phospholipids [PL]) to normal pooled plasma (NPP) in the absence/presence of anti-TF antibodies. Saliva was collected from healthy individuals in the morning, afternoon and evening.

Results:
Addition of saliva to NPP induced TG curves similar to those induced by r-TF and PL. Moreover, addition of anti-TF antibodies abolished saliva-induced TG, indicating TF-dependence. A large inter-individual variability (peak CV 31%, range 73- 220 nmol/L thrombin) in saliva-induced TG was observed. Interestingly, within subjects, saliva-induced TG was significantly ($P = 0.009$) increased in the morning (167 ± 40 nmol/L thrombin) compared to the afternoon (124 ± 39 nmol/L thrombin) and evening (123 ± 38 nmol/L thrombin). This diurnal variation was not attributable to gingival stimulation or damage induced by tooth brushing.

Conclusions:
We identified a diurnal rhythm in salivary TF activity that may have implications for tooth extraction and dental surgery, as performing invasive procedures in the morning may be beneficial for rapid coagulation. Future studies should correlate salivary TF to clinical outcome (ie, postextraction bleeding) and assess a possible relation with bacterial status in the oral cavity.

Keywords:
dentistry, diurnal rhythm, saliva, thrombin generation, tissue factor

Introduction

Postextraction bleeding is a frequently encountered (incidence up to 26%) complication in dental practice, defined as continuous bleeding for more than 8-12 hours after dental extraction.[1,2] Upon tooth extraction, extravascular tissue factor (TF) is released from damaged endothelium of the wound. TF is known as the key initiator of the coagulation cascade to arrest bleeding. Besides blood, another autologous body fluid, namely saliva, is in constant contact with the wound. Saliva was previously demonstrated to contain extracellular vesicle-derived TF[3,4] capable of triggering coagulation, as apparent from a shortened clotting time of autologous plasma and whole blood.[5] TF in saliva is bound to the membrane of exosomes released from epithelial cells.[5] Since the duration of post-extraction bleeding is highly variable between patients, we hypothesized that, besides the influence of blood-borne coagulation factor levels, comorbidities, and medication,[6] there may be variation in salivary TF-induced clotting activity. To obtain more mechanistic insight into the procoagulant effect of saliva, we measured thrombin generation (TG). TG is more informative than conventional clotting assays, which only reflect a small part of the coagulation process; more than 95% of all thrombin is formed after the initiation phase, when plasma has already clotted. In contrast, in TG measured by calibrated automated thrombinography (CAT) the entire process of the formation of the bulk of thrombin via feedback on factors V, VIII, and XI, as well as the effects of anticoagulant pathways and thrombin inhibition by protease inhibitors is visualized in the thrombogram.[7,8] Therefore, in the current study we used a modified TG assay to assess the variability of saliva-induced TG in a healthy population.

Materials and Methods

Saliva sample collection

Saliva was collected from 13 healthy individuals, who did not either eat, drink, or smoke for 30 minutes and thoroughly rinsed their mouths with water prior to collecting ~1 mL of saliva into a plastic sterile tube. Saliva samples were immediately placed on ice and stored at −20°C until use. For the intra-individual variation in saliva-induced TG, samples were collected at three time points, in the morning (7-9 am), afternoon (11 am-1 pm), and evening (5-7 pm).

Saliva-induced TG

TG was measured using the CAT method (using recombinant [r-]TF) as previously described by Hemker et al.[9] Directly prior to addition to the measurement wells, saliva was homogenised by vortexing at 850 rpm for 5 minutes and serially diluted 10-, 5-, 3.75-, or 2.5- fold (for dose response curve) or diluted 5-fold (for inter- and intra- individual variation experiments) with bovine serum albumin (BSA) buffer (5% BSA, 20 mmol/L Hepes, 140 mmol/L NaCl, 0.02% NaN3, pH 7.35). To study saliva-induced TG, 20 µL diluted saliva (known to contain phospholipids (PL)[10] was added to 80 µL normal pooled plasma (NPP), in the absence or

presence of 100 µg/mL anti-TF anti- bodies (rabbit polyclonal antibodies against TF were a generous gift of Dr. W. Kisiel, University of New Mexico, Albuquerque, NM, USA). Regular TG curves were obtained by addition of 20 µL CAT trigger solution containing 1 pmol/L r-TF (Innovin, Dade-Behring, Germany), of which concentration and activity were previously determined by comparison to recombinant TF with known activity (kindly provided by Dr. Y Nemerson [Mount Sinai Medical School, New York, NY, USA] obtained as described in[11] and 4 µmol/L PL.

Results and Discussion

Addition of saliva to NPP dose-dependently induced TG curves similar to those induced by the combination of r-TF and phospholipids (Figure 1A). Based on comparison of TG curves induced by saliva or r-TF, we estimated the concentration of TF in saliva to be in the order of 1-2 ng/mL, consistent with literature.[5,12]

To confirm that TF was indeed the procoagulant factor in saliva that triggered TG, we compared TG from the same saliva sample (from five donors) in the absence and presence of anti-TF antibodies (Figure 1B). Although thrombin was still generated in the presence of the antibodies, the lag time of this TG curve was substantially prolonged. The residual TG was comparable to that in the absence of tissue factor, and can hence be attributed to contact activation. Altogether we concluded that the saliva-induced TG is indeed TF-dependent. Moreover, the large variation between TG curves observed for the five donors in this experiment led us to further explore the inter- and intra-individual variation of saliva-induced TG curves.

In 13 healthy individuals, a large inter-individual variability in saliva-induced TG was observed, with coefficients of variation (CVs) of 31% for peak (range 68-242 nmol/L thrombin), 6% for ETP (range 1271-1678 nmol/L thrombin·min) and 24% for lag time (range 2.67-7.33 minutes). Saliva was collected from these individuals at three time points, in the morning (7-9 am), afternoon (11 am-1 pm), and evening (5-7 pm). Interestingly, we found that, within individuals, the saliva-induced TG occurred significantly faster (P = 0.03 for lag time) and was significantly increased (P = 0.009 for peak) in the morning (lag time 3.9 ± 1.0 minutes, peak 186 ± 40 nmol/L thrombin) com- pared to the afternoon (lag time 5.3 ± 1.9 minutes [not significantly increased], peak 124 ± 39 nmol/L thrombin) and evening (lag time 5.6 ± 1.2 minutes, peak 120 ± 43 nmol/L thrombin) (Figure 1C, D).

Our findings raised the question what could cause a higher salivary TF concentration in the morning compared to the rest of the day. One factor that we hypothesized could cause the diurnal rhythm in saliva-induced TG was toothbrushing, as this could stimulate and/or damage the gingiva to release TF.

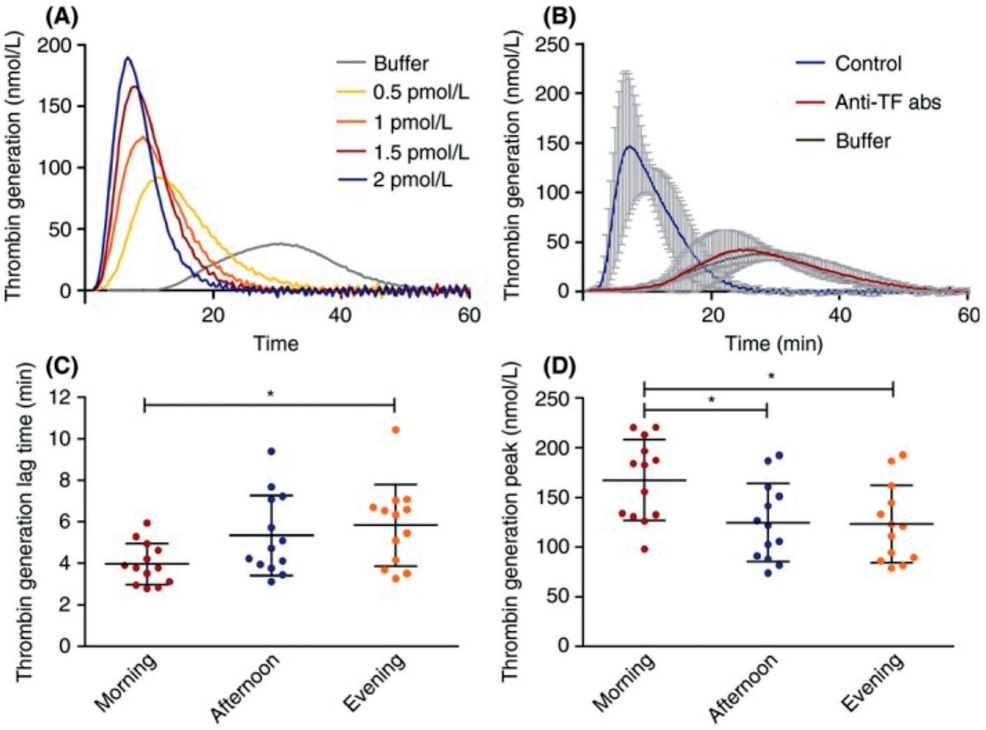

Figure 1. Saliva-induced TG in healthy individuals. (A) Saliva dose-dependently induces TG curves. Estimated concentrations of TF were based on comparison with curves generated from r-TF in conventional TG. Saliva dilutions were: 1/120, 1/60, 1/45, and 1/30 for the estimated 0.5, 1, 1.5, and 2 pmol/L TF indicated in the figure, respectively. Buffer condition does not contain saliva, TG for this control is induced by contact activation. (B) Addition of anti-TF antibodies (abs, 100 µg/mL) abolishes (TF-dependent) TG, demonstrating TF-dependency of saliva-induced TG. Buffer condition does not contain saliva, TG for this control is induced by contact activation. (C, D) TG induced with saliva samples collected from 13 healthy donors in the morning (7-9 am), afternoon (11 am-1 pm) and evening (5-7 pm) shows large interindividual variation and a diurnal rhythm. The TG parameters lag time (C) and peak (D) are presented as mean ± SD and were compared by one-way ANOVA with post hoc Bonferroni test if normally distributed, or by non-parametric Kruskal-Wallis with concurrent post hoc Dunn's test

To investigate this, 13 healthy individuals donated saliva before and after tooth brushing (without toothpaste and with concurrent rinsing with water), at the three time points mentioned earlier. Again, we observed a significant (P = 0.002) difference in TG between morning (lag time 4.0 ± 1.0 minutes, peak 168 ± 41 nmol/L thrombin) and evening (lag time 5.8 ± 1.9, peak 123 ± 39 nmol/L thrombin), but TG parameters did not differ before and after toothbrushing (Figure 2).

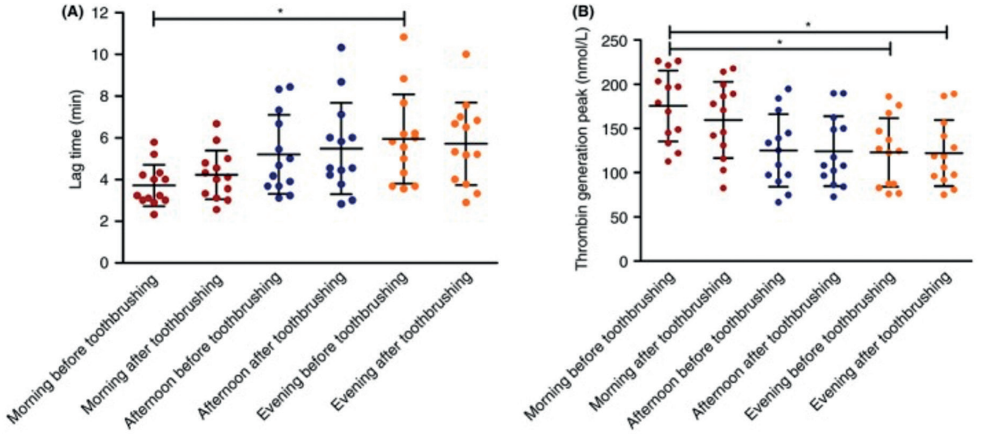

Figure 2. Toothbrushing does not influence TF-induced TG. TG induced with saliva samples collected from 13 healthy donors before and after toothbrushing at three time points (morning 7-9 am, afternoon 11 am-1 pm, and evening 5-7 pm) shows that (A) TG lagtime is shorter and (B) TG peak is higher in the morning compared to the evening but does not change as a result of gingival stimulation/possible damage induced by toothbrushing. Lag time (A) and peak (B) are presented as mean ± SD and were compared by one-way ANOVA with post hoc Bonferroni test if normally distributed, or by non-parametric Kruskal-Wallis with concurrent post hoc Dunn's test.

When humans or animals have a wound, they instinctively expose their blood to saliva (eg, by licking). Saliva contains growth factors and histatin, which promote wound healing[13,14] and provide antimicrobial activity.[15] In addition, saliva can serve as an additional source of extravascular TF to promote hemostasis. Besides reducing blood loss, rapid clot formation contributes to innate immunity and host defense, by decreasing the risk of pathogens entering the blood.[5]

A previous study by Hell et al.[16] reported on the procoagulant effect of extracellular vesicles in amniotic fluid, as measured by a modified TG assay. The current report is the first we are aware of that evaluated thrombin generation induced by saliva. A potential cause for the observed increased salivary TF activity early on the day may be that morning saliva is concentrated, as no food or beverages are consumed during the night, comparable to albumin levels in urine.[17] However, in the current study, subjects were asked to rinse their mouth before collecting saliva, also in the morning. Therefore, the contribution of this overnight concentration effect on morning salivary TF concentration is questionable.

A diurnal rhythm as observed for TF in saliva, but with a morning nadir and an increase towards the evening, is well known from several hormones (eg, cortisol),[18] peripheral blood cells (eg, lymphocytes and monocytes),[19] and inflammatory cytokines (eg, IL-6).[20] Moreover, various hemostatic factors are known to be subject to circadian variations. For instance, FVII and TF pathway inhibitor (TFPI) peak in the morning.[21] Hence, evidence suggests the existence of a relatively procoagulant status in the morning, supported by a preponderance of thromboembolic events early on the day.[22] Further studies are required to study possible

associations of salivary TF levels and activity with factors potentially causative of this within-day variation, such as melatonin, cortisol, and adrenaline. Another interesting question for further studies is whether the coagulation potential of saliva depends on the type of stimulus inducing its secretion, for instance chemosensory (ie, smell of food) and masticatory stimuli or anxiety (which induces hyposalivation).[23]

A final factor to consider as a cause for increased TF activity in the morning is the oral microbiome. Oral streptococci were previously demonstrated to induce (endothelial) tissue factor activity.[24] During the day, toothbrushing and food intake are known to alter the composition of the oral biofilm compared to the night, during which the low saliva flow creates a hospitable environment for, amongst others, streptococci.[25,26] In support of this hypothesis, a previous study reported that the use of prophylactic antibiotics prior to tooth extraction was independently associated with a higher risk of postoperative bleeding.[6] Therefore, it would be interesting to assess a possible relation between salivary TF concentration and bacterial status in the oral cavity.

From a clinical perspective, the observed diurnal rhythm in salivary TF activity may have implications for tooth extraction and other dental surgery, as performing invasive procedures in the morning may be beneficial for rapid coagulation. This may be particularly relevant for patients with a known bleeding tendency, for instance those on oral anticoagulants, to reduce the risk of prolonged post procedure bleeding. Future studies should assess whether bleeding after dental procedures varies depending on the time of day, and if so, correlate salivary TF activity to post procedure bleeding.

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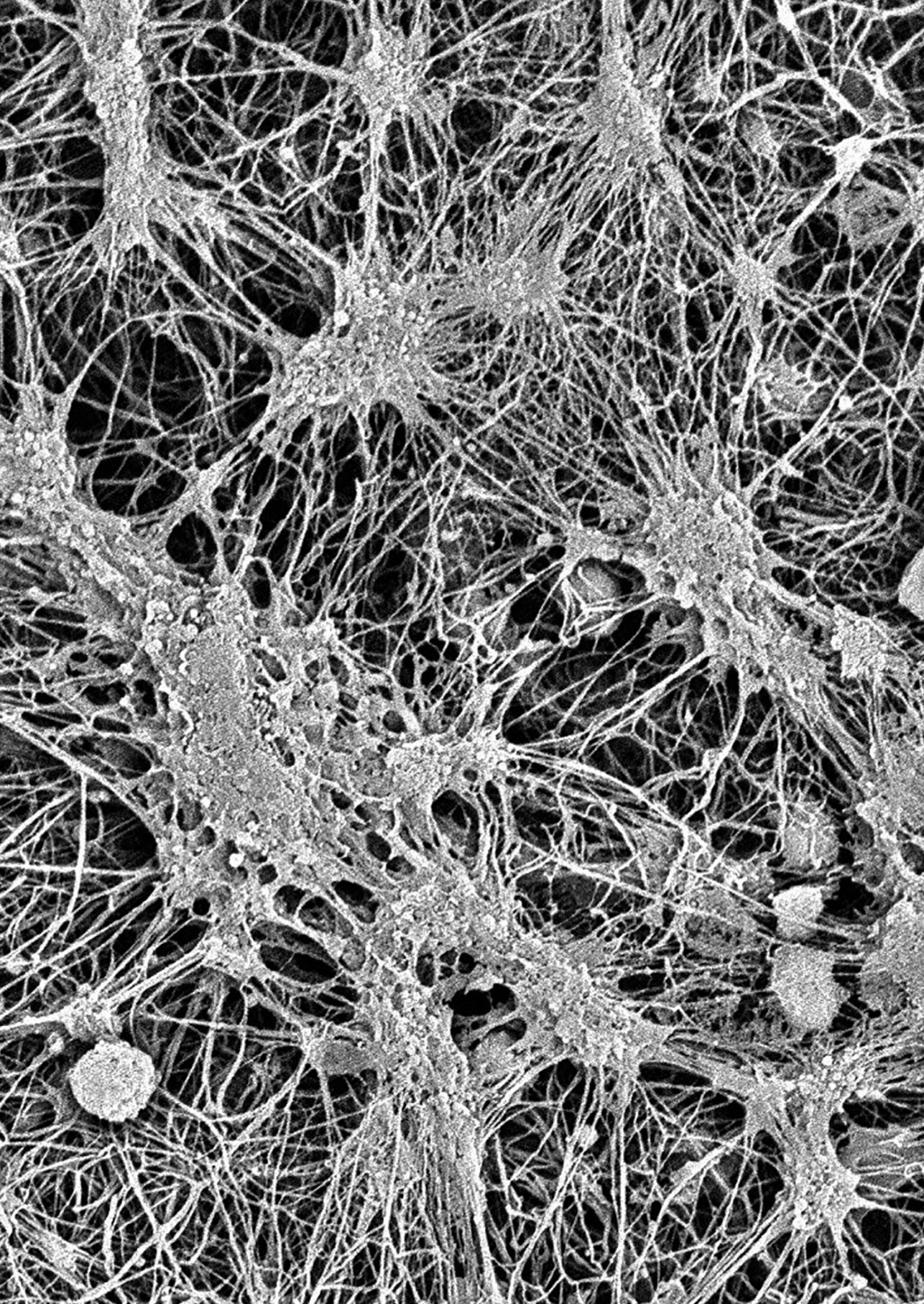

Chapter 8

Summary, discussion and future projects

Summary

Introduction

Tooth loss can occur for many different reasons such as periodontal diseases, failed endodontic treatment, vertical root fracture, infection, and trauma. Ultimately, we will end up with reduced bone height and width. This lack of hard tissue, and as a consequence also lack of soft tissue, is nowadays of major concern in the dental field.[1] Often patient's needs can be solved using oral implants. Prerequisites for long term success of oral implants are sufficient bone volume and soft tissue dimensions. To overcome the limitations of bone loss, guided bone regeneration, alveolar ridge preservation and sinus floor elevation were introduced. Although these procedures provide predictable outcomes, there is a demand to enhance bone regeneration and wound healing either after dental extraction or during implant placement. The local application of growth factors and scaffolds are supposed to enhance wound healing and bone regeneration.

Autologous preparation of growth factors containing cells from patient's whole blood needs no formal approval. Platelet-rich fibrin is prepared from the patient's own blood after a centrifugation protocol and claims to be suitable for oral and maxillofacial bone regeneration.[2] Over time there are different centrifugal protocols presented in the literature. Unfortunately, there is too much bias in the literature regarding the above-mentioned criteria. Researchers are comparing totally different protocols, with totally different devices and hardware, which leads to misunderstanding and misappreciation of the treatment method. This together with the different types of venal blood drawing tubes and different sorts of centrifuges led us to the believe that to obtain answers it is essential to perform with only one type of centrifuge, one centrifugal protocol and one type of collecting tubes.

Platelet-rich fibrin in combination with DBBM

In Chapter 2 we started a pilot case to explore the possibilities of the use of platelet-rich fibrin (PRF) in an extremely difficult esthetic case. As mentioned before, we only used throughout the complete study one protocol regarding G-force (200 G) and centrifugal time (8 minutes). To create the PRF membranes we used the PRF-Box and left the clots for exactly 60 seconds under the lid of the box.

In this case, where the buccal bone plate was totally lost due to a field hockey accident, and as a result of this an extreme soft tissue deficiency, we showed that PRF in combination with a bovine bone space maintainer is a promising treatment option for buccal bone augmentation as well as soft tissue restoration.

Bone augmentation was achieved with enough bone and bone density in order to reach sufficient primary implant stability. In a two-and-a-half-year follow-up we could demonstrate that the patient had a good functional and esthetic outcome.

Implant stability

Primary implant stability is one of the key factors for success and the survival of oral implants.[3-7] Especially in immediate loading cases primary stability is of utmost importance.[8-11] Several methods, invasive and non-invasive, have been described in the literature to measure implant stability.[5,12-15]

Resonance frequency analysis has been introduced in 1996 by Meredith and co-workers.[16] In 1999 the Osstell Company brought the idea of Meredith to the market and translated the kHz readings into a 1-100 scale which was named the ISQ (Implant Stability Quotient). This device made it possible to measure the primary implant stability in a non-invasive manor and monitor the development of osseointegration.

The first generation Osstell measuring tool was used with an L-shaped transducer which had a cable connection to the measuring device. The transducer had to be screwed on the implant and there was only a one direction measurement possible, mostly in buccal oral direction. This was a very time-consuming procedure with a lot of space for error, e.g. tongue position, contact from the transducer with a neighboring tooth, relatively big transducer, difficult to position etc.

After having been using this measuring device for a few years we decided to start an investigation on the reliability and validity of the instrumental assessment of implant stability as described in Chapter 3. The aim of the research was to determine the intra- and interobserver reliability and validity of the instrumental assessment of primary implant stability using resonance frequency analysis (RFA). We concluded in this research that primary stability of oral implants can be assessed reliable with RFA measurements.

During the years following the introduction of the first Osstell device the company introduced different devices with different specifications. The successor of the first device was the Osstell Mentor, a cordless device that used a non-cable fixed transducer, called the SmartPeg.

This device was the first that was able to measure in two different directions. After the introduction of the Osstell Mentor the company introduced the Osstell ISQ. In 2015 Integration Diagnostics Sweden launched the Penguin^{RFA}, an uncomplicated and affordable RFA measuring tool, which is also cordless and uses Multipegs which can be sterilized and reused.

Since we had both devices, Osstell ISQ and the Penguin[rfa], in our clinic we decided to investigate the differences of both devices in a retrospective cohort pilot study (Chapter 4). The purpose was to compare the implant stability quotient (ISQ) by resonance frequency analysis (RFA), recorded with two different devices after implant placement. We concluded that from a clinical point of view, both devices reveal functional differences in terms of handling and sustainability. SmartPegs of the Osstell device are merely for single use, while MultiPegs of the Penguin device are reusable items, due to their sterilizability. Due to the cable connection of the Osstell, handling with cordless Penguin was stated as much easier, based on the experience we have made during the experimental part of our present investigation. Finally, there is an obvious difference in price between both measuring instruments, with Penguin and reusable Multipegs being advantageous from an economical point of view. Reusabiliy of Multipegs may offer an additional benefit with regard on ecological aspects.

Implant stability is associated with peripheral blood cells and coagulation factors

PRF consists of a slowly polymerized fibrin network with enmeshed cytokines, structural glycoproteins, platelets and leucocytes.[17,18] The fibrin network has a key role in early wound healing and functions as a scaffold for ingrowing cells and as a reservoir of cytokines.[19]

Essential for successful bone and soft tissue regeneration is good vascularization to supply nutrients and oxygen into the wound. There is a specific role for the fibrin matrix in angiogenesis. Van Hinsbergh and co-workers[20] concluded from their research that a better insight into the role of fibrin in angiogenesis could contribute to a better management of the angiogenic process. Fibrinous exudate provides additional scaffolding for the invasion process and as such accelerates neovascularization.

In our investigations we hypothesized that peripheral blood cell composition and coagulation factor levels could influence PRF membrane characteristics (Chapter 5). We found a significant inverse correlation between red blood cell count and PRF membrane length. PRF membranes prepared from patients' blood with a higher erythrocyte count in peripheral blood (and higher hematocrit) resulted in shorter PRF membrane length than patients with a lower erythrocyte count. Conversely there were no significant associations between platelets and PRF size parameters. FVIII and active VWF levels correlated significantly with PRF membrane area, whereas fibrinogen levels did not correlate with PRF membrane area or length.

Implant stability, following alveolar ridge preservation with PRF and bovine bone substitute, is associated with several hematological parameters. Our results suggest that fibrin structure levels of (active) VWF and FXIII, more than platelet and leucocyte count, may be determining factors for PRF membrane characteristics and implant stability. However, we do not know

whether the concentration of these factors in the PRF membrane or the concentration in the blood of the patient is the determining factor.

Histological evidence

We assessed histologically the effect of pre-implantation extraction sockets grafting materials, PRF and bovine bone substitute, on bone regeneration in 19 cases. The extraction sockets contained deproteinized bovine bone mineral and autologous PRF membrane supplemented with autologous plasma growth factor concentrate. The socket was closed using a PRF membrane, on top of the graft, in an envelope technique. At 14 ± 2.5 weeks post extraction, biopsies were harvested from the surgical site. These biopsies were imbedded in paraffin, followed by de-calcification and haematoxylin-eosin staining. We found osteoid matrix deposited at the borders of the remnants of PRF and bovine bone, and dense cellular osteoblasts lined in fronts residing in connective tissue. (Chapter 6) These findings suggest that PRF and bovine bone have osteoconductive effects as early as 14 weeks post-PRF treatment. However, larger studies are necessary to show the exact effect of RPF treatment on bone formation.

Salivary tissue factor

Post-extraction bleeding has an incidence of up to 26.0 % in dental practice. The definition of post-extraction bleeding is continuous bleeding for more than 8-12 hours after dental extraction.[21,22]

Coagulation is initiated by extravascular tissue factor (TF). Additionally, saliva is in constant contact with the wound and contains extracellular vesicle-derived procoagulant TF. Since the duration of post extraction bleeding is highly variable between patients, we hypothesized that this may be caused by variation in saliva derived TF-induced clotting activity. The aim was to access the variability of saliva-induced thrombin generation (TG) in healthy individuals. Saliva was collected from healthy individuals in the morning, afternoon and evening. We observed a large inter-individual variability in saliva-induced TG(Chapter 7). Interestingly, within subjects, saliva-induced TG was significantly increased in the morning compared to the afternoon and evening. We identified a diurnal rhythm in salivary TF activity that may have implications for tooth extraction and dental surgery, as performing invasive procedures in the morning may be beneficial for rapid coagulation.

Future prospects

During the research of the present thesis, we investigated several centrifugal conditions and concluded that the use of the process for PRF preparation includes a centrifuge step with 200 G (1300 rpm) and a centrifugation time of 8 minutes was sufficient reliable for use in our research. By means of SEM (Scanning Electron Microscopy) we investigated the composition of the PRF membrane after different centrifugal protocols.

Recently a research from Richard Miron[23] described 24 different centrifugal protocols, which reveals the differences between the different G- forces and centrifugal time and ends up with a recommendation of a protocol to be used.

Unfortunately, in this research they did not use the glass tubes from the Process for PRF Company, but instead they used plastic collection tubes (Chixin Biotech, Wuhan, China).

There is no description of the coating of the different tubes used in this research.

We presume that the plastic tubes are covered with silica in order to enable the clotting of the blood samples. As we know from the literature, silica coated plastic tubes can lead to problems in the PRF clot, such as contamination of silica microparticles in A-PRF matrices,[24] Hideo Masuki and co-workers stated in their article "Acute cytotoxic effects of silica microparticles used for coating of plastic blood-collection tubes on human periosteal cells".[25] In this study, they obtained clear evidence that silica micro-particles derived from commercially available blood collection tubes exert toxic effects on human periosteal cells by absorbing on the plasma membrane and inducing apoptosis.

The influence of a different centrifugal protocol with the use of a horizontal centrifuge, upfront blood testing for patients, different collecting tubes for patients own blood collection (standardized) and G-force adaption in combination with the outcome of the blood composition per patient could lead to a new standardized, reproducible and predictable protocol. PRF in combination with sophisticated methods like (3D)-printed scaffolds could lead to more predictable bone- and soft-tissue regeneration. An upfront point of care test of the patients' whole blood composition could maybe be of help.

The studies described in the present thesis contribute to a further knowledge of the mechanisms involved in the interaction of platelet-rich fibrin in oral implant dentistry. The role of von Willebrand factor and factor XIII should be investigated extensively hereafter. It is to be expected that factor XIII stabilizes the fibrin clot, the scaffold for wound healing, and it might link growth factors to the fibrin clot. The role of von Willebrand in bone generation is less clear, the best explanation could be that von Willebrand localizes platelets around the wound and that the platelets release their content of growth factors. However, platelet number does not correlate with stability. It is possible that the platelet number is not a limiting factor, we have more than enough platelets in our circulation for optimal haemostasis. Nevertheless, we have found any role for platelets in the stimulation of bone regeneration.

One of the problems with these studies is that we cannot differentiate between the composition of the components in the PRF and in the circulation of the patient. It is unlikely

that the influence of hematocrit on stability is due to the composition of the PRF because red cells were excluded for the PRF. So, the number of red cells in the circulation seems to be an important factor in the stability of the implant. Whether von Willebrand factor in the circulation or von Willebrand factor in PRF is important for the stability, we do not know. For factor XIII it seems logic to suppose that factor XIII inside RPF is the important factor because factor XIII is only active after thrombin is formed. Further studies should differentiate between the role of the composition of the circulation blood and the composition of PRF. This will not be an easy task.

The next few years the focus should be on the importance of tissue factor in the process of tissue regeneration. Tissue factor activity results in thrombin generation and thrombin is a growth factor for many different cell types. Thrombin formation is essential for stopping the bleeding after extraction but it could also influence wound healing and probably bone generation.[26] A correlation between tissue factor activity in saliva and implant stability could answer this question. Moreover, the presence of different bacterial strains, such as P. gingivalis, Acinobaccillus actinomycetemcomitans and treponema denticola, can influence tissue factor levels in saliva and thus the bone regeneration process.[27] Bacteria can induce infection and infections will result in bleeding. More bleeding will result in more thrombin formed; however, infections will also result in degradation of bone. An accurate measurement of the different bacterial strains will help to answer this question. The composition of the gingival crevicular fluid may play an important role in understanding tissue factor levels and the influence of the different bacteria strains in the saliva on the tissue factor levels. Future research should help to elucidate success and failure of the different approaches and find an objective approach for further development of the platelet-rich fibrin technique.

Nederlandse samenvatting

Introductie

Het verlies van tanden en kiezen kan verschillende oorzaken hebben, zoals parodontale aandoeningen, falende wortelkanaalbehandelingen, verticale wortelbreuk, infectie of trauma. Dit leidt uiteindelijk tot een verminderde bothoogte en botbreedte van de kaak. Deze vermindering van het harde weefsel en als gevolg daarvan ook van het zachte weefsel, is tegenwoordig een belangrijk aandachtspunt in de esthetische tandheelkunde[1]. Dikwijls kunnen verloren gegane tanden of kiezen van patiënten vervangen worden door orale implantaten. Voorwaarden voor succes op lange termijn van orale implantaten zijn echter voldoende botvolume en de voldoende hoeveelheid zacht weefsel. Om de beperkingen van botverlies te ondervangen werden behandelmethodes zoals geleide botregeneratie, alveolaire kampreservatie en sinusbodem elevatie geïntroduceerd. Hoewel deze procedures voorspelbare resultaten opleveren, blijft de vraag bestaan naar betere en snellere botregeneratie procedures en wondgenezing, zowel na het verwijderen van een tand of kies als tijdens het plaatsen van implantaten. De lokale toepassing van groeifactoren en dragermaterialen(scaffolds) lijkt ervoor te zorgen dat de wondgenezing en botregeneratie verbeteren.

Wondgenezing is een dynamisch fysiologisch proces dat de normale architectuur en functionaliteit van het beschadigde weefsel hersteld. De wondgenezing bestaat uit drie opeenvolgende fasen van (1) Bloedstelping (haemostase) en daaropvolgend acute ontsteking (minuten, uren, dagen na de verwonding); (2) proliferatie en nieuwe weefselvorming (dagen, weken); en (3) remodeling (weken, maanden, jaren)[2,3].

Bloedplaatjes spelen een cruciale rol in de primaire haemostase en steeds meer klinische en experimentele gegevens wijzen erop dat deze geënucleërde cellen ook relevante modulatoren zijn van andere pathofysiologische processen, waaronder ontsteking en weefselgeneratie. De groeifactoren die aanwezig zijn in de alfa granula van de bloedplaatjes en die vrijkomen bij de activering, zijn mediatoren die de weefselgeneratie kunnen bevorderen[4].

Door bloedplaatjes te gebruiken van de patiënt zelf is er geen gevaar voor immunologische reacties of ziekteoverdracht. Met bloedplaatjes verrijkte materialen zijn in het laatste decennium belangrijk geworden en vormen een groeiend onderwerp van klinisch en experimenteel onderzoek in relatie tot wondgenezing en botregeneratie. Bloedplaatjes (trombocyten) concentraten worden daarom steeds vaker gebruikt om de wondgenezing en botregeneratie te stimuleren. Plaatjes Rijk Fibrine (PRF) bestaat uit een langzaam gepolymeriseerd fibrinenetwerk met daarin ingesloten cytokinen, structurele glycoproteïnen, bloedplaatjes en leukocyten[5,6]. Het fibrinenetwerk speelt een sleutelrol in de vroege wondgenezing en fungeert als een drager voor ingroeiende cellen en als een reservoir van cytokinen[7].

Autologe bereiding van groeifactoren met cellen uit volbloed van de patiënt behoeft geen formele goedkeuring[8]. Bloedplaatjes-rijk fibrine wordt bereid uit het eigen bloed van de patiënt na een centrifuge protocol en lijkt geschikt te zijn voor orale en maxillo-faciale botregeneratie[9]. In de loop van de tijd zijn er verschillende centrifuge protocollen gepresenteerd in de literatuur. Helaas is er te veel bias in de literatuur met betrekking tot de bovengenoemde criteria[10]. Onderzoekers vergelijken totaal verschillende protocollen, met totaal verschillende hulpmiddelen en hardware, hetgeen leidt tot misverstanden en een verkeerde waardering van de behandelmethode met wisselende uitkomsten. Dit, samen met de verschillende soorten veneuze bloedafnamebuizen en verschillende soorten centrifuges, heeft ons tot de overtuiging gebracht dat het voor het verkrijgen van antwoorden van essentieel belang is gestandaardiseerde studies uit te voeren met slechts één soort centrifuge, één centrifuge protocol en één soort afnamebuizen.

Bloedplaatjesrijk fibrine in combinatie met DBBM

In hoofdstuk 2 beschrijven wij een case-report om de mogelijkheden van het gebruik van bloedplaatjes-rijk fibrine (PRF) in een uitzonderlijk moeilijke esthetische casus te onderzoeken. In deze casus was de buccale botplaat volledig verloren gegaan als gevolg van een veldhockeyongeval. Dit resulteerde in een extreem tekort aan weke delen. In dit case-report tonen wij aan dat PRF in combinatie met een boviene bot substituut (DBBM, Deproteinized Bovine Bone Mineral) een veelbelovende behandeloptie is voor zowel buccale botaugmentatie als herstel van de weke delen. Gedurende de behandeling werd één protocol gebruikt met betrekking tot G-kracht (200 G) en centrifuge tijd (8 minuten). Voor het maken van de PRF-membranen werd gebruik gemaakt van de PRF-Box (Process for PRF, Nice) waarin de fibrine stolsel exact 60 seconden uitgeperst werd onder het deksel van de box.

De botaugmentatie resulteerde in voldoende botvolume en botdichtheid om uitstekende primaire implantaatstabiliteit te bereiken. In een follow-up van tweeëneenhalf jaar konden we aantonen dat de patiënt een goed functioneel en esthetisch stabiel resultaat heeft wat betreft botvolume en weke delen hoeveelheid en contour.

Implantaatstabiliteit

Primaire implantaatstabiliteit is een van de belangrijkste factoren voor het succes en de overleving van orale implantaten[11-15]. Vooral in gevallen van directe belasting is primaire stabiliteit van het grootste belang[5-18]. In de literatuur zijn verschillende methoden, invasief en niet-invasief, beschreven om de stabiliteit van implantaten te meten[13,20-23].

Resonantiefrequentieanalyse is in 1996 geïntroduceerd door Meredith en medewerkers[24]. In 1999 bracht de firma Osstell het idee van Meredith op de markt en vertaalde de kHz-metingen in een schaal van 1-100 die de naam ISQ (Implant Stability Quotient) kreeg. Dit

apparaat maakte het mogelijk om de primaire implantaatstabiliteit op een niet-invasieve manier te meten en de ontwikkeling van osseointegratie te volgen.

De eerste generatie Osstell meetinstrumenten werd gebruikt met een L-vormige transducer die via een kabel verbonden was met het meetapparaat. De transducer moest op het implantaat worden geschroefd en er was slechts een meting in één richting mogelijk, meestal in bucco-orale richting. Dit was een zeer tijdrovende procedure met veel ruimte voor fouten, b.v. tongpositie, contact van de transducer met een naburig gebitselement, relatief grote transducer, moeilijk te plaatsen etc.

Na de eerste generatie ISQ-meter een paar jaar gebruikt te hebben besloten we een onderzoek te starten naar de betrouwbaarheid en validiteit van de instrumentele beoordeling van de implantaatstabiliteit zoals beschreven in Hoofdstuk 3. Het doel van het onderzoek was het bepalen van de intra- en inter-observer betrouwbaarheid en validiteit van de instrumentele beoordeling van de primaire implantaatstabiliteit met behulp van Resonantie Frequentie Analyse (RFA). Wij concludeerden in dit onderzoek dat de primaire stabiliteit van orale implantaten betrouwbaar kan worden beoordeeld met RFA-metingen.

In de jaren na de introductie van de eerste generatie Osstell meter introduceerde het bedrijf verschillende apparaten met verschillende specificaties. De opvolger van het eerste apparaat was de Osstell Mentor, een snoerloos apparaat dat gebruik maakte van een wegwerp transducer zonder snoerverbinding, de SmartPeg genaamd.

Dit apparaat was het eerste dat in staat was om in twee verschillende richtingen te meten (bucco-oraal en mesio-distaal). Na de introductie van de Osstell Mentor introduceerde het bedrijf de Osstell ISQ.

In 2015 lanceerde Integration Diagnostics Sweden de Penguin^{RFA}, een ongecompliceerd en betaalbaar RFA-meetinstrument, dat ook snoerloos is en gebruik maakt van Multipegs die kunnen worden gesteriliseerd en hergebruikt.

Om het proces te standaardiseren hebben wij gekeken naar de prestaties van twee verschillende ISQ meters. Wij hadden de Osstell ISQ en de Penguin^{RFA} tot onze beschikking en besloten de verschillen van beide apparaten te onderzoeken in een retrospectieve cohort pilotstudie met PRF als behandelmethode (Hoofdstuk 4). Het doel was om de verschillende implantaat stabiliteitsquotiënten (ISQ) met elkaar te vergelijken door middel van resonantiefrequentie analyse (RFA), geregistreerd met twee verschillende apparaten na implantatie. We concludeerden dat, vanuit klinisch oogpunt, beide apparaten functionele verschillen vertonen op het vlak van hanteerbaarheid en duurzaamheid. SmartPegs van het Osstell apparaat zijn slechts voor eenmalig gebruik, terwijl MultiPegs van het Penguin apparaat herbruikbare items zijn, vanwege hun steriliseerbaarheid. Door de kabelverbinding

van de Osstell werd de bediening van de draadloze Penguin als gemakkelijk ervaren, gebaseerd op de ervaring die wij hebben opgedaan tijdens het experimentele deel van ons onderzoek. Tenslotte is er een duidelijk prijsverschil tussen beide meetinstrumenten, waarbij de Penguin en de herbruikbare Multipegs uit economisch oogpunt voordelig zijn. De herbruikbaarheid van Multipegs kan een bijkomend voordeel bieden met betrekking tot ecologische aspecten.

De stabiliteit van het implantaat is geassocieerd met perifere bloedcellen en stollingsfactoren

In Hoofdstuk 5 stelden wij de hypothese dat de samenstelling van perifere bloedcellen en het niveau van stollingsfactoren de eigenschappen van het PRF-membraan zouden kunnen beïnvloeden. Vijftig patiënten werden geïncludeerd in het onderzoek in de periode van 2015-2017. PRF-membranen werden van het autologe bloed bereid en tevens werd het aantal bloedcellen en stollingsfactoren gemeten. De restalveole werd na extractie opgevuld met DBBM en PRF en afgesloten met een PRF-membraan. Wij vonden een significante inverse correlatie tussen het aantal rode bloedcellen en de lengte van de PRF-membranen. PRF-membranen bereid uit bloed van patiënten met een hoger erytrocyten aantal in het perifere bloed (en een hogere hematocriet) resulteerden in kortere PRF-membraanlengtes dan patiënten met een lagere erytrocyten aantal. Omgekeerd waren er geen significante associaties tussen bloedplaatjes en PRF-membraan grootte parameters. FVIII- en actieve VWF-spiegels correleerden significant met PRF-membraanoppervlak, terwijl fibrinogeenniveaus niet correleerden met PRF-membraanoppervlak of -lengte.

Implantaatstabiliteit, na alveolaire botpreservatie met PRF en boviene bot substituut, is geassocieerd met verschillende haematologische parameters. Onze resultaten suggereren dat de fibrinestructuur in combinatie met (actief) VWF en FXIII, meer dan het aantal bloedplaatjes en leukocyten, bepalende factoren kunnen zijn voor PRF-membraankarakteristieken en implantaatstabiliteit.

Histologisch bewijs

Gezien de veelbelovende effecten van de behandeling van PRF bij socket preservatie, werd in hoofdstuk 6 onderzocht of er hiervoor histologische aanwijzingen zijn.

Wij includeerden 19 patiënten behandeld met PRF en DBBM in deze studie. De extractie sockets bestonden uit gedeproteïniseerd boviene botmineraal en een in kleine deeltjes geknipte autologe PRF-membraan aangevuld met autoloog plasma-groeifactor-concentraat. De alveole werd gesloten met een PRF-membraan, bovenop het bot transplantaat en in een enveloptechniek in-gehecht. Op 14 ± 2,5 week na extractie werden biopten genomen van de geheelde alveole. De biopten werden afgenomen met een 2mm trepaanboor, kleiner dan diameter van het uiteindelijke geprepareerde implantaatbed. Deze biopten werden ingebed in paraffine, gevolgd door ontkalking en hematoxyline-eosine kleuring.

We vonden een osteoblasten hechting op de grenzen van de in kleine deeltjes geknipte PRF-membraan en het boviene bot en een dichte cellulaire osteoblasten activiteit in het bindweefsel (Hoofdstuk 6). Deze bevindingen suggereren dat PRF en boviene botsubstituut al vanaf 14 weken na de PRF-behandeling osteoconductieve effecten hebben. Grotere studies zijn echter nodig om het exacte effect van PRF-behandeling op botvorming te onderzoeken.

Stollingsactivator in speeksel

Een bloeding na een extractie is een vaak voorkomende complicatie (incidentie tot 26%) in de tandartspraktijk[25]. De definitie van een post-extractie bloeding is een continue bloeding gedurende meer dan 8-12 uur na een tandheelkundige extractie[26,27].

Bloedstolling wordt geïnitieerd door extravasculaire weefselfactor (Tissue Factor). Speeksel bevat eveneens extracellulaire vesicle-afgeleide procoagulant TF. Aangezien speeksel voortdurend in contact met de wond komt en de duur van de bloeding na extractie sterk varieert tussen patiënten, veronderstelden wij dat dit veroorzaakt zou kunnen worden door variatie in TF-geïnduceerde stollingsactiviteit in speeksel. Het doel was om de variabiliteit van speeksel-geïnduceerde trombine generatie (TG) bij gezonde personen te onderzoeken. Speeksel werd verzameld bij gezonde personen in de ochtend, middag en avond. We constateerden een grote inter-individuele variabiliteit in speeksel-geïnduceerde TG (Hoofdstuk 7). Interessant is dat binnen de proefpersonen het speeksel-geïnduceerde TG significant verhoogd was in de ochtend vergeleken met de middag en de avond. Dit dagritme in speeksel TF-activiteit kan implicaties hebben voor tandheelkundige extracties en tandheelkundige chirurgie, omdat het uitvoeren van invasieve procedures in de ochtend gunstiger kan zijn voor een snelle coagulatie.

Toekomstperspectieven

De studies beschreven in dit proefschrift dragen bij tot een verdere kennis van de mechanismen betrokken bij de interactie van bloedplaatjes-rijk fibrine in de orale implantologie. Verder onderzoek naar de rol van von Willebrand factor en factor XIII is van groot belang. Het is te verwachten dat factor XIII het fibrinestolsel, het dragermechanisme voor de wondgenezing, stabiliseert en het zou groeifactoren kunnen koppelen aan het fibrinestolsel. De rol van von Willebrand bij de aanmaak van bot is minder duidelijk; de beste verklaring zou kunnen zijn dat von Willebrand bloedplaatjes rond de wond lokaliseert en dat de bloedplaatjes hun inhoud aan groeifactoren vrijgeven.

Onlangs heeft een onderzoek van Richard Miron[28] 24 verschillende centrifuge protocollen beschreven, waarbij de verschillen tussen de verschillende G-krachten en centrifugale tijd aan het licht komen en eindigt met een aanbeveling om één duidelijk protocol te gebruiken. Helaas hebben zij in dit onderzoek niet de glazen venapunctie buizen van de Process for PRF Company gebruikt, maar in plaats daarvan plastic venapunctie buizen (Chixin Biotech, Wuhan, China). De

coating van de verschillende buizen die in dit onderzoek zijn gebruikt, is niet beschreven in dit onderzoek. Wij veronderstellen dat de plastic buizen met een silica coating behandeld zijn om de stolling van de bloedmonsters mogelijk te maken. Zoals wij uit de literatuur weten, kunnen met silica gecoate plastic buizen leiden tot problemen bij het stollen van de PRF. Tsujino en medewerkers[29] constateerden dat er verontreiniging op kan treden van silica microdeeltjes in PRF-matrices. Hideo Masuki en medewerkers beschreven in hun artikel "Acute cytotoxic effects of silica microparticles used for coating of plastic blood-collection tubes on human periosteal cells" [30] het toxische effect van losgeraakte silica deeltjes. In deze studie verkregen zij duidelijke bewijzen dat siliciumdioxide microdeeltjes afkomstig van in de handel verkrijgbare bloedafnamebuizen toxische effecten hebben op menselijke periostale cellen door absorptie op het plasmamembraan en het induceren van apoptose.

De invloed van een ander centrifugeprotocol met gebruikmaking van een horizontale centrifuge, het vooraf testen van het bloed van patiënten, verschillende opvangbuizen voor de bloedafname van patiënten (gestandaardiseerd) en aanpassing van de G-kracht in combinatie met de uitkomst van de bloedsamenstelling per patiënt zou kunnen leiden tot een nieuw gestandaardiseerd, reproduceerbaar en voorspelbaar protocol.

PRF in combinatie met geavanceerde methoden zoals (3D) -geprinte scaffolds zou kunnen leiden tot meer voorspelbare bot- en zacht-weefselregeneratie. Een voorafgaande point of care test van de (optimale) samenstelling van het bloed van de patiënt zou wellicht van nut kunnen zijn.

De komende jaren moet de aandacht worden gericht op het belang van weefselfactor (TF) in het proces van weefselregeneratie. Weefselfactoractiviteit resulteert in trombine vorming en trombine is een groeifactor voor veel verschillende celtypes. Trombine vorming is essentieel om het bloeden na een extractie te stoppen, maar het zou ook de wondgenezing en waarschijnlijk ook de botaanmaak kunnen beïnvloeden[31]. Een correlatie tussen weefselfactoractiviteit in speeksel en implantaatstabiliteit zou deze vraag kunnen beantwoorden. Bovendien kan de aanwezigheid van verschillende bacteriestammen, zoals P. Gingivalis, Acinobaccillus Actinomycetemcomitans en Treponema Denticola, van invloed zijn, niet alleen op ontstekingsschade maar ook op het gehalte aan weefselfactor in speeksel en daarmee op het botregeneratieproces[32]. Bacteriën kunnen een infectie induceren en infecties zullen leiden tot bloedingen. Meer bloeding zal resulteren in meer trombine vorming; infecties zullen echter ook resulteren in afbraak van bot. Een nauwkeurige meting van de verschillende bacteriestammen zal helpen om deze vraag te beantwoorden. De samenstelling van de creviculaire vloeistof kan een belangrijke rol spelen bij het begrijpen van de weefselfactor niveaus en de invloed van de verschillende bacteriestammen in het speeksel op de weefselfactor (TF) niveaus. Toekomstig onderzoek moet helpen om succes en falen van de verschillende benaderingen op te helderen en een objectieve benadering te vinden voor de verdere ontwikkeling van de bloedplaatjes-rijke fibrine techniek.

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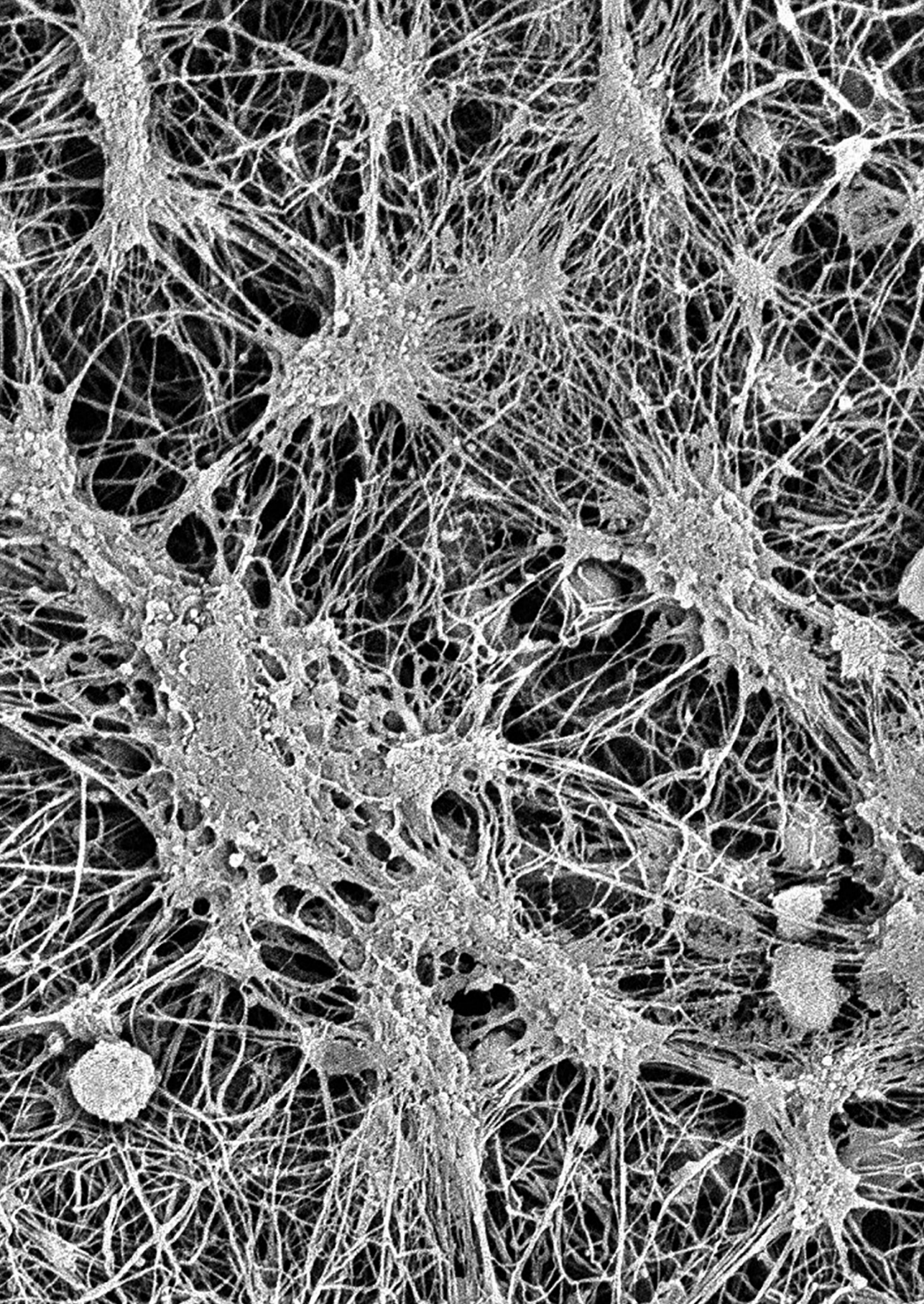

Appendices

Appendix I Impact Statement

With our population getting older and older, loss of teeth will be a growing problem. An incidental circumstance is a simultaneous loss of bone. One of the challenges of artificial teeth implantation is to develop methods to stop this loss of bone and to stimulate bone regeneration, to allow stable implants. The experiments described in this thesis are the first steps to understand the process of bone regeneration with the help of blood clots and to start to develop uniform guidelines for treatment.

Loss of soft- and hard tissue is a major problem in oral implant therapies. Although autologous bone and soft tissue grafts still are "gold" standards[1,2] in oral implant surgery, there is growing interest in alternatives to these invasive surgical procedures. The need of a second surgical site in soft- and hard tissue augmentation procedures with autologous materials and the limited amount of harvestable material, has significant disadvantages.[3] Platelet-rich fibrin can contribute to less pain after surgery, less swelling i.e., a less traumatic and faster treatment process4. Application of platelet-rich fibrin clots in combination with bone substitutes in oral implantology is a potential alternative treatment. Platelet-rich fibrin, prepared from autologous patient blood, is a platelet concentrate which could have significant potential if used with a proper standardized protocol. There are currently several treatment protocols in the implantology market with untenable promises creating confusion by a continuous adaptation of protocols, which may influence clinical outcomes. There is a need for standardized procedures, in order to be able to establish a solid scientific base obtaining optimal clinical outcomes. Therefore, we used in the present research standardized protocols to study the effects of platelet-rich fibrin in oral implantology.

Key contributions of this thesis

In 2015 we introduced the application of platelet-rich fibrin in our clinic and since then we use it on a daily basis. Using platelet-rich fibrin in our clinic for different indications, revealed the potential in clinical outcome of this autologous platelet product. These findings were the basis to the underlying research project.

First, we started to developed an optimal protocol for the use of platelet-rich fibrin in challenging cases by testing different centrifugal protocols in vitro[5]. During our research we used the same standardized platelet-rich fibrin generation protocol.

We aimed to show the importance of the use of platelet-rich fibrin in combination with DBBM (demineralized bovine bone matrix) in esthetically demanding oral implant cases, which we demonstrated in the case report study[6].

Oral implant stability is a promisable tool to measure the clinical outcome of platelet-rich fibrin treatment in patients. We validated two different RFA (Resonance Frequency

Analysis) measurement devices[7]. Our study adds insights in current measurement methods, comprising the comparison of the accuracy of measurement between the two currently available devices for implant stability measurement. The ISQ (Implant Stability Quotient) value is an important parameter for success of oral implant therapy[8,9]. We tested both devices after conventional implant placement of self-tapping dental implants in post-extraction sites with ridge preservation. The ridge preservation was conducted following a standardized platelet-rich fibrin preparation protocol in combination with DBBM. Our findings support the application of both measurement devices as reliable and useful tools to determine implant stability.

There is a variability in clinical outcomes (e.g., bone formation, gingiva generation, implant stability) in patients treated with platelet-rich fibrin. Not only through different centrifugal protocols, also due to the use of different materials used to collect patients' blood. One of the major variables may be the composition of the patients own whole blood[10,11]. Therefore we studied the association between dental implant stability and peripheral blood cell composition and levels of coagulation factors in patients treated with alveolar ridge preservation with platelet-rich fibrin and bovine bone substitute[12]. We found that erythrocyte count was inversely associated with platelet-rich fibrin membrane length, but not with implant stability. Conversely, platelet count did not correlate with membrane size, but inversely correlated with implant stability at 7 and 17 weeks. In addition, we found that implant stability was directly correlated with levels FXIII, active von Willebrand Factor and total von Willebrand. In conclusion, alveolar ridge preservation with platelet-rich fibrin and demineralized bovine bone matrix is associated with circulating blood cells and coagulation factors. In particular, fibrin structure, von Willebrand Factor and Factor XIII may be important modulators of implant stability.

Another variable parameter which may influence the oral implantology treatment with platelet-rich fibrin may be the contribution of saliva. Since it has been shown that saliva contains tissue-factor (TF) and thereby may influence hemostasis and wound healing. Since there is a high variability in post-extraction/ post-surgical bleeding in patients, therefore we hypothesized this may be caused by a variation in saliva-derived TF-induced clotting activity. In the present thesis we describe our findings that saliva induces thrombin generation in plasma and showed to be tissue-factor dependent. Interesting were our findings on the inter-individual variability in saliva induced thrombin generation[13]. Interestingly, within subjects, saliva-induced thrombin generation was significantly increased in the morning compared to the afternoon. These findings could help clinicians in the decision making of the timepoint of treatment, for instance patients on oral anticoagulant therapy which cannot be stopped temporarily. Further research should reveal if treatment in the morning, higher TF level in saliva, leads to less bleeding after surgery. A possible cause for increased TF activity in the morning is the oral microbiome. Oral streptococci were previously demonstrated to induce

(endothelial) tissue factor activity[14]. In this context it could be interesting to access a possible relation between salivary TF concentration and bacterial status in the oral cavity.

Conclusion and prospects

Guidelines have to be developed in how to correctly prepare autologous blood concentrates for specific indications. Also, we have to improve the quality of the starting material, i.e., the composition of the whole blood, quality of the collecting tubes, centrifuges, etc. Upon screening the composition of the patient's whole blood before treatment, we may develop a personalized protocol for specific patients in the future generation tailor-made precision medicine. Future research should have their focus on these possibilities. International research collaboration in the field of oral implantology is essential to develop predictable protocols for platelet-rich fibrin in different indications.

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Appendix II Curriculum vitae

J.E.I.G. (Joost) Brouwers was born on November 16, 1956 in Heerlen (the Netherlands). He finished secondary school (VWO, Bernardinuscollege) in 1977. In 1979 he started his study Dentistry at the Rijksuniversiteit te Utrecht. In 1986 he received his master degree in dentistry. From 1987 until 1993 he worked as a general practitioner in his private practice in Werl in Germany. In 1994 he moved back the Netherlands and started his private practice in Beverwijk, from 1994 until 2003. In 2001 he started as a researcher in the Department of Prosthetic Dentistry and Oral Implantology at the Academic Center for Dentistry Amsterdam. In 2007 he received his Master of Science degree in Oral Implantology at the same university. Also, in 2001 he joined Han van Dijk at the Kliniek Implantologie Amersfoort until today. In 2015 a collaboration was established with dr. J.A. Remijn (Department of Clinical Chemistry, Gelre Hospitals Apeldoorn/Department of Clinical Chemistry, Meander Medical Center) and prof. dr. Ph.G. de Groot and dr. B. de Laat (Maastricht University/Synapse Research Institute Maastricht) on the effect of Platelet-rich Fibrin in oral implantology. This research resulted in the thesis Platelet-rich Fibrin interactions in Oral Implantology.

Appendix III List of Publications

Publications in this thesis

J.E.I.G. Brouwers, Sharon Buis, Rianne Haumann, Philip Ph G de Groot, Bas de Laat, Jasper A Remijn. Successful soft and hard tissue augmentation with platelet-rich fibrin in combination with bovine bone space maintainer in delayed implant placement protocol in the esthetic zone. Clinical Case Reports 2019 May 8;7(6):1185-1190.

J.E.I.G. Brouwers, Frank Lobbezoo, Corinne M. Visscher, Daniel Wismeijer, Michiel Naije. Reliability and validity of the instrumental assessment of implant stability in dry human mandibles.
Journal of Oral Rehabilitation 2009 36;279-283

J.E.I.G. Brouwers, S. Buis, Ph.G. de Groot, B. de Laat, J.A. Remijn.
Resonance Frequency Analysis with two different devices after conventional placement of self-tapping dental implants in post-extraction sites with ridge preservation.
Clinical Implant Dentistry and Related Research 2021;1-11

Joost E.I.G. Brouwers, Lisa N. Van der Vorm, Sharon Buis, Rianne Haumann, Joke Konings, Philip G. de Groot, Bas de Laat, Jasper A. Remijn. Implant stability in patients treated with platelet-rich fibrin and bovine bone substitute for alveolar ridge preservation is associated with peripheral blood cells and coagulation factors. Clin Exp Dent Res. 2020; 6:236-243

Joost E.I.G. Brouwers, Lisa N. Van der Vorm, Sharon Buis, Rianne Haumann , Joke Konings, Philip G. de Groot, Bas de Laat, Jasper A. Remijn. Histological evidence for osteoconductive effects of autologous platelet-rich fibrin in oral implantology. Journal of Dentistry and Oral Biology 2020: 5(4): 1174

van der Vorm LN, **Brouwers JEIG**, Mondria C, de Laat B, de Groot PG, Remijn JA. Salivary tissue factor induces thrombin generation in a diurnal rhythm. Res Pract Thromb Haemost. 2018;2:757-761

Other Publications

JEIG Brouwers, R Buchner, HW van der Glas, F Bosman. The post-stimulus electromyographic complex of jaw elevator muscles following a transcient mandibular load in patients with myogenic craniomandibular disorders. Journal of Oral Rehabilitation, 1988, Volume 15, 187.

R Buchner, **JEIG Brouwers**, HW van der Glas, F Bosman. The bilateral amplitude imbalance in the jaw-jerk reflex after a transcient mandibular load in patients with craniomandibular disorders. Electromyography of jaw reflexes in man. D van Steenberghe & A de Laat: University Press, Leuven 1989.

R Buchner, HW van der Glas, **JEIG Brouwers**, F Bosman. Electromyographic parameters related to clenching level and jaw-jerk reflex in patients with a simple type 0 myogenous cranio-mandibular disorder. Journal of Oral Rehabilitation, 1992, Volume 19, 495-511.

F Lobbezoo, **JEIG Brouwers**, MS Cune, M Naeije. Dental implants in tooth grinders. Nederlands Tijdschrift Tandheelkunde. 2004 Mar;111(3):85-90.

F Lobbezoo, **JEIG Brouwers**, MS Cune, M Naeije. Dental implants in patients with bruxing habits. Journal of Oral Rehabilitation. 2009 Apr;36(4);297-83.

Oral and poster presentations

15 Oct 2016 The formation of Platelet Rich Fibrin (PRF) is not only dependent on the centrifugal preparation method, but also related to the composition of peripheral blood cells (Poster ENHD (Enhanced Natural Healing in Dentistry) Leuven, Belgium)

12 July 2017 Treatment of patients with autologous Platelet Rich Fibrin is dependent on the composition of peripheral blood cells: dental implant stability is associated with red blood cells and not with platelets. Poster presentation. International Society on Thrombosis and Haemostasis , Berlin, Germany

07 Oct 2017 Treatment of patients with autologous Platelet Rich Fibrin is dependent on the composition of peripheral blood cells: dental implant stability is associated with red blood cells and not with platelets (II). Poster presentation. European Association for Osseointegration, Madrid, Spain.

12 Oct 2018 Saliva-derived tissue factor induces thrombin generation in a diurnal rhythm. Oral Communication European Association for Osseointegration, Vienna, Austria.

26 Sept 2019 Treatment with Platelet Rich Fibrin results in increased oral implant stability, which is associated with peripheral blood cell- and coagulation parameters. Poster presentation. European Association for Osseointegration, Lisbon, Portugal.

Appendix IV Dankwoord

Promoveren is als het proeven van wijn. In het prille begin kun je alleen aangeven of de wijn smaakt of niet. Gaandeweg ontwikkel je je neus en je smaakbeleving op de tong en dat maakt je steeds kritischer bij het proeven. Je gaat indringende discussies voeren met de echte kenners over het glas, de temperatuur, de geur, de smaak, de kleur en de afdronk. Je ontwikkelt misschien wel voorkeuren voor een land, gebied, cépage, terroir, kleur, etc.
Eenmaal je favorieten gevonden kan het leiden tot overmatig drankgebruik (zeker in een promotietraject), met als gevolg hevige hoofdpijn.
Voor mij was dit promotietraject niet echt veel anders, ik heb veel van gedachten mogen wisselen met echte kenners en heb daar enorm veel van geleerd met af en toe een beetje hoofdpijn.

Graag wil ik iedereen die op wat voor wijze dan ook een bijdrage geleverd heeft aan dit promotieonderzoek bedanken.
Een aantal mensen wil ik in het bijzonder bedanken.

Allereerst mijn promotoren Prof. Dr. H. ten Cate, Prof. Dr. Ph. G. De Groot, Dr. J.A. Remijn en Dr. B. De Laat.

Hugo, bedankt dat je mij de kans gegeven hebt om bij jou te mogen promoveren. Vooral veel dank voor je snelle reacties en alle hulp die ik van jou heb mogen ontvangen in het traject.

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Jasper, zonder onze "toevallige" ontmoeting had dit avontuur nooit plaats kunnen vinden. Ik denk dat het onze tweede ontmoeting was waarbij wij onze gezamenlijke interesse in "Platelet-rich fibrin" ontdekten. Vanaf dat moment zijn wij in een soort "rollercoaster" terecht gekomen. Ik kijk met veel plezier terug op alle mooie meetings in Amersfoort, een goed glas wijn en goede ideeën uitwerken. Onze gezamenlijke trips naar de verschillende internationale congressen. Voor mij heel bijzonder om in de wereld van de haematologie een kijkje te mogen nemen. Fantastisch dat jij meeging naar de orale implantologie congressen. Kortom ik heb genoten van deze tijd. Maar het is gelukkig nog niet voorbij, het volgende project is alweer opgestart. Wij verheugen ons op de volgende stap en gaan er weer voor met elkaar.

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Leden van de beoordelingscommissie, prof. dr. C.P.M. Reutelingsperger, prof.dr. E.A.M. Beckers, prof. dr. J.C. Sluimer, prof. dr. H. Weber, prof. dr. F. Beuer, hartelijk dank voor het zorgvuldig lezen en beoordelen van mijn proefschrift en de bereidheid om zitting te nemen in mijn corona.

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Sharon, zonder jou was dit project veel lastiger geworden. Je kwam als stagiaire voor je afstudeerproject bij ons en nu meer dan zeven jaar later ben je een volwaardige implantologie/ok assistente met bijzondere affiniteit voor het onderzoek. Onvoorstelbaar hoe jij je ontwikkeld hebt door de jaren heen, chapeau! Ik wil je graag bedanken voor je eeuwigdurende opgewektheid, altijd lachend en vooral je accuratesse waarmee jij alle data rangschikte en verwerkte. Ook voor je reizen naar Apeldoorn om droogijs te halen of samples te brengen, altijd per openbaar vervoer, voor je enorme snelle manier van typen (ik ben echt jaloers) en het direct mailen van de notulen van onze vergaderingen. Kortom ik hoop dat wij nog jaren gezamenlijk interessante onderzoeksprojecten mogen doen. Dank je wel Sharon.

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Zat ik in de put omdat het niet liep zoals ik wilde dan had jij altijd een opkikker “komt tijd, komt raad”.
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Drs. R. Sinke, beste Robert, paranimf en "sportmaat",
Tja, het opstel (zoals onze personal trainer Gerco het altijd noemde), is eindelijk af.
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