IN VITRO EVALUATION OF THE EFFECTS OF LEUKOCYTE-PLATELET RICH FIBRIN ON DISINFECTION OF A ROUGH IMPLANT SURFACE

by

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Abstract

Objectives: Peri-implantitis is a frequent and serious clinical problem affecting between 1 and 47% of implants. Bacterial contamination of the roughened implant surface plays a major role in the etiology and progression of the disease. Successful treatment of peri-implantitis requires disinfection of the rough implant surface. There is no generally accepted protocol for implant disinfection. Autologous leukocyte and platelet-rich fibrin (L-PRF) membranes can be produced from autologous human blood via a one-step centrifugation procedure. It was hypothesized that the antimicrobial defense system of L-PRF may decontaminate the SLA® implant surface. The objective of this study was to test the efficacy of L-PRF for SLA® implant surface disinfection.

Methods: Collagen-coated SLA® (sand blasted, large grit acid etched) titanium discs were inoculated with dispersed dental plaque with a minimum bacterial cell concentration of $3.2 \times 10^7$ CFU/ml. After 21 days of anaerobic incubation at 37°C, discs were rinsed with 12 ml 0.9% NaCl to remove unattached biofilm, and exposed for 48 hours to Leukocyte-Platelet Rich Fibrin (L-PRF) in DMEM. Disks with or without rinsing with 12 ml of 0.9 % NaCl were fixed for SEM. Bacterial counts and perforations in bacteria were quantified from standardized scanning electron micrographs of the implant surface. The rinsing solution was collected and Western blot analysis was performed. L-PRF disks were compared with the control group (rinse).

Results: Difference in presence of bacteria displaying perforation of the cell wall between cell–rich L-PRF treated samples and rinsed control group was statistically significant ($p < 0.0001$, Fisher’s Exact Test). Western blot analysis of the rinse fluid demonstrated presence of Platelet Factor-4. Activated platelets in intimate contact with bacteria were detected on SEM images. SEM analysis demonstrated a statistically significant reduction of residual bacteria in the lacunae of the rough SLA® surface after L-PRF treatment. ($p<0.05$, Kruskal-Wallis).

Conclusions: Autologous L-PRF may have potential as a biological means to decontaminate rough implant surfaces, possibly by exploiting the antimicrobial effects of platelets.
Lay Summary

Millions of dental implants are being placed annually to replace missing teeth. Peri-implantitis is a frequent problem affecting dental implants. It can lead to loss of the implant due to damage to the supporting bone. There has been limited success with current treatment modalities. One of the factors that complicate treatment is the rough implant surface which cannot be easily disinfected. Lower infection rates have been reported when blood concentrates are used during oral surgery. This study found that Leukocyte-Platelet Rich Fibrin (L-PRF), a blood concentrate, can reduce bacteria when applied to a contaminated rough implant surface. Platelets may be involved in the effect of the L-PRF. Further research in needed to determine if this can impact the success of peri-implantitis therapy if applied clinically.
Preface

This dissertation is an original intellectual product of the author, Luisa Antonia Schuldt. The University of British Columbia’s Research Ethics Board [certificate number H15-01881] approved all experimental methods. Luisa Antonia Schuldt and Jirau Bi performed all experimental manipulation and prepared the samples for SEM imaging. Luisa Antonia Schuldt and Dr. G. Owen carried out SEM imaging. Luisa Antonia Schuldt proceeded with the image analysis for SEM images, prepared the corresponding figures and all statistical analysis.
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List of Abbreviations

ADP: adenosine diphosphate
BHI: Brain-heart infusion medium
BoP: Bleeding on probing
CHX: Chlorhexidine
EMD: Enamel matrix derivative
FDP: Fibrinogen degradation products
GBR: Guided bone regeneration
GDP: Guanosine diphosphate
L-PRF: Leukocyte-platelet rich fibrin
L-PRP: Leukocyte-platelet rich plasma
PBS: Phosphate buffered saline
PDGF: Platelet derived growth factor
PD: Probing depth
PF-4: Platelet factor 4
PIVES: Piperazine-N,N'-bis(2-ethanesulfonic acid)
PmP: platelet microbicidal protein
PPP: Platelet poor plasma
P-PRF: Pure platelet-rich fibrin
P-PRP: Pure platelet-rich plasma
Sa: Surface area roughness
SD: Standard deviation
SEM: Scanning electron microscopy
TGF-β: Transforming growth factor-β
TPS: Titanium plasma-sprayed
VEGF: Vascular endothelial growth factor
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Dedication

I dedicate not only this work, but also myself, to my little Nicholas.

I pray to be provided the wisdom to instill in you a love of learning and the will to never give up,

as my mom did with me.

I love you more than I could have ever imagined.
Chapter 1: Introduction

Dental implants have become the standard of care in modern dentistry for the replacement of missing teeth with success rates around 95% commonly reported in several studies. Implants have been placed in increasing numbers worldwide for the last 30 years and have reached an estimated 1,260,000 dental implant procedures performed in 2013 in the United States alone. The number of implants placed annually is expected to double in 7 years reaching 2,540,000 implants in the United States. In spite of reported high success rates and similarities between peri-implant and periodontal diseases, implants have been found to be more susceptible to peri-implant disease than teeth are to periodontal disease. Frequency of peri-implantitis has been reported to be between 6.47% up to 56% depending of definition criteria and study protocols.

Peri-implant disease, if left untreated, can lead to continued bone loss and ultimately implant failure. The associated bone loss can make replacement of the affected implant non-plausible, unpredictable or only possible after extensive bone grafting. Considering the large number of potentially affected dental implants and the effects that failure can have on a patient’s oral health, improved treatment modalities are needed.

The rough implant surface has been considered a breakthrough innovation in Implant Dent significantly increasing implant success rates, shortening healing times and in consequence, allowing the development of novel treatment modalities. Unfortunately, when this surface becomes exposed to the oral cavity it facilitates bacterial adhesion and acts as a niche for bacteria. Management of this bacterial contamination is considered to be an important part of the treatment of peri-implant diseases. Currently disinfection techniques are mechanical or chemical in nature and are ineffective in removing the biofilm. Platelets are the first responders to vessel wall breaches. Their primary function is to patrol the vasculature and seal any breaches to the vessel walls to limit blood loss. Also, platelets and the growth factors they release have an essential role in the regulation of the cellular events that follow tissue damage.
They have the capability to adhere, aggregate and form a fibrin mesh. During their function, they release a large variety of substances including cytokines, growth factors, catecholamines, serotonin, osteonectin, von Willebrand factor and proaccelerin.¹²

Platelet concentrates can be prepared from whole blood quickly using simple methodology. They can be used to potentiate healing and tissue regeneration. More recently an additional advantage to their use has been proposed. Blood concentrates have been found to provide antimicrobial properties.¹²,¹³ Although these properties are not yet fully understood, antimicrobial peptides, essential elements of the innate immune system, such as defensins may be involved. The potential applications of these concentrates in the management and prevention of infections have not been fully explored.
Chapter 2: Review of the Dental Literature

2.1 Peri-implant Diseases

Dental implants were introduced in the 1980’s by P.I. Brånemark, as a highly researched technique with a strict treatment protocol. At that time, clinicians were in awe of the concept of osseointegration. Currently, due to modern biomaterials, rougher implant surfaces and improved clinical procedures achieving osseointegration is no longer the only key issue in oral implantology. The focus has shifted to improving the long-term predictability of implant therapy, particularly to achieving peri-implant bone stability.

It was initially thought that implants were lost due to occlusal overload or poor surgical technique. Over the last three and a half decades many modifications have been made to the original treatment protocol. The technique is now used for single implants, bridges and full mouth restorations. Restorations over implants can be fixed or removable. Multiple surgical protocols are also in place including immediate, early and delayed placement. Along with these advances in implant protocols, problems have also been detected. Possibly, most alarming are the highly prevalent peri-implant diseases. These can negatively impact dental implants causing failure of the dental implant itself and its overlying prosthesis.

The definition of success versus survival complicates the determination of treatment outcomes. Many papers report on implant survival, which is frequently considered as the continued presence of the implant in the mouth regardless of its condition. Surviving implants may not be successful as the criteria for success are stricter including lack of infection, minimal to no bone loss, no signs of inflammation and the implant must be restorable. Again, these criteria vary widely from study to study.

Implant success rates have been reported to be as high as 99% in the mandible to 93% in the maxilla after 5 years. The Albrektsson criteria for success, used to determine the aforementioned
success rates, and referenced in many studies since, include: absence of implant mobility; no evidence of peri-implant radiolucency; vertical bone loss less than 0.2 mm annually following the implant's first year of service; and absence of signs and symptoms such as pain, infections, neuropathies, paresthesia or violation of the mandibular canal. These criteria do not take into account esthetic results or presence of peri-implant disease, although minimal progressive bone loss independent of the cause is considered. Smith and Zarb suggested the addition of several conditions to implants being evaluated including that the implant be under functional load at the time of evaluation.

In an estimation made by Tarnow, at least 10% of all implants placed can be expected to present peri-implant disease after 10 years. He calculates that if over 1 million implants were placed worldwide in 2015, 100,000 of these implants would require treatment for peri-implant diseases.

2.1.1 Definition and Diagnosis

At the 1st European Workshop on Periodontology in 1993, it was agreed that peri-implantitis should be used specifically to describe destructive inflammatory processes around osseointegrated implants in function that lead to peri-implant pocket formation and loss of supporting bone. Peri-implant mucositis has been defined as inflammation of the soft tissues surrounding the dental implant with no signs of loss of supporting bone and peri-implantitis as inflammation affecting the supporting bone. Implant bone loss is associated with exposure of the rough surface of the implant to the oral cavity.

Putting these definitions into practice has become a controversial matter. When studying prevalence, incidence and risk factors of a disease, a precise definition of any pathological condition is required. Sound cut-off points defining presence and absence of the disorder, definitions of disease severity and an appropriate research methodology are of the utmost importance. The same applies in a clinical setting. A clear cut-off point for diagnosis allows for the selection of appropriate treatment modalities. The
definition criteria for peri-implant diseases are less than clear.\textsuperscript{25} Criteria used to define peri-implantitis vary widely from study to study,\textsuperscript{26} including different measurements for bone loss. Consideration may or may not be given for initial bone remodeling. Some authors include suppuration among the definition criteria.\textsuperscript{24, 27}

Clinical parameters similar to those used for the monitoring of periodontal health have been suggested. Probing should be considered an important diagnostic parameter in the monitoring of peri-implant tissues.\textsuperscript{28} Progressive probing depths (PD) and bleeding on probing (BoP) are commonly used criteria for the diagnosis of peri-implant conditions.

Animal studies have shown that progressively increased PD are associated with attachment loss and bone loss.\textsuperscript{29, 30} BoP around dental implants indicates the presence of inflammation\textsuperscript{29} but can also be a sign of disease progression at site when BoP is present at more than half of the recall visits over a 2-year period.\textsuperscript{31} It is safe and necessary to probe around dental implants and their associated restorations,\textsuperscript{32, 22, 33} as the healing of the epithelial attachment is complete at 5 days after probing.\textsuperscript{34} Suppuration has been associated with bone loss around implants resulting in exposure of 3 or more threads.\textsuperscript{35}

In clinical practice, diagnosis of peri-implantitis can be just as complex as in the research setting. Radiographic evaluation of crestal bone levels over time seems to be the most reliable tool to identify those implants undergoing continuous bone loss.\textsuperscript{36} Based on longitudinal studies and in agreement with the Consensus for the 6\textsuperscript{th} European Workshop on Periodontology, time of prosthesis installation has been suggested to establish a baseline for bone level.\textsuperscript{28} There are limitations to attempting to establish a baseline in this manner. Radiographs at time of restoration are not always available as a baseline, and quality of these radiographs including vertical angulations can significantly alter their efficacy as diagnostic aids. In the absence of an adequate baseline radiograph the use of a threshold vertical distance of 2 mm from the expected marginal bone level following re-modeling post-implant placement has been suggested.\textsuperscript{37, 38} Bone loss on the buccal or lingual aspects of the implant cannot be observed radiographically limiting their sensitivity.\textsuperscript{38} Certain protocols, such as with immediate loading, may require more time for an adequate baseline to be made.\textsuperscript{25}
Clinical signs indicating the presence of peri-implant mucositis include BoP and/or suppuration, usually associated with PD less than or equal to 4 mm and no evidence of radiographic loss of bone beyond bone remodeling. A critical parameter in the diagnosis of peri-implant mucositis is BoP. Gentle probing with pressures of <0.25 N to 0.15 N have been suggested. The absence of BoP is an indicator for stable peri-implant conditions. Etter et al evaluated the healing following standardized peri-implant probing using a force of 0.25N and observed complete reformation of the mucosal seal after 5 days.

In counterpoint, Coli et al, in their 2017 review, state that periodontal indices, such as PD and BoP, are not sensitive for identification of peri-implant disease and future risk of peri-implant crestal bone loss when used as standard diagnostic measures. PD of healthy peri-implant mucosa can be far deeper than 4 mm. The use of probing pocket depth and BoP assessments alone can lead to over-diagnosis and overtreatment of assumed peri-implantitis lesions. Patient’s symptoms (discomfort, pain, etc.), presence of swelling, redness and pus and significant crestal bone loss over time, as verified with radiography should be considered prior to making the diagnosis of peri-implant disease and performing treatment.

Albrektsson, T., author of the previously mentioned implant success criteria, along with multiple other highly respected authors of implant literature, came together in 2013 in a meeting sponsored by multiple implant companies. The group brought to light several limitations of current definitions for peri-implant disease stating that crestal bone loss presents for reasons other than infection. In consequence, peri-implantitis is an inadequate term for all crestal bone loss and a limited amount of crestal/marginal bone loss may be part of a biologic response to implant placement. It is also stated that many factors can contribute to crestal bone loss including implant-, clinician- and patient related factors, such as: material, surface properties, surgical and prosthodontic experience and skills of the clinician, patients oral and systemic health, behavior (eg. smoking, oral hygiene and maintenance) and site related factors. These authors also go as far as to suggest the following alternate definition for peri-implantitis: infection with suppuration associated with clinically significant progressing crestal bone loss after the adaptive phase of implant placement.
In response, Froum et al.\textsuperscript{42} state that although peri-implantitis is an unsuitable term to describe all crestal bone loss, to define peri-implantitis as an infection with suppuration and to determine that therapeutic intervention is only necessary when combined with crestal bone loss, is contrary to much published data. Peri-implantitis, which has been defined as inflammation of the peri-implant mucosa accompanied by bone loss does not require suppuration to be present. Similarly, active bone loss around a tooth with periodontitis is often seen without suppuration. Many experienced clinicians believe that BoP and increasing PD along with crestal bone loss beyond physiologic normal adaptive changes are diagnostic for peri-implantitis.

According to Albrektsson et al.\textsuperscript{22} states that marginal bone loss around implants is usually associated with immune-osteolytic reactions\textsuperscript{43} opposing the widely accepted concept of peri-implant infection resulting from a disturbance of the balance between the microbiologic challenge and host response;\textsuperscript{22} and again displaying the lack of consensus in the definition and diagnosis criteria for peri-implant diseases.

2.1.2 Epidemiology

Many studies have been conducted to investigate the prevalence of peri-implant diseases. Limited information is available with regard to the incidence, prevalence and risk factors of peri-implant diseases.\textsuperscript{21,24,28} Unfortunately, as described previously, there is not yet clear consensus with respect to the definition of peri-implantitis; and the criteria used in studies also vary, impacting the results of the research. Tarnow\textsuperscript{16} provided the example of a study by Koldsland\textsuperscript{44}, in which altering the definition criteria from 0.4 mm bone loss to 3 mm the prevalence changes from 47% to 11%.

A similar example is mentioned by Coli et al.\textsuperscript{36} regarding the work of Jemt et al.\textsuperscript{45} and Fransson et al.\textsuperscript{46}. Although the authors described the same group of patients the follow-up findings make it clear that
current definitions for disease are not useful in predicting further breakdown. Fransson et al. defined peri-implantitis as any implant that presented BoP and bone loss at any time between 1 year and the 23 years follow-up and determined a prevalence of 28% (182 patients). Fransson also found that at implant level, the presence of pus, soft tissue recession and PD of 6 mm or more were more common around implants with radiographically evidence of progressive bone loss than around implants without bone loss. When 145 of these peri-implantitis patients were evaluated by Jemt et al. 9 years later, 65 of the patients had been referred to an oral hygienist and 80 had not. Bone loss for these patients were 0.5 mm (SD 0.80) and 0.1 mm (SD 0.62) respectively. 39 patients had received surgical treatment for peri-implantitis and of these 5 had implant failures. Among the 106 patient’s that did not receive surgical therapy 11 had implant failures. 97 patients presented little to no problems, indicating that a large number of patients in the “peri-implantitis group” had no further bone loss. It was also concluded that patients treated by oral hygienists and/or had experienced peri-implantitis surgery did not show any more favorable progression of bone loss as compared with non-treated patients.

Roos-Jansaker et al. reported on 294 patients after 9 to 14 years of function and described prevalence at 76.6% and 16% for peri-implant mucositis and peri-implantitis at the patient level, and at the implant level at 48% and 6.6%, respectively. Definitions used in this study were: peri-implant mucositis, ≥4 mm and BoP; and peri-implantitis, bone loss ≥1.8 mm compared with 1-year data, combined with BoP or suppuration. The same group of authors found that smoking was associated with mucositis and peri-implantitis, and that peri-implantitis was related to a previous history of periodontitis.

Mombelli et al. reviewed epidemiology of peri-implant disease and determined a prevalence of peri-implantitis of 10% implants and 20% patients during 5 –10 years after implant placement. It was also mentioned that the individual reported figures are rather variable, not easily comparable and not suitable for meta-analysis. The factors that may have affected the prevalence are the disease definition, the differential diagnosis, the chosen thresholds for PD and bone loss, differences in treatment methods and aftercare of patients, and dissimilarities in the composition of study populations.
A 2013 meta-analysis estimates a prevalence of peri-implant mucositis as 30.7% of implants, while those presenting peri-implantitis was estimated at 9.6% of implants. In this study peri-implant mucositis was defined as inflamed mucosa presenting a bleeding index of $\geq 2$ and or suppuration; and peri-implantitis was defined as presence of inflamed mucosa with a positive BoP, PD of $\geq 5$ mm, cumulative bone loss of $\geq 2$ mm and/or $\geq 3$ threads of the implant. A greater frequency of occurrence of peri-implant diseases was recorded for smokers with an estimate of 36.3%. Higher prevalence numbers over 40% have been reported in individual studies, depending on threshold used for disease classification.

Another meta-analysis conducted in 2016 at the XI European Workshop on Periodontology, addressing prevalence extent and severity of peri-implant diseases, recognized the need for consistent definition criteria and that the variations of these criteria in research further illustrate the current lack of consensus. The findings, based on 11 included studies (15 articles), indicated prevalence ranging from 19-65% for peri-implant mucositis and 1-47% for peri-implantitis. Extent and severity of the disease are rarely reported.

Among the risk factors identified for peri-implant mucositis, in addition to smoking, are history of periodontal disease, lack of professional maintenance, poor oral hygiene, diabetes, radiation therapy, limited keratinized tissue. There is evidence that the lack of annual supportive therapy in patients diagnosed with peri-implant mucositis is associated with increased risk for conversion of mucositis to peri-implantitis.

Other factors that may also be responsible for the reported variations of peri-implantitis prevalence are implant type (including the differences in implant surface), clinician experience and systemic health of the implant patients.
A positive relationship has also been identified between excess cement and peri-implant disease. Wilson\textsuperscript{54} found that excess dental cement was associated with signs of peri-implant disease in 81\% of the cases included in this study. After removal of the excess cement, signs of peri-implant disease were no longer detected clinically in 74\% of the treated implants. Linkevicius\textsuperscript{55} found that not all patients respond equally to the presence of excess cement. Patients with a history of periodontitis are more likely to present peri-implantitis at implants with cement remnants than their non-periodontitis counterparts. Other studies have shown that there is no difference between peri-implantitis prevalence in patients with cemented or screw-retained restorations.

### 2.1.3 Pathogenesis

Although it has been suggested that initiation of peri-implant disease is the result of a multifactorial process, in which iatrogenic, mechanical, anatomic, immunologic, environmental, genetic and microbiologic factors play a role\textsuperscript{56}, strong evidence indicates that bacteria are the key etiological factor in peri-implant mucositis and peri-implantitis development\textsuperscript{25,28}. While peri-implant diseases may clinically resemble gingivitis and periodontitis, there are large differences between these diseases.\textsuperscript{57} Periodontitis and its counterpart around implants, peri-implantitis, have many features in common as well as critical histopathological differences between them.\textsuperscript{58} For peri-implant disease the misbalance in the host–parasite interaction is key in the pathogenesis of the tissue destruction.\textsuperscript{59} Peri-implantitis is characterized by more extensive inflammatory infiltrate and innate immune response, increased severity of tissue destruction and more rapid progression.\textsuperscript{60}

As with gingivitis, a cause and effect relationship between biofilm formation on implants and peri-implant mucositis was demonstrated when using the experimental gingivitis model\textsuperscript{61–63} developed by Löe et al.\textsuperscript{64} Mucosa around implants and gingiva around teeth have a similar response to early plaque formation.\textsuperscript{65} These differences and similarities are further described in a review by Berglundh et al.\textsuperscript{58} For both teeth and implants, in animals, placement of ligatures in a submarginal position with plaque formation results in loss of
supporting tissues and the establishment of large inflammatory cell infiltrate. This is where the similarities end.

Following ligature removal, a “self-limiting” process occurred around teeth resulting in the formation of a protective connective tissue capsule that separated the inflammatory cell infiltrate from bone at 1 month after ligature removal. This protective process did not take place in peri-implant tissues. Experimental peri-implantitis sites, exhibit signs of acute inflammation and large amounts of osteoclasts lining the surface of the bone crest at varying periods after ligature removal. Also, experimental peri-implantitis models demonstrated that the lesions produced from ligature-induced breakdown and plaque formation also progressed with additional bone loss after the removal of ligatures. Similar effects have not been demonstrated for periodontitis. 58

Other findings, describing some level of similarity and as well and pronounced differences, are described in humans by Salvi. 61 After 3 weeks of undisturbed plaque formation in 15 partially dentate patients with dental implants an inflammatory response could be detected both around teeth and dental implants, with the response in peri-implant soft tissues being more pronounced. After 3 weeks of resumed plaque control tissues presented improved clinical characteristics but longer healing periods are needed for both gingival and peri-implant tissues to fully recover.

Similarly to the established lesion of periodontal disease 66, the presence of biofilm on implants during 6 months provoked an inflammatory lesion in the connective tissue of the peri-implant mucosa that was dominated by plasma cells and lymphocytes. 67 Both periodontitis and peri-implantitis lesions are characterized by an infiltrate of large inflammatory cells adjacent to the pocket epithelium, although peri-implantitis lesions contain a greater proportion of neutrophils and macrophages. A more pronounced apical extension going beyond the pocket epithelium is present in peri-implantitis. 68 58 Peri-implantitis associated bone loss, like periodontitis, is of non-linear progression but with the rate of loss increasing over time. 69

Granulation tissue from peri-implantitis sites exhibits higher mRNA expression of pro-inflammatory cytokines Interleukin IL-6, IL-8 and (TNF)-α compared to matched tissue from periodontitis sites. 60
Factors other than plaque have been implicated in peri-implant inflammation. It is possible that some of these factors can heighten the inflammatory response in the peri-implant tissues leading to greater breakdown in the presence of peri-implant disease. Wilson et al\textsuperscript{70} describe the presence of radiopaque foreign bodies in 34 of 36 biopsies taken of peri-implant soft tissue of implants clinically determined to have peri-implantitis. Peri-implantitis was defined as: clinical signs of inflammation, including BoP, suppuration, increased PD, abscess, pain, erythema, edema and radiographic signs of severe progressive bone loss.

2.1.4 Microbiology

Peri-implantitis is considered an infectious disease\textsuperscript{25}, characterized by BoP and progressive marginal bone loss.\textsuperscript{22,25} The composition of the subgingival microbiota in experimental periodontitis and peri-implantitis is similar, namely an anaerobic proteolytic flora.\textsuperscript{71,72,73} Both diseases are associated with polymicrobial, anaerobic and primarily Gram-negative infections.\textsuperscript{74,75} Some studies suggest that peri-implantitis microbiota displays less variability than that of its periodontal counterpart.

The sequence of microbial colonization on dental implants is similar to that of teeth.\textsuperscript{5,71} Shortly after installation an implant sub-mucosal microbiota is established.\textsuperscript{59} Early colonizers have been found to be oral bacteria such as \textit{P. intermedia}, \textit{P. micros} and \textit{F. nucleatum}.\textsuperscript{76} A complex microbiota including species from the red and orange complexes can be detected in the peri-implant sulcus as early as 2 weeks.\textsuperscript{77}

The microbiological pattern of experimental peri-implantitis closely resembles that of periodontitis as revealed by Leonhardt et al\textsuperscript{71} in an experimental microbiological study. It was also found that the succession of events from aerobic to anaerobic flora around implants was strongly linked to the presence of pathology. Mombelli and Lang\textsuperscript{78} conclude that there is a clear microbiological distinction between stable implants and
implants with peri-implant pathology; and that gram-negative anaerobic bacteria, such as spirochetes, are involved in pathological developments around dental implants. The presence of *Parvimonas micro* in the peri-implant pocket has been found to be a highly accurate predictor of peri-implantitis. 79

Shilbi et al 80 found that the bacteria associated with periodontitis are commonly found in peri-implantitis including *Bacteroides, Campylobacter, Eubacterium, Fusobacterium,* and *Treponema* species and that the microbiota associated with peri-implantitis was comprised of periodontal pathogenic bacterial species including *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia.* Da Silva et al 81 found that biofilm associated with peri-implantitis harbored more bacterial species from the orange complex, 82 such as *Fusobacterium nucleatum, Parvimonas micro,* *Prevotella intermedia,* and *Campylobacter gracilis* and significantly higher percentages of strains of Desulfovulbus species, *Dialister* species, *Filifactor* species, *Fusobacterium* species, *Mitsuokella* species and *Porphyromona* species in comparison to healthy implants. Persson and Renvert 83 found nineteen bacterial species at higher counts on implants with peri-implantitis including: *Aggregatibacter actinomycetemcomitans, Campylobacter gracilis, Campylobacter rectus,* *Campylobacter showae,* *Helicobacter pylori,* *Haemophilus influenzae,* *Porphyromonas gingivalis,* *Staphylococcus aureus,* *Staphylococcus anaerobius,* *Streptococcus intermedius,* *Streptococcus mitis,* *Tannerella forsythia,* *Treponema denticola,* and *Treponema socranski.*

*Filifactor alocis* has recently been proposed as a potential periodontopathogen. 84 This gram + rod has been detected in the sulcus of implants presenting peri-implantitis. 85

Peri-implant bone loss has been found to be associated with the submucosal presence of the putative periodontopathogens *Tannerella forsythia, Campylobacter* species, and *Peptostreptococcus micros* while pain was associated with *P. micros, Fusobacterium* species, and *Eubacterium* species. 74
2.1.5 Dental Implant Surfaces

Dental implant surfaces have been modified over the years with the objective of facilitating bone ingrowth into minor surface irregularities and increasing surface area, in consequence, improving bone-to-implant contact and osseointegration.\(^5\) Multiple methods of increasing the roughness of the surface have been developed including additive (titanium-plasma spray) and subtractive (sandblasting and acid etching) techniques. Roughness can be expressed as surface area roughness (Sa). Sa values are between 1.0 and 2.0 \(\mu m\) for the TiOblast\(^{TM}\), SLA\(^{®}\) and TiUnite\(^{®}\) implants, while it values between 0.5 and 1.0 \(\mu m\) are present on the machined or turned type of implants. Moderately rough surface implants (Sa between 1.0 and 2.0 \(\mu m\)) have shown a stronger bone response than other surfaces, improving the survival rates.\(^4\) The surface irregularities, while on the one hand improve implant-to-bone contact, have also been proposed as a potential niche for bacteria if exposed, for example, due to marginal bone loss or poor surgical technique. Rougher implant surfaces facilitate bacterial adhesion.\(^6\) Literature is inconclusive as to the effect of implant surface roughness on long-term success and peri-implant bone loss.\(^{15,86}\) There is insufficient evidence linking implant surface characteristics to the initiation or peri-implantitis and there is limited evidence suggesting that these features may have an effect on the progression of established peri-implant disease.\(^{87}\) Quirynen et al\(^{88}\) suggests that implants with increased surface roughness are more prone to peri-implant bone loss and subsequently late implant failure. In counterpoint, Chappuis et al\(^{89}\) found that rough surface implants (Titanium plasma-sprayed, TPS) can be successful (success rate was 89.5%), presenting minimal peri-implant bone loss after 20 years. In this study, the bone level was stable in 92% of the surviving implants (78/85, displaying less than 1 mm peri-implant bone loss over the 20-year observation period). Becker describes similar a success rate for 388 TPS surface implants followed for 12-23 years. 9.7% of the surviving implants were diagnosed with peri-implantitis.\(^{90}\)

Berglundh et al,\(^{91}\) explores the differences in the response of moderately rough (SLA\(^{®}\), large grit sandblasted and acid etched) and polished implant surfaces to ligature induced peri-implantitis in animals.
Findings included stabilization of radiographic bone loss and soft tissue parameters after removal of the ligature in machined implants. In the case of the moderately rough implant surface, progressive bone loss continued, thus reinforcing the concept that the rough surface can act as a niche if exposed.

Implant surface may also impact the result of peri-implantitis therapy.92

2.1.6 Current Treatment Modalities

As with any disease, treatment should include the identification of etiological and risk factors and the management or elimination of these. Elimination of plaque, smoking cessation, management of underlying disease, such as poorly controlled diabetes, are critical components of peri-implantitis prevention and treatment. Successful treatment requires commitment to meticulous home care and compliance with the professional hygiene maintenance program.

Several protocols have been suggested for the management of peri-implant diseases, including the CIST Protocol,93 the Decision Tree for Management of Peri-implant Diseases94 and the decision tree for ailing and failing implants. 95 Although they all suggest conservative treatment for peri-implant mucositis and surgical procedures for peri-implantitis, the details regarding how these treatments are to be performed are not mentioned.

Although current evidence does not allow for firm recommendations for non-surgical or surgical therapies for the treatment of peri-implant disease,96 certain elements seem to provide some benefit. Oral hygiene instruction, smoking cessation, assessment of the prosthesis to allow access for plaque control (and possibly removal or adjustment of the prosthesis), non-surgical debridement with the use or not of local antimicrobials may be beneficial. If these non-surgical therapies do not provide resolution of the signs and symptoms of the disease surgical procedures may be indicated including open flap debridement and
resective or regenerative therapies. Maintenance care is an important part of peri-implantitis prevention and therapy. 97

Five considerations have been suggested in the therapy of peri-Implantitis: 1. Disturbance or removal of biofilm from the pocket; 2. Decontamination of the implant surface; 3. Correction or reduction of sites that cannot be easily maintained; 4. Establishment of an adequate maintenance regimen; 5. Regeneration of bone and re-osseointegration. 98, 99

Non-surgical therapies:

Multiple technologies have been proposed for the mechanical debridement of dental implants including the use of curettes (carbon fiber, titanium, stainless steel), 7,100,101,102 abrasives (air-polishing with glycine powder with or without tricalcium phosphate), 103,104,105,106 ultrasonic devices, 100, 59 lasers, 107,102 and photodynamic therapy, 108,109

The ability of these technologies to successfully debride the implant surface is limited by access, surface characteristics, implant design and the clinician’s skill. 106 Success rates of nonsurgical therapies in the treatment of peri-implantitis are limited and a surgical approach will frequently be required. 110 In addition to mechanical methods, chemical methods have been suggested, including, the use of antiseptics and antibiotics have been proposed to complement mechanical debridement in an attempt to further reduce the bacterial load. These include the use of doxycycline, minocycline, chlorhexidine. 111,8

Implantoplasty is a technique which consists in flattening exposed threads and smoothing the contaminated implant surface with the use of rotary instruments.
Surgical therapies

Flap surgery may be required to facilitate access to the implant surface and bone defect. The aims of these procedures include: improved decontamination of the implant surface and performing resective or regenerative therapy to correct the bone defect.

Resective surgery includes of bone recontouring and modification of the implant surface (removal of implant threads and smoothening of the rough implant surface), while regenerative therapy attempts to recreate the lost hard tissue structure along with the bone to implant contact. In the case of an infrabony defect regenerative techniques may be considered, especially in the esthetic zone.

2.1.7 Disinfection Techniques

Biofilm plays a significant role in the initiation and progression of peri-implant diseases. A contaminated implant surface presenting bacterial by-products is believed to leads fibrous encapsulation as opposed to re-osseointegration.\textsuperscript{112,113}

Current disinfection techniques, including mechanical and chemical treatments, are ineffective in removing the biofilm from the rough implant surface,\textsuperscript{8,10,9} a step considered to be of great importance in the management of these diseases and required for effective wound healing including reattachment of the soft tissues and regeneration of bone. Clinicians have used different chemotherapeutic agents, such as chlorhexidine (CHX), tetracycline paste and/or phosphoric and citric acid gels as well as locally administered antibiotics (minocycline and doxycycline) as adjuncts to mechanical debridement to decrease the microorganisms to a level compatible with health.\textsuperscript{9,10,114,115,116} Laser therapy,\textsuperscript{110} photodynamic therapy,\textsuperscript{108-110} pumice,\textsuperscript{112} implantoplasty\textsuperscript{112} and air abrasives (glycine powder)\textsuperscript{104,106,117} have also been utilized in an attempt to remove biofilm from the rough implant surface. Saline has been suggested in combination with other
agents.\textsuperscript{112} There is a lack of evidence supporting any single approach for surface decontamination.\textsuperscript{118,119,120} Overall, these treatments have been found to be ineffective in removing the biofilm from the contaminated rough implant surface.\textsuperscript{9}

Rubbing the contaminated titanium surface with a cotton pellet soaked in sterile saline for 1 minute has been reported to reduce lipopolysaccharide levels significantly.\textsuperscript{112,121} Although saline solution has been proposed as part of many disinfectant protocols, few studies have assessed the effectiveness of saline solution alone. It is more frequently studied in combination with other agents. Dostie et al, describes the effectiveness of saline solution applied with a syringe to the contaminated rough implant surface \textit{in vitro}. Saline significantly reduced the bacterial load and none of the chemotherapeutic agents studied further reduced the bacterial contamination.\textsuperscript{10,9}

Schwartz et al was unable to demonstrate a significant impact of the method of surface disinfection of the clinical outcome following surgical therapy for the treatment of peri-implantitis when comparing disinfection with curettes and saline versus Er:YAG laser.\textsuperscript{119} The lack of impact in disinfection method is supported as well by Schou et al.\textsuperscript{122} In this animal study, no differences were detected among the application of air-powder abrasive, citric acid, saline, or chlorhexidine in different combinations when performing regenerative surgery in the treatment of peri-implantitis lesions.

Air polishing has been introduced as an alternative for the management of biofilm supra and subgingivally on natural dentition and dental implants. Air polishing with glycine has been found to be of advantageous in the prevention and management of peri-implant mucositis and peri-implantitis.\textsuperscript{106} In vitro studies have found that it can reduce the biofilm without causing pronounced changes to the implant surface.\textsuperscript{106}
Due to the adverse effect of chlorhexidine on the titanium implant surface, rendering it no longer biocompatible, it has been suggested that its use is no longer recommended for implant surface decontamination. Saline solution, citric acid and EDTA are suggested.\textsuperscript{123}

2.1.8 Success Rates

Froum et al\textsuperscript{124} reports elevated survival rates from 2-10 years (98.9%), bone gain, reduction in PD and BOP. The protocol includes open flap, debridement, disinfection with minocycline and chlorhexidine, saline spray, application of enamel matrix derivative (EMD) (Emdogain) or PDGF (GEM21), and guided bone regeneration (GBR = allograft and membrane). The need for 2 -3 retreatments for management of inflammation, suppuration or to halt progression of bone loss has been reported. In a 2014 systematic review, success rates for treatment of peri-implant mucositis and peri-implantitis vary from 0% to 100\%\textsuperscript{96}

2.2 Autologous Platelet-Rich Preparations

The ability to heal is one of the most important processes that an organism can perform and is essential to survival. Wound healing presents several stages: i. hemostasis; ii. inflammation; iii. proliferation; and iv. maturation. After injury, hemostasis, the first step in the healing process, is triggered. Epinephrine is released to minimize bleeding and platelets are deployed forming a plug. Fibrin, another major factor in coagulation and healing process, forms a clot.

In the 1970’s, the development of blood concentrates began with the creation of a surgical adjuvant rich in this component denominated fibrin glue.\textsuperscript{125} Fibrin glue (also called fibrin sealant or fibrin adhesive) is a protein-based product developed for tissue hemostasis and sealing.\textsuperscript{126} Currently, fibrin glue is prepared from platelet-poor plasma and there are several protocols for its preparation with variations in the composition of
Multiple blood concentrates rich in platelets have since been developed with one common goal: to reinforce the natural process of healing by enhancing every stage wound healing. Additional advantages of platelet concentrates are simple collection, easy bedside preparation, and clinical application without the risks associated with allogenic products due to their autologous nature.

Due to the import role of platelets in the healing process (as described in section 2.2.4), platelet-rich preparations have been developed for various applications. Platelet concentrates are blood extracts obtained after processing of a whole blood sample, mostly through centrifugation. These extracts are used as surgical adjuvants or regenerative medicine preparations in several medical fields, particularly sports medicine and orthopedic surgery and more recently in aesthetic plastic surgery. The objective of the processing is to separate the blood components in order to discard elements considered not to be beneficial in the healing process (mostly the red blood cells, which can be easily separated) and to collect and concentrate the elements that may be of use for therapeutic applications aimed at better healing outcomes (platelets, leukocytes and other forms of circulating cells; fibrinogen/fibrin, growth factors). Platelets contain high quantities of wound healing-related growth factors, such as PDGF-AB (platelet-derived growth factor AB), TGFβ-1 (transforming growth factor β-1) and VEGF (vascular endothelial growth factor), that are able to stimulate cell proliferation, matrix remodeling and angiogenesis.

There is great variability in the processing, definitions and classification of these preparations. The literature available highlights the lack of consensus in protocols, preparations and definitions and resulting in confusing and often contradictory data. Therefore, it is difficult for the clinician to determine which particular product offers greater clinical advantage in the area of oral surgery.
2.2.1 Classification

The current consensus is based on a simple classification system proposed in 2009 dividing the many products into four main families, based on their fibrin architecture and cell content (mostly presence of leukocytes): Pure Platelet-Rich Plasma (P-PRP); Leukocyte- and Platelet-Rich Plasma (LPRP); Pure Platelet-Rich Fibrin (P-PRF); Leukocyte- and Platelet-Rich Fibrin (L-PRF). These four families of products present different biological signatures and mechanisms, and differences in clinical application. Among these products, the L-PRF is the newest invention.

Pure Platelet-Rich Plasma (P-PRP)

In all PRP techniques blood is collected with anticoagulant just before or during surgery and is immediately processed by centrifugation. A first centrifugation step is designed to separate the blood into three layers: 1. Red blood cells (RBCs) found at the bottom; 2. Acellular plasma (PPP, platelet-poor plasma) at the top; 3. ‘Buffy Coat’ layer, rich in platelets, appears in the middle. The next steps vary among the numerous protocols but all attempt to discard the RBCs and the PPP, collecting only the ‘buffy coat’. Finally, the concentrate is applied to the surgical site with a syringe, together with thrombin and/or calcium chloride (or similar factors) to trigger platelet activation and fibrin polymerization. These products are, hence, without leukocytes and with a low-density fibrin network and can be liquid solutions or can be in an activated gel form. Two examples of PRP are the a manual method known under the commercial name PRGF [Plasma Rich in Growth Factors or Preparations Rich in Growth Factors or EndoRet, Biotechnology Institute BTI (dental implant company), Vitoria, Spain] and an automated method: Vivostat PRF (Platelet-Rich Fibrin, Vivostat A/S, Alleroed, Denmark).

Leukocyte- and Platelet-Rich Plasma (L-PRP)

The products are preparations with leukocytes and with a low-density fibrin network after
The initial objective of developing alternative easy-to-handle methods was to make it possible to use platelet concentrates in daily practice without having the support of a transfusion laboratory. Without a cell separator, elimination of leucocytes becomes more difficult, and the resulting platelet concentrates therefore, contain a high quantity of leucocytes. There are two general protocols for this technique:

**Manual protocols:**

This method consists of a first centrifugation step, which separates the blood components into three layers of RBCs, 'buffy coat' and PPP. The PPP and buffy coat layers are then carefully collected, avoiding RBC contamination, and transferred to another tube, where they are subjected to a second centrifugation step at high speed separating the sample again. After the second centrifugation step, most of the PPP layer is discarded manually. The PRP concentrate obtained with this method is composed of a high quantity of platelets, leucocytes and circulating fibrinogen and residual RBCs.

Curasan, Friadent-Schütze, Regen and Plateltex systems correspond to this protocol.

**Automated protocols:**

These protocols have been developed as PCCS (Platelet Concentrate Collection System) by 3I (Palm Beach Gardens, USA) and SmartPReP by Harvest Corp (Plymouth, USA). The automated techniques are similar to the manual protocol but involve less manipulation by the clinician. In PCCS, the separation of components after the first and second centrifugation is performed automatically using air pressure to move the desired components from one chamber to another. The SmartPreP system separates based on variations in weight and centrifugation speed.

Two other systems that are included in this classification are the Magellan APS (Autologous Platelet Separator) by Medtronic (Minneapolis, USA) and the GPS (Gravitational Platelet Separation System) by
Biomet Biologic (Warsaw, USA). PRGF developed by Anitua et al, is a minor variation of the L-PRP protocol consisting of a one-step centrifugation process.

**Pure Platelet-Rich Fibrin (P-PRF)**

The Fibrinet PRFM (Platelet Rich Fibrin Matrix) kit by Cascade Medical (New Jersey, USA), contains two tubes, one for blood collection and another for PRFM clotting, together with a transfer device. This protocol is similar to PRP protocols, with the main difference being the very low amounts of leucocytes collected due to the specific separator gel used. The fibrin matrix in Fibrinet PRFM is denser and more stable than that in PRPs.

**2.2.2 Leukocyte- and Platelet-Rich Fibrin (L-PRF)**

L-PRF preparations contain platelets, leukocytes within a high-density fibrin network and only exist in the form of dense, crosslinked membranes. These membranes are durable and strong and offer, therefore, a slow release of many growth factors for longer periods. The first of these techniques is Choukroun’s A-PRF protocol (classified as an L-PRF preparation), a simple technique developed in France. It is considered a second-generation platelet concentrate, different from previous protocols in that it is produced without any anticoagulants or gelifying agents. In this technique, three layers are produced by centrifugation, namely the red blood cell base layer, acellular plasma top layer and a PRF clot in the middle. The L-PRF clot forms a strong fibrin matrix in a complex three-dimensional architecture, in which most of the platelets and leucocytes from the harvested blood are retained and concentrated. Platelets and leukocytes are collected with high efficiency in this method.

A-PRF by Choukroun and CGF by Medifuge are examples of this kind of preparation.
In comparison to L-PRP, L-PRF has a greater release of TGF-β, a long-term release of growth factors, and stronger induction of cell migration.127

2.2.3 Biologic Functions

Various aspects of L-PRF membranes have been studied in an attempt to better understand the biological functions of these concentrates, including the role of platelets139 and leukocytes (particularly monocytes)140, the 3-dimensional architecture of the fibrin network132, growth factors (concentration, activity and release)141, and promotion of angiogenesis142.

Platelets

About 75 to 200 x 10^10 platelets are circulating in the blood stream of a healthy individual at any given time.11,143 They are discoidal, anuclear cell fragments formed in bone marrow from megakaryocytes. In spite of the multitude of functions attributed to platelets beyond the traditional processes of thrombosis and hemostasis,143,144,145 the recognition of these as cells is controversial due to their lack of a nucleus.146 Their life span is 8 to 10 days.139 Although neutrophils, basophils and eosinophils are considered to be the essential human granulocytes, platelets contain three types of granules143,147 that can be liberated intra- or extracellularly qualifying them as "granulocytes" as well.148

Dense δ-granules: contain mediators of vascular tone such as nucleotides (such as ADP and GTP), bioactive amines (such as histamine and serotonin) and bioactive ions (such as Ca^{2+} and PO_{3}^{-}).

Alpha α-granules: contain adhesion molecules, platelet microbicidal proteins (PmPs), kinocidins, mitogenic factors, coagulation factors and protease inhibitors.
Lysosomal \(\lambda\)-granules: contain enzymes including proteases and glycosidases.

The primary function of platelets is to patrol the vasculature and seal any breaches to the vessel walls to limit blood loss.\(^{11}\) In the early stages of wound healing, platelets are activated by the coagulation cascade. Activation results in the release of the contents of platelet cytoplasmic granules which contain at least 300 proteins\(^{145,147}\) including \(\beta\)-thromboglobulin, fibronectin, thrombospondin, fibrinogen, other factors of coagulation, growth factors, and immunoglobulins, all which contribute to the wound healing process. Cytokines with the capacity to stimulate cell migration and proliferation, events that are important in the first stages of healing, are also released.

Among the growth factors released by platelets are:

a. **Transforming growth factor-\(\beta\) (TGF-\(\beta\))**: TGF-\(\beta\) is a superfamily of more than 30 proteins. Of these, the most produced form in platelet concentrate is TGF\(\beta\)-1. In vitro, this growth factor has been found to have varying effects depending on the cell type and environment, for example, being able to stimulate or inhibit osteoblast proliferation. It exerts strong anti-inflammatory actions,\(^{139,142}\) serves as a chemoattractant for many cell types (such as monocytes and macrophages)\(^{144}\) and promotes matrix synthesis in osteoblasts and fibroblasts.\(^{139,142}\)

b. **Platelet-derived growth factors**: These are regulators of migration, proliferation and survival of mesenchymal cell lineages (such as osteoblasts and fibroblasts).\(^{10}\) These polypeptide growth factors are released from activated platelets during blood clotting and are powerful cytokines considered to play important roles in wound healing. PDGR-BB has been proposed to enhance blood vessel formation and mesenchymal stem cell/pericyte recruitment stimulating bone formation. PDGF-based products have been developed for clinical applications with some positive outcomes. Currently in clinical use are Regranex gel (Systagenix Wound Management Inc.) that has been
approved as an adjunct therapy for the treatment of diabetic neuropathic ulcers\textsuperscript{149} and GEM21S gel (Osteohealth) that has been approved for periodontal regeneration. GEM21S contains 1000 times as much platelet derived growth factor (PDGF) as platelet rich plasma.\textsuperscript{150}

c. **Insulin-like growth factor-I (IGF-I):** This growth factor is released from platelet alpha granules upon activation. It stimulates keratinocyte, fibroblast and osteoblast proliferation and matrix production.\textsuperscript{139}

In L-PRF, platelets have been found to accumulate in the lower part of the fibrin clot, mainly at the junction between the red clot and the L-PRF itself.\textsuperscript{132}

**Leukocytes**

The L-PRF clot contains more than 50\% of the leukocytes from the initial blood harvest.\textsuperscript{142} The presence of leukocytes has a substantial impact on the biology of these products due to their immune and antibacterial properties. PMN leucocytes are crucial cells during early healing to prevent infection and initiate the pro-solving stage of wound healing.\textsuperscript{129} Also they are considered turntables of the wound healing process and the local factor regulation.\textsuperscript{131} Supporters state that the effect on inflammation is beneficial, while opponents notice negative effects.\textsuperscript{133} Leukocytes have effects on the reduction/prevention of infection, on wound healing and on multiple tissues such as bone and fibroblasts. Some of these effects are achieved through signaling molecules such as interleukins and cytokines.

**Inflammatory cytokines**

a. **Interleukin-1\beta (IL-1\beta):** is a key mediator in inflammatory control. Stimulates T-helper lymphocytes.

Has effects on bone metabolism in combination with TNF-a, IL-1.\textsuperscript{140,142}

b. **Interleukin 6 (IL-6):** has multiple functions including B cell differentiation factor which induces the
final maturation of B cells into antibody-producing cells, stimulating the secretion of antibodies by 120–400 times. Also, IL-6 is essential for T-cell activation and proliferation.

c. **Tumor necrosis factor α (TNF-α):** stimulates tumor necrosis and regression. Bacterial endotoxin stimulates its release. TNF-α activates monocytes, stimulates remodeling capacities of fibroblasts, increases phagocytosis, neutrophil cytotoxicity and modulates the expression of key mediators such as IL-1 and IL-6.

**Healing cytokines**

a) **Interleukin 4 (IL-4):** induces differentiation of naive helper T cells into T_{H2} cells, supports proliferation and differentiation of the activated B cells and is a potent inducer of Interleukin-1 receptor antagonist (IL-Ra), which contributes to its anti-inflammatory actions by neutralizing the biological effects of IL-1.

b) **Vascular endothelial growth factor (VEGF):** is considered as a master regulatory molecule for angiogenesis-related processes. It is produced by multiple cells, including leukocytes, macrophages and platelets. Factors like IGF-I and IL-1b regulate angiogenesis by upregulating expression of VEGF.

Another potential property of L-PRF related to leukocytes is their antibacterial function. Neutrophils, the most common form of leukocytes, have strong phagocytic properties and are considered the first line of defense. The cytoplasm of the neutrophil granulocytes contains numerous granules:

a) **primary (azurophil) granules:** connected to bacterial destruction, containing multiple bactericidal factors including defensins, cathelicidins, serprocidins, bactericidal/permeability-increasing protein
(BPI) of gram-negative bacteria, myeloperoxidase and cytoplasmic calprotectin.

b) **Secondary granules**: are also rich in antibacterial proteins such as lysozyme, collagenase, gelatinase, lactoferrin, phospholipase A2, transcobalamin-I and membrane proteins.¹³³

**Fibrin Network**

Fibrin is formed from fibrinogen during blood clotting. Fibrinogen is present in plasma and platelet granules. Fibrinogen is transformed into fibrin and cross-linked to fibronectin by thrombin and factor XIII, in the presence of calcium ions and is the final product of the coagulation reaction. Fibrin-fibronectin copolymer constitutes the first wound matrix of the injured site and supports cell adhesion.¹³²

In Choukroun's A-PRF (classified as L-PRF)¹³⁰,¹³⁶, the platelets come into contact with negatively-charged glass tube walls and this activates the coagulation cascades. Fibrinogen initially is concentrated in the high part of the tube before it is transformed into fibrin. The fibrin clot descends to the middle of the tube between the red cells in the bottom and the acellular plasma at the top.¹³²

Fibrin is considered the natural guide of angiogenesis, support to immunity and guide for wound coverage. It also serves as a net to stem cells.

a) **Angiogenesis**: Several properties of L-PRF, including the fibrin structure, promote angiogenesis. Angiogenesis, cells and scaffold are major factors in tissue regeneration.¹²⁵ In an animal study, using a modification of Choukroun's technique, a PRF membrane was produced to study angiogenic ability on microvasculature both in vivo and in vitro. PRP and PRF were found to induce angiogenesis in this model.¹⁵¹ Several properties of L-PRF can potentially be involved in the promotion of angiogenesis. Properties proposed include the characteristics of the fibrin network¹³⁰,¹³⁷ and high levels of growth factors such as VEGF.¹²⁹ Promotion of angiogenesis in early wound healing will increase the arrival, via
blood flow, of multiple cells including stem cells and defense cells, further promoting wound healing and infection reduction.

b) **Immunity:** Fibrin and fibrinogen degradation products (FDP) stimulate the migration of neutrophils and increase expression of CD11c/CD18 receptor. This allows adhesion of the neutrophil to endothelium and fibrinogen, as well as transmigration of neutrophils. Phagocytosis of neutrophils and enzymatic degradation are also modulated by FDP.

c) **Wound coverage:** The fibrin matrix guides the coverage of injured tissues, affecting the metabolism of epithelial cells and fibroblasts. Epithelial cells at the wound margin lose their polarity and produce basal and lateral extensions toward the wound. Fibrin, fibronectin, PDGF and TGF-β are essential for modulation of integrin expression, fibroblast proliferation and cell migration.\(^{130}\)

### 2.2.4 Platelets in Microbial Defense

Under normal conditions platelets circulate in the bloodstream without interacting with endothelium or other blood cells such as leukocytes in spite of the diverse array of highly responsive membrane receptors. Upon tissue injury platelets are activated. At this point, their function in maintaining hemostasis and their contribution in wound healing become of the utmost importance. The antimicrobial functions of platelets and their role in antimicrobial defense have been greatly underappreciated in the past.\(^{152}\) Platelets are the first responders to vessel wall breaches placing them at the front line of antimicrobial host defense and in a key position to orchestrate immune responses.\(^{11}\) Following tissue damage platelets are activated. Due to this activation they can adhere, aggregate, form a fibrin mesh and release a wide array of substances.\(^{13}\) Platelets release granules rich in a plethora of proteins including growth factors, cytokines, inflammatory mediators and antimicrobial proteins.\(^{11,13,152}\) Also, platelets express receptors on their surface that are involved in platelet-bacteria interactions.\(^{11}\)
The primary function of platelets is to patrol the vasculature and seal any breaches to the vessel walls to limit blood loss. An association between platelets and bacterial infection has been suspected as early as 1887, as reported in Yeaman’s review. At that time Fodor described the bactericidal effect of heated sera. The molecule responsible for this effect was identified and named β-lysine. It was determined to be of platelet origin due to its presence in coagulated plasma and its absence in the other blood cells. In 1901, platelets again were described to have interaction with bacteria, due to the formation of aggregates by platelets when in contact with Vibrio cholerae.

More recently, it has been found that platelets are involved in the earliest detection of microbial pathogens, as well as, in the activation and recruitment of complementary host defenses, such as neutrophils. Platelets are capable of binding, aggregating and internalizing microorganisms. The binding of platelets to microorganisms may take place directly through the platelet Fc receptor or indirectly through plasma protein bridges. Platelets possess cytotoxicity functions allowing them to participate in the destruction of protozoal pathogens. Platelets have direct antimicrobial functions that are mediated by the secretion of antimicrobial effector molecules, including platelet microbiocidal proteins (PmPs) and kinocidins. Among PmPs are Platelet Factor 4 (PF-4) and fibrinopeptide B. Also released are chemotactic agents such as PDGF, 12-hydroxyeicosatetraenoic acid and platelet-derived histamine releasing factor, TGF-β; and antimicrobial peptides such as beta-lysin, neutrophil activating peptide and RANTES. Antimicrobial peptides (AMP) are essential elements of the innate immune system. Human defensins are a subfamily of AMP and kill microbes by destroying their cell membrane without the need of the adaptive immune system. These 4-5 kDa, open-ended, cysteine-rich peptides, classified as α- and β-defensins, were originally isolated from human skin. The expression of these antimicrobial peptides can be induced by different tissues under appropriate stimulation. Platelets have been found to release β-defensin 2.
Other antimicrobial properties of platelets include generation of oxygen metabolites, initiation and amplification of complement fixation in the presence of microorganisms and synergism with leukocytes in vitro.\textsuperscript{153,154} The ability of platelets to express a wide variety of potential bacterial receptors including complement receptor, FcyRII and Toll-like receptors which provide them with the capacity to participate directly and indirectly in microbial defense. Additional evidence supporting the importance of platelets in host defense includes the increase in susceptibility to and severity of infections in thrombocytopenia.\textsuperscript{153}

The presence of platelets in the setting of infections had historically been interpreted to suggest that platelets facilitate infection. Due to current and compelling evidence, platelets are presently believed to have an important role in antimicrobial host defense.\textsuperscript{144,145,147,152,153,155} In fact, they may also participate in diverse diseases such as atherosclerosis, autoimmune disorders, inflammatory lung and bowel disease, host-defense responses, sepsis\textsuperscript{147} and periodontal disease.\textsuperscript{156,157}

As a result of these antimicrobial properties, new clinical applications of PRP and other blood concentrates have been proposed.\textsuperscript{13}

\textbf{2.2.5 Current Applications of L-PRF}

Rational for the use of blood concentrates is the release of growth factors and other bioactive molecules released by platelets upon activation. Autologous platelet concentrates may possess anti-inflammatory and antimicrobial properties as beneficial effects on postoperative pain and infection have been reported.\textsuperscript{154} Although blood concentrates have been shown to have antimicrobial properties \textit{in vitro},\textsuperscript{12,13,154,158} the mechanisms of this antibacterial activity is still poorly understood.\textsuperscript{158}

There is a lack of consensus regarding the role of leukocytes in platelet concentrates. Some authors
mention they can increase the antimicrobial potential\textsuperscript{159}, regulate the inflammatory response and improve the scaffold, while others recommend removing the leukocytes due to concern regarding the secretion of hydrolases and pro-inflammatory proteases.\textsuperscript{154}

In dentistry, multiple applications have been suggested including the application of PRP to periodontal surgical sites due to its regenerative potential and its antibacterial effects;\textsuperscript{160} in extraction sockets;\textsuperscript{161,162} in periodontal\textsuperscript{163,164,159} and peri-implant\textsuperscript{165} defects; in sinus lift procedures, in conjunction with soft tissue grafting procedures;\textsuperscript{163,166,167} and in endodontic procedures.\textsuperscript{168}

Anwandter et al\textsuperscript{161} report benefits of L-PRF in socket preservation. In this human study, several L-PRF clots were placed in a fresh extraction socket and were covered by an L-PRF membrane. Volumetric bone changes were found to be comparable to those reported in other studies for xenografts and allografts.

In a systematic review, Castro et al\textsuperscript{163} report enhanced wound healing with increased pocket reduction, radiographic bone fill and clinical attachment level gain when compared to open flap debridement. In the context of furcation defects significant reductions in PD and increased clinical attachment gain and radiographic bone fill were also detected. When compared to connective tissue grafting procedure, comparable results in root coverage, clinical attachment gain and keratinized tissue width are reported. The results of a systematic review by Miron et al are consistent with these findings, although the lack of randomized trials and the existence of conflicting studies is highlighted.\textsuperscript{167}

You et al\textsuperscript{172} report improved re-osseointegration with PRP, when combined with autogenous bone, in the treatment of peri-implantitis in a dog model.
Chapter 3: Aims & Hypothesis

3.1 Aims

Although widely in clinical use with reported favorable clinical results, little is known about the effect of L-PRF on the treatment of peri-implantitis. This in vitro study on a peri-implantitis model will allow the evaluation of an important aspect of peri-implantitis treatment: disinfection of the contaminated rough implant surface by L-PRF.

The goal is to contribute knowledge that may lead to the establishment of evidence-based guidelines for the clinical application of L-PRF Medifuge (Silfradent, USA) preparations in the treatment of peri-implantitis.

3.2 Hypothesis

1. Application of L-PRF membrane will significantly reduce or eliminate bacteria when applied to a rinsed rough implant surface contaminated with multispecies biofilm.

The null hypothesis is that the application of L-PRF membrane will not further reduce the bacteria on the rinsed rough SLA® implant surface contaminated with multispecies biofilm.
Chapter 4: Materials & Methods

4.1 Implant Surface Biofilm Model

Biofilm model was performed as described in Dostie 2015.10,9 Sterile SLA® implant disks (5 mm diameter; 1 mm thick) (Straumann®, Basel, Switzerland) were rinsed in Phosphate Buffered Saline (PBS) and then coated with bovine dermal collagen type I (10 μg/mL collagen in 0.012 N HCl in water) (Cohesion, Palo Alto, CA). The coating process consisted of overnight incubation at 4°C in the wells of a 24-well tissue culture plate containing 2 ml of the collagen solution. After incubation, the discs were rinsed with 2 ml of sterile phosphate-buffered saline (Sigma-Aldrich, Saint Louis, MO). The implant discs were placed in the wells of a 24-well tissue culture plate containing 2 ml Brain-heart infusion medium (BHI). Each well was inoculated with dispersed dental plaque, collected from subgingival sites of healthy volunteers, containing a minimum bacterial cells concentration of $3.2 \times 10^7$ CFU/ml. The discs were incubated under anaerobic conditions (AnaeroGen; Oxiod, UK) at 37°C for 21 days changing medium once a week.

4.2 Treatment Protocol

A set of disks with mature biofilm was fixed (Baseline) ($n=\ldots$). Implant SLA disks with established mature biofilms were subjected to a saline rinse (0.9% NaCl, 12 ml) to remove loose microorganisms. A set of rinsed disks was fixed for SEM (see below) prior to any additional treatment (Control Group A). L-PRF membranes were prepared from volunteers’ peripheral venous blood donations, as described by manufacturer of Medifuge MF200 (Silfradent, Italy). Approval was obtained from the University of British Columbia Clinical Research Ethics Board (CREB). All volunteers signed informed consent in accordance with the Declaration of Helsinki. The blood donation was obtained by venipuncture with 21G x 3/4x12” butterfly
blood collection set (Vaculet, Exel International, USA) and collected into Serum Plus Blood collection 10 ml tubes (BD Vacutainer, USA). Blood donor inclusion criteria were: healthy, non-smokers, no medications. Donors who did not meet these criteria were excluded. 11 donors (5 males, 6 females) were included. Mean age was 33.81 year (SD±6.81, max=43 min=26). Multiple donors participated more than once. Donor information can be viewed in Appendices A and B. 3 tubes per donor were placed into the Medifuge MF200 until the completion of the automated CGF cycle (Table 1). The content of each tube was placed into the L-PRF preparation box and red corpuscule portion was separated. (Figs, 1, 2 and 3) The preparation box lid was placed over the L-PRF clot for 5 minutes, after which the flattened clots were divided into two portions: the cell-rich portion (buffy coat), closer to the red corpuscule base; and the cell-poor portion. (Fig. 4) Separation of L-PRF sections was performed preserving approximately 1 mm of the red blood cell base layer and then dividing the L-PRF portion in half. For test groups the cell-rich portion was applied to the surface of the SLA disk for 48 hours at 37°C under aerobic conditions in 2 ml DMEM (Dulbecco’s Modified Eagle Medium). As 3 tubes were obtained at each time of donation, test samples were done in triplicate. A group of disks was placed in DMEM for 48 hours without application of the L-PRF membrane (Control group B).

<table>
<thead>
<tr>
<th>Time</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 seconds</td>
<td>Acceleration</td>
</tr>
<tr>
<td>2 minutes</td>
<td>2,700 rpm/735 g</td>
</tr>
<tr>
<td>4 minutes</td>
<td>2,400 rpm/580 g</td>
</tr>
<tr>
<td>4 minutes</td>
<td>2,700 rpm/735 g</td>
</tr>
<tr>
<td>3 minutes</td>
<td>3,300 rpm/905 g</td>
</tr>
<tr>
<td>33 seconds</td>
<td>Deceleration and stop</td>
</tr>
</tbody>
</table>

Experiment 1: disks were treated with L-PRF for 48 hours, L-PRF membrane was removed and the disks were immediately fixed for SEM without rinsing.

Experiment 2: disks were treated with L-PRF for 48 hours, L-PRF membrane was removed, disks were rinsed with 12 ml saline solution and were fixed for SEM. (Illustration 1)
Figure 1. Tubes of volunteer blood after the centrifugation cycle in Medifuge MF200 (Silfradent, Italy) containing acellular plasma, fibrin clot (L-PRF), and red corpuscule base.

Figure 2. Content of tube placed into L-PRF preparation box. Fibrin clot (L-PRF) and red corpuscule base are present.

Figure 3. Red corpuscule base separated from fibrin clot (L-PRF).
Figure 4. L-PRF membranes in preparation box. Separation of cell-rich and cell poor portions.

Illustration 1. Flow chart of experimental procedures.
4.3 Sample Preparation for SEM

The disks were submerged into wells containing 1 ml of 0.1M piperazine-N,N’-bis(2-ethanesulfonic acid) PIPES (pH 7.4) (Sigma-Aldrich, Saint Louis, MO) for 2 minutes. PIPES was removed and followed by 1 ml of 2.5% glutaraldehyde in 0.1M PIPES (pH 7.4) for 30 minutes for protein fixation. The disks were again submerged in 1 ml of 0.1M PIPES (pH 7.4) for 5 minutes. Lipid fixation was performed with 1 ml of 1% osmium tetroxide in 0.1M PIPES (pH 6.8) for 60 minutes. For removal of the osmium solution disks were submerged into 2 ml of double distilled water 3 times for 5 minutes. Dehydration was performed by placing disks into wells in successive increasing concentrations of ethanol (EtOH; Electron Microscopy Sciences, Hatfield, PA) for 5 minutes each at 50%, 60%, 70%, 80%, 90% and 3 times 5 minutes at 100%. Once dehydrated, the samples were dried by the critical point drier (Tousimis Samdri®-795 Critical Point Dryer, Rockville MA, USA).

To facilitate coating, the disks were attached to metallic stubs using electrical conducting double-sided adhesive. In order to improve electrical conductivity, the contour of each disk and stud was painted with colloidal silver and was allowed to air dry. (Fig. 5) Samples were coated with 8 nm of iridium using the Leica EM MED020 Coating System (Leica Microsystems, Wetzlar, Germany).

4.4 Scanning Electron Microscopy (SEM)

Each sample was examined using scanning electron microscopy (Hitachi SU3500 Scanning Electron Microscope (SEM), Etobicoke ON, Canada). Images of the center of each disk were taken at a voltage of 1 kV at 2 magnifications providing an overview image with an 80 μm × 65.25 μm field of view representing a surface area of 4500 μm², and a detailed image with a 23.97 μm × 16.85 μm field of view representing a
surface area of 403.89 µm². These fields of view correspond magnifications of 1500 and 5000 times respectively. Additional select images were obtained with Helios NanoLab 650 Focused Ion Beam SEM, Oregon, USA. Assessment of the images was performed with ImageJ 1.47v software (National Institute of Health, Bethesda, MD).

4.5 Western Blotting

The rinsing solution from experiment 2 was collected and analyzed by Western Blot with anti-Platelet Factor-4 (PF-4). Saline solution served as negative control. Whole platelet lysate, known to contain PF-4, served as positive control. Predicted molecular weight is 11 kDa. The secondary antibody was conjugated with IRdye (1:10,000; LI-COR Biosciences).

4.6 Statistical Analyses

For experiment 1, for analysis of the presence of perforations of bacterial cell wall on test samples versus control B1 Fisher’s exact test was performed. Distribution of the number of residual bacterial per disk in Experiment 2 was assessed by Kolmogorov-Smirnov Goodness-of-Fit Test. As distribution was not normal, data was analyzed by Kruskal-Wallis test. Significance level was set at 0.05 and confidence interval at 95%. Pairwise analysis was performed. (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY) Effect size was measured by Cohen’s D.
Chapter 5: Results

A total of 92 disks were examined under SEM. Groups are as follows:

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Characteristics</th>
<th>Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control B1</td>
<td>Rinsed 12 ml, DMEM 48 hours</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Test 1</td>
<td>Rinsed 12 ml, 48 hours in DMEM with cell-rich portion of L-PRF</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Control B2</td>
<td>Rinsed 12 ml, DMEM 48 hours, 12 ml rinse</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Test 2</td>
<td>Rinsed 12 ml, 48 hours in DMEM with cell-rich portion of L-PRF, 12 ml rinse</td>
<td>12</td>
</tr>
</tbody>
</table>

In group Test 2, one disk in one set of triplicates was excluded. The disks present either a depression or a laser etched marking on the non-treatment side. The sample was detected to be upside down during L-PRF treatment.

Samples in Biofilm groups A (Fig. 6) exhibited biofilm consisting of coccoid and filamentous bacteria over a rough implant surface and served as a baseline. Control Groups A (Fig. 7), B1 and B2 exhibited a rough implant surface with primarily coccoid bacteria present in the pits of the rough implant surface.
Figure 5. SEM of baseline biofilm at 3 weeks consisting of coccoid and filamentous microorganisms on SLA implant surface. (Hitachi SU3500)

Figure 6. SEM of Control A. Residual coccoid microorganisms on SLA surface after 12 ml saline rinse. (Hitachi SU3500)

Figure 7. SEM of Control A. Residual coccoid microorganisms on SLA surface after 12 ml saline rinse. Higher resolution of center of disk in Figure 7. (Hitachi SU3500)
5.1 Test 1

A total of 24 L-PRF treated disks (without rinsing after 48-hour L-PRF treatment) were examined under SEM (Hitachi SU3500 Scanning Electron Microscope (SEM), Etobicoke ON, Canada). The purpose of test 1 was to determine whether any cells originating from the L-PRF were present at the implant surface after the 48-hour treatment and to evaluate the effect of L-PRF on the bacteria. Images of the center of the disks were obtained at 1500x and 5000x magnification. The images displayed cells measuring 4-7 µm presenting >20 finger-like extensions. Helios SEM images of these samples at high magnification (5000x-20,000x) displayed close contact between these cells and coccoid microorganisms. The microorganisms appeared larger than in control groups (>1 µm in test group 1 vs. <.5 µm in control) and present multiple perforations of the cell wall. Vesicles appeared to be released from the extensions of the larger cells. (Figures 8-10).

The presence of bacteria with perforations on biofilm, test 1 and control samples A and B was assessed. Bacteria detected to be displaying one or more perforations were counted as bacteria with perforations. While the SEM images of the samples in Biofilm group, group A and B presented bacteria, none of these samples presented bacteria with perforations. Biofilm group presented a total of 7785 bacteria, no perforations were detected. Control group A presented 1595 bacteria and no perforations were detected. Control group B presented 733 bacteria. No bacteria with perforations were detected in this group. Of the 24 samples in test group 1, 7 did not present bacteria. In consequence, these 7 samples could not be assessed for perforations. On the 17 remaining samples a total of 405 bacteria were present (168 presented perforations; 237 did not present perforations). (Fig. 11) (Table 3) (Illustration 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Total bacteria</th>
<th>Bacteria without perforations</th>
<th>Bacteria with perforations</th>
<th>% with perforations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm</td>
<td>9</td>
<td>7785</td>
<td>7785</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control A</td>
<td>8</td>
<td>1595</td>
<td>1595</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control B</td>
<td>4</td>
<td>733</td>
<td>733</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Test 1</td>
<td>17</td>
<td>387</td>
<td>219</td>
<td>168</td>
<td>43.4%</td>
</tr>
</tbody>
</table>

Note: Although there were 24 samples in Test group 1 bacteria was not detected on 7 images. These were excluded. The 17 samples on which bacteria was detected were included. Total bacteria in the summation of all disks in the corresponding group.
Figure 8. Experiment 1. SEM of Test sample of control A. Coccoid bacteria without perforations are present. (Hitachi SU3500)

Figure 9. Experiment 1. SEM of sample of Test. Coccoid bacteria with perforations are present. (Hitachi SU3500)

Figure 10. Experiment 1. SEM image of sample in Test group displaying multiple 4-7 µm “cells” with extensions. Coccoid microorganisms with perforations. (Helios SU3500)
Figure 11. Experiment 1. SEM image of sample in Test group displaying cells with extensions in close contact with perforated coccoid microorganisms. (Helios SU3500)

Figure 12. Experiment 1. SEM image of sample of Test group displaying cells with extensions and vesicles. (Helios SU3500)

Figure 13. Experiment 1. SEM of sample of Test group. Assessment of cells with perforations (yellow) and without perforations (orange). (Hitachi SU3500)
Illustration 2. Experiment 1: Bacteria on disks were assessed for presence of cell wall perforations. Test 1 = cell-rich L-PRF in 48 hours DMEM, Control B1 = 48 hours DMEM

Fisher’s exact test was performed for Test group 1 and Control B resulting in p<0.0001. (Illustration 3). The result was statistically significant. (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY) (Appendix C)

<table>
<thead>
<tr>
<th>perforations</th>
<th>cont</th>
<th>test</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>733</td>
<td>219</td>
<td>952</td>
</tr>
<tr>
<td>yes</td>
<td>0</td>
<td>168</td>
<td>168</td>
</tr>
<tr>
<td>Total</td>
<td>733</td>
<td>387</td>
<td>1120</td>
</tr>
</tbody>
</table>

Illustration 3. Contingency table for Fisher’s exact test. (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY)

5.2 Test 2

Next, the biofilm contaminated disks were treated with the L-PRF membraned and rinsed after the 48-hour treatment to quantify the residual bacteria on the disks. The images of 35 L-PRF treated disks were used for bacterial counts. Counting was performed on ImageJ. The mean number of residual bacteria was 12.65 (SD 17.21) in the test group. In this group 13 disks displayed no residual bacteria. (Illustration 4) The baseline biofilm disks presented a mean of 865.00 bacteria (SD±455.71). Control group A presented a mean of 169.00 (SD150.71) and for control group B2, the mean was 151.25 (SD± 81.51). (Illustration 5) (Fig, 15-20)
Table 4. Experiment 2: Residual bacteria

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 2</td>
<td>12</td>
<td>12 ml saline rinse, Cell-rich L-PRF 48 hours</td>
<td>12.65 (SD±17.21)</td>
</tr>
<tr>
<td>Biofilm</td>
<td>9</td>
<td>Biofilm</td>
<td>865.00 (SD±455.71)</td>
</tr>
<tr>
<td>Control A</td>
<td>6</td>
<td>12 ml saline rinse</td>
<td>169.00 (SD±150.71)</td>
</tr>
<tr>
<td>Control B2</td>
<td>12</td>
<td>12 ml saline rinse, 48 hours DMEM</td>
<td>151.25 (SD±81.51)</td>
</tr>
</tbody>
</table>

Illustration 4. Experiment 2. Chart of Residual bacteria on samples in Test and Control B.

Figure 14. Experiment 2. SEM of sample of baseline Biofilm. (Helios SU3500)
Figure 15. Experiment 2. SEM of sample of control group A. 12 ml saline rinse. (Helios SU3500)

Figure 16. Experiment 2. SEM of sample of control group B. 12 ml saline rinse and 48 hours in DMEM. (Helios SU3500)

Figure 17. Experiment 2. SEM of Test group sample. Cell-rich L-PRF. (Helios SU3500)
Figure 18. Experiment 2. SEM of sample of test group. Cell-rich L-PRF. Cell count performed with ImageJ. (Hitachi SU3500)

Figure 19. Experiment 2. SEM of sample of control group. Cell-rich L-PRF. Cell count performed with ImageJ. (Hitachi SU3500)

Illustration 5. Experiment 2: Box plot of test group and control. (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY)
The data was determined to be not normally distributed by Kolmogorov-Smirnov Goodness-of-Fit Test (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY). (Appendix 4). Statistically significant differences were found between Baseline (Biofilm) and Control A when analyzed by Mann-Whitney U (p<0.001). The initial 12 ml rinse significantly reduced the bacterial load as compared to baseline. Data for test and control groups was analyzed by Kruskal-Wallis and the difference was found to be statistically significant (p<0.001) (Illustration 6). Pairwise analysis determined that there were no statistically significant differences between Control groups A and B. Values were adjusted by Bonferroni correction for multiple factors. Control groups A and B presented statistically significant differences with Test group (Illustration 7). There was no statistically significant reduction in bacterial load due to the placement of samples in DMEM for 48hrs. Cohen’s D was performed using mean and standard deviation of Control group B and Test. Effect size was determined to be high (d=2.3528).

<table>
<thead>
<tr>
<th>Null Hypothesis</th>
<th>Test</th>
<th>Sig.</th>
<th>Decision</th>
</tr>
</thead>
<tbody>
<tr>
<td>The distribution of bacteria is the same across categories of group.</td>
<td>Independent-Samples Kruskal-Wallis Test</td>
<td>.000</td>
<td>Reject the null hypothesis.</td>
</tr>
</tbody>
</table>

Asymptotic significances are displayed. The significance level is .05.

Illustration 6. Experiment 2: Kruskal-Wallis Test, significance. (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY)

<table>
<thead>
<tr>
<th>Sample1-Sample2</th>
<th>Test Statistic</th>
<th>Std. Error</th>
<th>Std. Test Statistic</th>
<th>Sig.</th>
<th>Adj.Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test-Control A</td>
<td>13.583</td>
<td>4.01</td>
<td>3.086</td>
<td>.002</td>
<td>.006</td>
</tr>
<tr>
<td>Test-Control B</td>
<td>15.083</td>
<td>3.594</td>
<td>4.197</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>Control A-Control B</td>
<td>-1.500</td>
<td>4.01</td>
<td>-.341</td>
<td>.733</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Illustration 7. Experiment 2. Kruskal-Wallis Test, pairwise comparisons. Significance values have been adjusted by the Bonferroni correction for multiple factors (IBM SPSS Statistics for Mac, Version 24.0; Armonk, NY)

The size of the cells detected on the implant surface (in direct contact with the bacteria) was consistent with the known size of platelets. The saline solution used for rinsing Test group 2 was collected and analyzed by Western blot for anti-platelet marker antibody (PF-4) to confirm that platelets were present.
on the disks. Western blot analysis of the rinse fluid showed presence of anti-PF-4 (Figure 21) which is consistent with the presence of platelets.

Figure 20. Western blotting with anti-Platelet Factor 4 antibody (PF-4 antibody) for rinse fluid from L-PRF treatment of SLA disks, positive control and negative control.
Chapter 6: Discussion

The use of implants to support dental prosthesis with the objective of restoring oral function and esthetics after tooth loss has become a common treatment modality with significant scientific evidence. Despite high success rates, these treatments are susceptible to mechanical and biological complications. These complications can impact the longevity of the prosthesis and of the implants themselves. Peri-implant mucositis and peri-implantitis are examples of biologic complications. Reported prevalence of these diseases varies greatly between studies due to a lack of consensus on definition criteria. None the less, prevalence and incidence are considered to be alarmingly high. Consensus does exist, however, on the important impact peri-implant disease can have. If left untreated, the bone loss that accompanies peri-implantitis can lead to failure of the dental implant and of the overlying prosthesis. Unfortunately, current treatment modalities have had limited success in halting the progression of bone loss. It has been hypothesized that one of the causes of poor success rates is the rough implant surface.

The introduction of the rough implant surface increased survival rates of dental implants dramatically. Modern implant surfaces allow for shorter healing times, increased bone to implant contact and the incorporation of treatment modalities that differ considerably from the original Brånemark protocol, such as the immediately provisionalized single implant. The rough implant surface is designed to be submerged within the bone. When the rough surface becomes exposed to the oral cavity it can act as a niche for complex biofilm capable of causing inflammation and further bone loss in the susceptible patient. There are many causes that can lead to exposure of the rough implant surface. Among these causes are iatrogenic factors such as poor implant placement, poor surgical technique, poor prosthetic design and presence of residual cement; environmental factors such as smoking; and host factors which can be genetic or acquired. Poor oral hygiene is an important risk factor. Irrespective of the cause for the initial exposure of the implant to the oral cavity, the rough implant surface becomes a niche for dental plaque. Management of this bacterial contamination is considered to be an important part of the treatment of peri-implant diseases.
Another factor to consider when selecting a treatment for implant surface disinfection is the effect this treatment will have on the implant surface. The ideal outcome after peri-implantitis treatment is re-osseointegration consisting of new bone to implant contact in the area of bone loss. There is limited information regarding the effect of chemical and mechanical treatments on the titanium implant surface and the effect of these treatments on re-osseointegration.

L-PRF has been reported to reduce postoperative infection rates. The two main cellular components of L-PRF, leukocytes and platelets, have important roles in host defense. The premise of this study was based on the biologic plausibility that these components or their products, applied to the contaminated implant surface could have the potential to reduce the bacterial count without causing significant damage to the implant surface.

For these reasons, this biologic implant surface disinfection protocol has been proposed. No other studies in which the cell-rich portion of the L-PRF membrane was applied to a contaminated implant surface were identified. Only one study was identified in which an autologous blood product, PRP (described as an autologous platelet-rich fibrin glue) was utilized in the treatment of experimental peri-implantitis. The use of PRP was proposed due to the platelet release of growth factors and the dense fibrin clot which facilitates management of the bone particulate. Improved re-osseointegration was detected in this animal study compared to the non-PRP test groups as determined histologically 6 months after regenerative implant treatment. The new evidence from this L-PRF in vitro study suggests that the favorable results obtained in the animal model may not only be due to the positive effects on wound healing of PRP. The favorable results may be produced by the bacteriolytic effects of the autologous platelet concentrate as well.

The biofilm model used for this study was developed and previously studied by Dostie et al, 2017. Incubation was performed at 37°C in anaerobic conditions mimicking the microenvironment present in the peri-implant crevice. Although the in vivo oral cavity is more complex, this biofilm model is an improved representation of the contaminated implant surface compared to the planktonic or immature biofilm used in
other implant decontamination studies. Mouhyi et al, developed a technique in which failed explanted implants were used to test decontamination techniques. This could potentially offer a more complex initial biofilm.

SEM microscopy of the mature biofilm at 3 weeks demonstrated a complex biofilm composed of a combination of coccoid and filamentous bacteria, as well as spirochetes. Disruption of this biofilm with saline solution (0.9% NaCl) reduced the biofilm to a primarily coccoid bacteria present in the lacunae of the rough surface. Similar results are described by Dostie et al, 2017, which also demonstrated that chemical agents cannot remove these residual bacteria. Other in vitro studies have also confirmed limited efficacy of chemotherapeutic agents in removal of biofilm.

Two experiments were conducted to examine the effects of the cell-rich portion of the L-PRF membrane on the residual bacteria on the rinsed rough implant surface. The first experiment was geared toward examining the effect of the membrane on the bacteria themselves; and the second allowed for evaluation of the reduction in residual bacteria after the L-PRF treatment.

Of note for experiment 1 are the presence of cells with extensions; and secondly, the presence of enlarged bacteria with multiple perforations. In several images, the release of vesicles from the extensions was also detected. Additional cells known to be present in L-PRF, such as leukocytes, which were not detected in the images may have been present during the treatment with L-PRF. It possible that some elements were washed away by the rinsing procedures and submergence of the disks in multiple preparation and fixation solutions.

Due to the size, shape, and the positive Western blot analysis, it was concluded that the cells with extensions are activated platelets. It is not known what could cause this level of activation of the platelets. It is possible that it is consequence of the L-PRF preparation itself, contact with the implant surface, contact with the bacteria or a combination of these. It had been proposed that the ability of L-PRF to reduce
infection (up to 9.5 fold in extraction sites) is due to the increase in white blood cells and macrophages capable of fighting infection; and that the use of L-PRF offers some antibacterial defense against incoming pathogens. The evidence presented in this study does not exclude a beneficial role of the leukocytes present in the cell-rich portion of the L-PRF membrane, but rather highlights that platelets themselves may be taking the mainstage in the lysis of residual bacteria on the implant surface.

Consideration should be given to the fact that Western blot is a highly specific and sensitive test. When an antibody with high specificity is used, it will only attach to the protein in question. The positive Western blot analysis indicates that platelets were present in the rinsing solution but it does not indicate directly that the cells detected on the SEM images are platelets nor does it rule out the presence of other cell types. Other cells could have been involved in activation of the platelets or the bacteriolytic effect detected on the samples.

With regard to the presence of enlarged bacteria presenting multiple perforations, direct contact between the platelets and some of these perforations was detected in the SEM images. Platelets have been found to participate in host defense in many ways including direct contact, release of antimicrobial proteins and reactive oxygen derivatives, internalizing pathogens, activation of other cells such as neutrophils and initiation of complement. This study was performed in vitro and in consequence, the bacteriolytic response is limited to the components of L-PRF. Other factors from the host that would participate in an in vivo defense process are not available in this setting.

One of the primary functions of the bacterial cell wall is to maintain the necessary levels of electrolytes. Damage to the cell wall, whatever the cause, leads to lysis due to the osmotic influx of water. The influx of water through the damaged bacterial wall leads to enlargement, as detected on the SEM images.
PmP1 and PmP2 (platelet microbicidal proteins 1 and 2) are examples of two platelet proteins capable of damaging the bacterial cell wall with the potential to lead to bacterial lysis. These are released under the induction of thrombin or bacteria. Once cleaved by thrombin, the two sub-units act autonomously but complementing each other and lead to alteration of the permeability of the bacterial wall. This is only one of the many mechanisms that may be at play in the cell-rich L-PRF samples involved in achieving the destruction of the bacterial wall. Other thrombin-releasable antimicrobial peptides from human platelets, which could potentially be involved in the antimicrobial effect of cell-rich L-PRF, include: PF-4, RANTES, connective tissue activating peptide 3 (CTAP-3), platelet basic protein, thymosin-4 (T-4), fibrinopeptide B (FP-B), and fibrinopeptide A (FP-A).

Host tissue toxicity has been described to be a side effect of the release of neutrophil defensins, while PmPs, on the other hand, have been hypothesized to have minimal host tissue toxicity due to their structural features. Unfortunately, platelets additionally can release β-defensin 2. This study does not provide evidence regarding the exact innate immune factors involved in the bacteriolytic effect or their side effects on host tissues.

An initial reduction in bacteria was achieved with the 12 ml saline rinse. The efficacy of this treatment was explored by Dostie et al. Due to the favorable effect achieved with saline as compared to other chemotherapeutic agents studied this step was incorporated into the present study.

In Experiment 1, platelets and bacteria with perforated cell walls were detected, in addition to the residual bacteria. For this reason, a 2ml rinse was incorporated into the protocol with the objective of removing loose debris including platelets and dead bacteria. In the clinical setting, this step of the protocol would not be recommended especially in the case of open flap procedures. A second surgery to access the area for a second time at 48 hours would not be reasonable. Fortunately, in vivo there is an important difference with this in vitro study: the host. The host defenses, including macrophages and neutrophils would be available to participate in the immune response and can potentially be called to the site through
chemotaxis. In lieu of the rinse, debris of cells that have undergone lysis could be removed from the implant surface in vivo through phagocytosis.

L-PRF is an autologous blood product, and as such, can vary from subject to subject eliciting varying responses. Substantial variation in the growth factor content of platelet concentrates, such as platelet-rich plasma, has been reported and the factors influencing this are unknown.\textsuperscript{176} Age and platelet count have been found to affect the growth factor levels of platelet concentrates.\textsuperscript{176} Gender, race and diet, are among the factors found to affect platelet functions in healthy subjects.\textsuperscript{177} Foods such as caffeine, alcohol and those rich in flavonoids such as chocolate, red wine and tea have been described to have an effect of platelet functions.\textsuperscript{178,179} Patients presenting peri-implantitis may have underlying diseases such as diabetes, or may be exposing themselves to environmental factors such as smoking. Poorly controlled diabetes, uncontrolled diabetes and smoking are considered risk factors for peri-implant diseases.\textsuperscript{22} Systemic disease, environmental factors, such as smoking, and medications may have an effect on platelet function. A classic example of a common medication that has effect on platelets is aspirin. Aspirin in known to cause platelet inhibition.\textsuperscript{180} The effects of aspirin, or any other medication, on L-PRF membranes and on the immune response of platelets is unknown. In this study only healthy non-smoking blood donors, taking no medications were included. The effect all these host factors have on L-PRF membranes is not understood, nor is the effect on platelets and their ability to participate in the innate immune response. There may be significant inter- and intra-individual variation on the characteristics of L-PRF membranes and platelet function; and the effect of this variation on the efficacy of cell-rich L-PRF membranes to disinfect contaminated implant surfaces is not known.

Our findings are consistent with the reported beneficial effect of platelet concentrates with regard to infection. Platelet concentrates been reported to have antimicrobial properties, and it has been proposed that they may represent a useful natural alternative for controlling or preventing postoperative infections at surgical sites.\textsuperscript{154} In vitro studies have also brought to light the antimicrobial properties of platelet-rich concentrates.\textsuperscript{12}
Experiments 1 and 2, were both conducted on the SLA implant surface. This is only one of the many commercially available implant surfaces on the market. It is feasible that the biofilm formation, platelet activation and subsequent disinfection present distinct features on other implant surfaces.
Chapter 7: Conclusions & Future Directions

7.1 Conclusions

Application of the cell-rich portion of the L-PRF to the contaminated SLA surface leads to platelet activation and to damage to the cell wall of residual bacteria.

Application of the cell-rich portion of the L-PRF membrane to the contaminated SLA implant surface further reduced the bacterial count as compared to 12 ml saline rinse in this in vitro model. Autologous L-PRF has potential as a biological means to decontaminate rough implant surfaces, possibly by exploiting the antimicrobial effects of platelets.

7.2 Future Directions

This in vitro study, as such, has limitations. Further in vivo studies are required to determine if this effect can be replicated in the clinical setting. If reduction in the bacterial load can be achieved in vivo further research would be required to determine if the improved disinfection of the implant surface has an impact on the success of peri-implantitis treatment and if this disinfection protocol can lead to re-osseointegration. The potential negative side effects of the immune response, such as tissue toxicity should also be investigated.

Further investigations would be required to determine the exact mechanism of the bacteriolytic process detected. If this process is, in fact, due to platelets it is possible that a specific platelet product or group of products is responsible. If so, it may be feasible for the substrates involved to be synthesized or extracted from platelets for use in implant disinfection or other settings involving bacterial contamination or infection.
Research would be required to determine if the immune response at play in the destruction of bacteria has, as well, potentially adverse effects on the host tissues such as toxicity.

Other benefits of L-PRF membrane have been described in the literature, especially in with regards to wound healing. Reduced postoperative discomfort and improved wound healing have been associated with the use of L-PRF membranes. The favorable effects of L-PRF on wound healing could potentially have a positive impact on peri-implantitis therapy. Studies to assess the benefits of L-PRF membrane on wound healing after peri-implantitis therapy are needed.

It is also possible that the bacteriolytic effect of L-PRF is not limited to this blood concentrate. The evidence obtained in this study suggests that this effect is a result of the innate immune functions of platelets. Platelets are found in high concentrations in P-PRP, L-PRP and P-PRF, as well as other commercially developed L-PRF products. Further studied are required to determine if other products can produce similar results or may even have enhanced effects on implant disinfection and wound healing.

L-PRF in an autologous blood product. Inter-individual variation can be expected. The effect of inter-individual variation in the disinfectant potential of cell-rich L-PRF and platelets is unknown. Further studies are needed to determine the effect of individual variation.

Other implant disinfection protocols used in the treatment of peri-implantitis have been suggested, such as air abrasives (glycine powder), photodynamic therapy and laser. These disinfections protocols should be examined and compared to the proposed biologic disinfection protocol.

In this study, the SLA implant surface was used as a substrate. Further research is required to determine if a comparable response presents on other implant surfaces.


Appendices

**Appendix A:** Experiment 1. Donor and Test Sample Data

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Bacteria with perforations/total bacteria</th>
<th>Mean</th>
<th>Gender</th>
<th>Age</th>
<th>Donor</th>
</tr>
</thead>
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<td>16/20</td>
<td>17.66/20 F</td>
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<tr>
<td>3</td>
<td>14/16</td>
<td>17/20</td>
<td>8/9</td>
<td>13/15 F</td>
<td>37</td>
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<tr>
<td>4</td>
<td>1/19</td>
<td>0/18</td>
<td>1/2</td>
<td>0.67/13 F</td>
<td>32</td>
</tr>
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<td>5</td>
<td>0/0</td>
<td>15/20</td>
<td>14/25</td>
<td>14.5/22.5 M</td>
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<td>6</td>
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<td>3/42</td>
<td>0/0</td>
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<tr>
<td>8</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>All samples excluded M</td>
<td>32</td>
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* Multiple donors participated more than once and can be identified by repeated donor number. Samples presenting no bacteria could not be assessed for perforations. These were excluded.
Appendix B: Experiment 2. Donor and Test Sample Data

<table>
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<th>Sample numbers</th>
<th>Residual Bacteria per sample</th>
<th>Mean</th>
<th>Gender</th>
<th>Age</th>
<th>Donor*</th>
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<td>3</td>
<td>F</td>
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<td>27</td>
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<td>0</td>
<td>9.66</td>
<td>F</td>
</tr>
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<td>4</td>
<td>0</td>
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<td>0</td>
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<td>F</td>
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<td>0</td>
<td>F</td>
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<td>8</td>
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<td>0</td>
<td>4.66</td>
<td>M</td>
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<td>99</td>
<td>1</td>
<td>5</td>
<td>35</td>
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<td>53.66</td>
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<td>8</td>
<td>5.5</td>
<td>F</td>
</tr>
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</table>

*Multiple donors participated more than once and can be identified by repeated donor numbers. Donor number are continued from Appendix A. Donor 4 appears with age 32 and age 33 due to birthday between donations. Donor’s age at time of first donation was used for donor descriptives (mean, standard deviation). Excluded sample was found to be upside down during treatment.
### Case Processing Summary

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<th>Valid Percent</th>
<th>Cases Missing N</th>
<th>Cases Missing Percent</th>
<th>Total N</th>
<th>Total Percent</th>
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<td>0.0%</td>
<td>1120</td>
<td>100.0%</td>
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### perforations * group Crosstabulation

**Count**

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<th>group</th>
<th>cont</th>
<th>test</th>
<th>Total</th>
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</thead>
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<td>168</td>
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### Chi-Square Tests

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<th>Asymptotic Significance (2-sided)</th>
<th>Exact Sig. (2-sided)</th>
<th>Exact Sig. (1-sided)</th>
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</thead>
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<tr>
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<td>.000</td>
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<td>Continuity Correction\textsuperscript{b}</td>
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<td>Fisher's Exact Test</td>
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<td>.000</td>
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<tr>
<td>N of Valid Cases</td>
<td>1120</td>
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\textsuperscript{a} 0 cells (0.0%) have expected count less than 5. The minimum expected count is 58.05.

\textsuperscript{b} Computed only for a 2x2 table
Appendix D: Statistical Analysis Experiment 2

<table>
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<tr>
<th>Group</th>
<th>N</th>
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<th>N</th>
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</thead>
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</tr>
<tr>
<td>Control A</td>
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<td>0.0%</td>
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<tr>
<td>Control B</td>
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<td>0.0%</td>
<td>12</td>
<td>100.0%</td>
</tr>
<tr>
<td>Test</td>
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</tr>
</tbody>
</table>

### Descriptives

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<tr>
<td></td>
<td>Upper Bound</td>
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</tr>
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</tr>
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### Tests of Normality

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* This is a lower bound of the true significance.

a. Lilliefors Significance Correction