Non-invasive measurements of oral mucosa blood flow in patients with various clinical conditions

PhD Thesis

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1 List of Abbreviations

BF – Blood Flow

CAF – Coronally Advanced Flap

CMBC – Concentration of Moving Red Blood Cells

CR – Coefficient of Repeatability

CTG – Connective Tissue Graft

CV – Coefficient of Variation

DALY – Disability Adjusted Life Years

DM – Diabetes Mellitus

DPU – Doppler Perfusion Unit

ECM – Extracellular Matrix

EXP. – Experiment

GBF – Gingival Blood Flow

GCF – Gingival Crevicular Fluid

GFPA – Gingival Flux Pulse Amplitude

GRD – Gingival Recession Depth

GRW – Gingival Recession Width

ICC – Intraclass Correlation Coefficient

JE – Junctional Epithelium

KT – Keratinized Tissue

LDF – Laser Doppler Flowmetry

LDI – Laser Doppler Imager

LSCI – Laser Speckle Contrast Imaging

LSPU – Laser Speckle Perfusion Unit

MAP – Mean Arterial Pressure

MARTD – Multiple Adjacent Recession Type Defects

MAX – Maximum Absolute Change

MCAT – Modified Coronally Advanced Tunnel

OPS – Orthogonal Polarized Spectral

PS – Periotron Scores

PRP – Platelet-rich Plasma

RBC – Red Blood Cells

REC – Recession Depth Reduction

RT – Recovery Time

RW – Recession Width Reduction

SDF – Sidestream Darkfield

SE – Standard Error

VEGF – Vascular Endothelial Growth Factor

2 Preamble

Microcirculation studies have had a long tradition at the Department of Conservative Dentistry, established by Professor Árpád Fazekas. This provided an opportunity to gain further knowledge in this field of science and planted the seed for further research. At the same time, new techniques in periodontology and oral surgery have developed, enabling graft materials and implant placement to become widespread in dentistry. The predictability of outcomes following surgical procedures has a fundamental importance in medicine, therefore, success can only be achieved if biological aspects and regeneration are taken into consideration. It has become inevitable to be familiar with the oral aspects of wound healing to be able to intervene in the process if difficulties occur. This requirement resulted in a demand for monitoring wound healing in an objective manner in addition to subjective methods. Because of the widespread application of surgical interventions, the observations were necessarily extended to various diseases and harmful behaviors, e.g. Diabetes Mellitus (DM), bisphosphonate-related osteonecrosis of the jaw (BRONJ) or smoking.

In the third year of my residency status at the Department of Conservative Dentistry, János Vág PhD offered me the opportunity to become a member of an emerging working group. As part of this group, I developed a particular interest in wound healing and microcirculation. Having been accepted to the Dental Research Program led by Professor Gábor Varga of the Semmelweis University School of Clinical Medicine, the initial objective of my PhD research was to investigate a new non-invasive method for measuring microcirculation in the oral cavity. After setting up a physiological test with Laser Doppler Flowmetry (exp. I–III) and getting familiar with the novel LSCI method for blood flow measurements (exp. IV–VII), I started to work with some of the best clinicians at Semmelweis University, including Bálint Molnár PhD from the Periodontal Department (exp. VIII).

3 INTRODUCTION

3.1 The significance of the vasculature in periodontal tissues

Periodontitis is a ubiquitous chronic inflammatory disease, initiated by the accumulation of pathogenic dental plaque biofilm above and below the gingival (gum) margin, and by microbial dysbiosis leading to a chronic nonresolving and destructive inflammatory response (1, 2). 75% of the population is affected based on a survey under the Global Burden of Disease 2010 study, which was compiled by the Institute for Health Metrics and Evaluation on a sample of 11% of the Earth's population (3). The frequency of severe periodontitis increases with age, and among 35–59 year-olds, it is the world's leading contributor to disability adjusted life years (DALY). It is also the major cause of tooth loss, nutritional compromise, altered speech or low self-esteem (4). Twenty years ago, 95% of the Hungarian population suffered from some type of periodontal disease (5) and that ratio was still 88% in 2009 (6). In addition to the local effects of periodontitis, it involves a significant risk factor for systemic diseases such as coronary heart disease, stroke, diabetes and osteoporosis (7, 8). In view of this, better understanding of the pathomechanisms of periodontitis and the development of treatment options are indispensable.

During inflammation, the bacterial biofilm adhering to the teeth produces bioactive substances (9), which induce a local immune response in the gingiva. The inflammatory reaction also involves local microvascular changes (10, 11), which also play a role in the progression of periodontitis according to some studies (12, 13). Several epidemiological studies confirm that there is a two-way relationship between periodontitis and diabetes mellitus (DM) (14-18). The pathomechanism in this interplay is not known. Considering the presence of severe microvascular damage in DM, it may be possible that the progression of periodontal disease is promoted by vascular effects (15, 19). There is very little data available on microcirculatory changes in periodontitis in diabetic patients, but animal experiments indicate a significant relationship (20).

Periodontal therapy frequently involves surgery. Taking the stages of wound healing into consideration, it is clear that blood flow is essential in this process. In the case of surgery, during the early-healing period, the transport of cellular and humoral factors, such as platelets, immune cells, oxygen and nutrients are critical to achieving an optimal process.

Reperfusion of the flap is a determinant of avoiding partial flap necrosis, especially in areas located distally from the flap basis (21). Postoperative reperfusion of the flap ultimately depends on the preservation of microcirculatory inflow (22). Nutrition is supplied to the distal part of the mucosal flap through extravasation in the early ischemic period of healing (23). Newly formed blood vessels in the provisional granulation tissue re-establish the microvascular network in the connective tissue and provide supply for healing (24).

3.2 The framework of wound healing

Wounds can be described as tissue disruption of normal anatomic structure with consecutive loss of function (25). Wound healing is a highly regulated process of cellular, humoral and molecular mechanisms which begins directly after wounding and might last for years. This complex process has been studied in great detail (26) and can be theoretically divided into three phases which overlap in time and space (Figure 1): inflammation (early and late phase), granulation tissue formation, and matrix formation and remodeling (27).

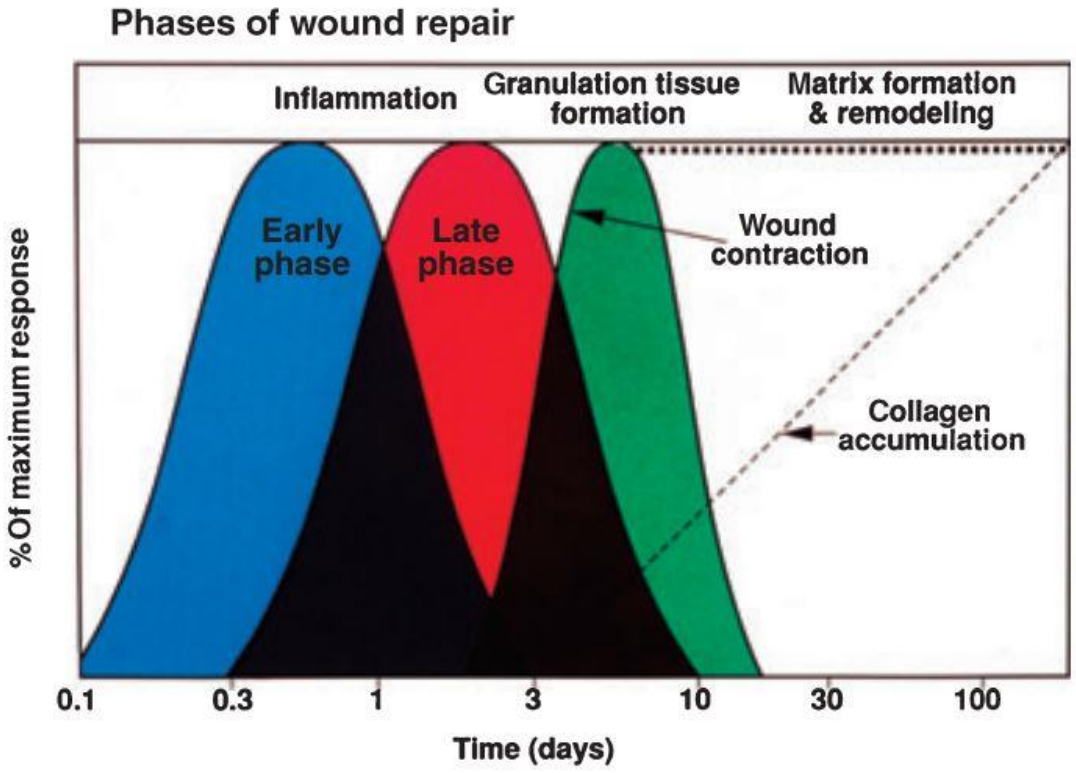

Figure 1: Phases of wound healing.

Model made for epidermal incisional wounds. The inflammatory phase (including an early and a late phase), granulation tissue formation and matrix formation & remodeling over time (27).

If an injury causes capillary damage and hemorrhage, blood clot is formed as the first step. This clot has two functions: it temporarily protects the denuded tissues and it serves as a provisional matrix for cell migration (28). Blood clot consists of all the cellular components of blood: fibrin molecules, fibronectin, vitronectin and thrombospondin, serving as a transitional matrix for the arriving leukocytes, keratinocytes, fibroblasts, endothelial cells and reservoirs for growth factors (29). Transient vasoconstriction is followed by vasodilation, with platelets overflowing the transitional matrix (30). Platelets produce cytokines and growth factors and attract leukocytes. Following this, leukocytes play a role in cytokine production as well, thereby stimulating collagen synthesis (FGF-2, IGF-1), the transformation of fibroblasts into myofibroblasts (TGF-β), the initiation of angiogenesis (FGF-2, VEGF-S, HIF-1α, TGF-β) and support for epithelialization (EGF, FGF-2, IGF-1, TGF-α) (31). This **inflammatory phase** can be divided into two phases: **early neutrophil invasion** and a **late phase** (31). Neutrophils are present after 2–5 days

of injury. Their phagocytic ability and protease secretion enables the destruction of the bacteria present and the breakdown of necrotized tissue. Protease secretion also acts as a chemoattractant for other inflammatory cells (32). About 3 days after injury (in the late phase), macrophages appear and they further support the process with phagocytosis, wound purification, growth factors and cytokines production (33).

The **proliferation phase, i.e. granulation tissue formation,** starts 3–10 days after the damage, when reepithelialization, angiogenesis and scar tissue formation begins. Reepithelialization is started at the wound edges by keratinocytes (34, 35, 30). Collagen synthesis increases on the entire surface of the wound (36). Molecular mediators of angiogenesis are VEGF, PDGF, FGF and serine protease thrombin, which bind to endothelial cells. The activated endothelial cells produce proteolytic enzymes to dissolve the basal lamina, which enables them to proliferate and migrate. The final step of the proliferation phase is scar tissue formation. Scar tissue is characterized by the accumulation of fibroblasts, granulocytes, macrophages, capillaries and collagen bundles. Fibroblasts have a decisive role in the formation of the extracellular matrix (ECM) (37, 38).

The last phase of wound healing is the **restorative phase, i.e. matrix formation and remodeling**. It begins around the 21st day and may take up to 1 year. Granulation tissue is eliminated by apoptosis. Components of the ECM undergo changes. Stronger collagen is formed. Once the collagen matrix has been synthesized, some fibroblasts undergo transformation into myofibroblasts and express α-smooth muscle actin. This transformation and synthesis is responsible for wound contraction (39, 33, 40). The time of angiogenesis is reduced and epithelialization is complete (31). In the skin, the tensile strength of the formed scar reaches 70% of the original tissue (26).

The healing of a wound serves the purpose of replacing it with similar, physiologically and functionally identical tissue. This process is called regeneration. Ideally, tissue deficiency is replaced by parenchymal cells; however, if total regeneration cannot be achieved for some reason, the parenchyma site becomes connective tissue scar, a process called reparation. The type of the damaged tissue and the nature of the injury determine which of the two processes occurs (41).

3.3 Wound healing in the oral mucosa

There are several types of tissues in the oral cavity, including soft tissues, muscles, bones, mucosa and teeth. In this milieu, wound healing and tissue regeneration require great coordination, since they involve structures that are close to each other in space, but are physiologically different (38). The general principles of healing and the associated cellular and molecular events were observed in non-oral sites, also applicable to the healing processes that take place following mucosal healing in the oral cavity. However, human oral mucosal wounds heal fast and with minimal scar formation as compared with the skin (42). This was demonstrated on experimental wounds created in the tongue and the buccal mucosa of rodents (41). Surgical wounds, especially in the oral keratinized attached gingiva and the palatal mucosa, heal with very little scar formation (43). Yet, there is a common belief (44) among clinicians alongside sparse documentation (45, 46) that improper incision and flap design may cause hypertrophic scar formation in the oral mucosa. However, this has not been systematically investigated.

The difference in the healing process is characterized by a lower inflammatory response in the mucosa. Studies have shown that in comparison to skin wounds, oral wounds exhibit lower neutrophil, macrophage and T-cell infiltration (47). Oral and skin wounds also exhibit differences in the expression of TGF-ß1, a pro-inflammatory, pro-fibrotic cytokine which has been implicated in the etiology of hypertrophic scars (48). Angiogenesis in oral wounds is less robust than in the skin (49) and the dominant mediator of wound angiogenesis, the production of Vascular Endothelial Growth Factor (VEGF), is significantly less pronounced in oral than in skin wounds (50).

Primary and secondary forms of wound healing are fundamentally different. Primary wound healing is usually observed in the oral cavity, with sharp, non-inflamed, clean wound edges. The epidermis perfectly covers the affected area, without reparation tissue. In secondary intention healing, wound edges do not meet and an inflammatory zone and tissue deficiency are found, with reparation scar tissue (51).

3.4 The effect of gender on wound healing

Clinical observations (52-55) and findings in an experimental excisional palatal wound model (56) suggest that mucosal wound healing is faster in males than in females. They

studied 3.5 mm circular wounds on the oral hard palate of 212 volunteers. Wound videographs were taken daily for 7 days after wounding to assess wound closure. Although all wounds were initially the same size, 24 hours after wounding, men had significantly smaller wounds. This difference was apparent until day 5 (Figure 2). In addition, the proportion of individuals considered healed was significantly higher among men than among women on days 5 and 6 (Figure 2). These results suggest that oral mucosal wounds heal more slowly in women than in men, regardless of age. Such gender differences in wound healing may be explained by sex hormones.

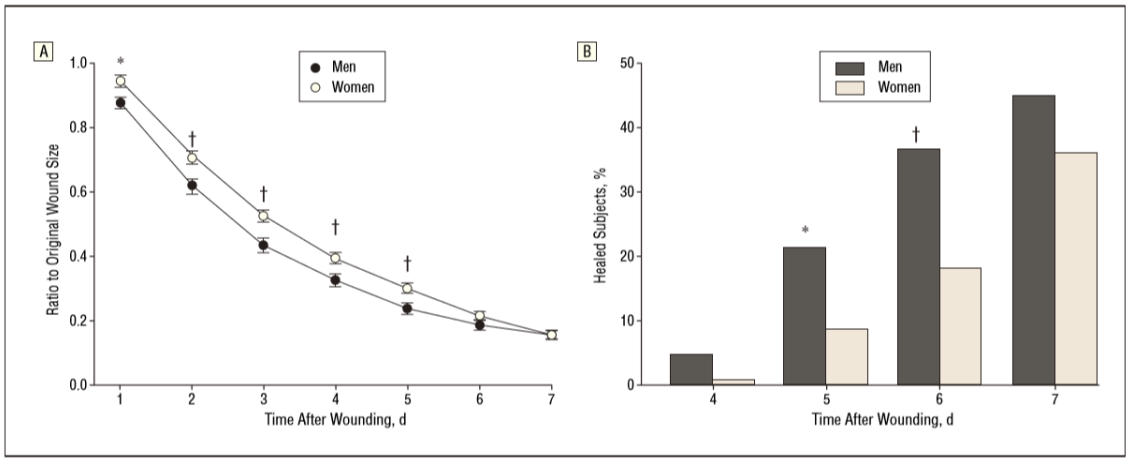

Figure 2: Gender-related changes in wound healing parameters.
Less wounds were considered healed in women on days 5 and 6 compared with men (56).

However, female hormones such as estrogens seem to have a favorable effect on acute wound healing in rodents and in the human skin (57, 58). In contrast to estrogens, testosterone delays wound healing in the skin (59-61). Just as in the case of cutaneous wound healing, testosterone levels negatively correlate with mucosal wound healing in adults, regardless of gender (62). In an ischemia reperfusion murine model, permeability and leukocyte infiltration in the intestinal mucosa increased more in males than in females (63). Overall, the aforementioned evidence suggests gender difference in oral mucosal wound healing, but the mechanism has not been clarified yet. Possible differences in the regulation of blood flow during healing cannot be excluded either.

3.5 Vascular changes in periodontitis

3.5.1 Changes in morphology and the number of vessels

Nuki and Hock studied the structure and organization of vessels in gingiva with no previous history of inflammation. They described a microvascular bed around the teeth, where capillaries predominated within the crestal gingiva and within the superficial buccal and crevicular networks. Precapillary arterioles and postcapillary venules were most common in the mid-gingival region. Small arterioles and venules were present in the apical gingiva (Figure 3).

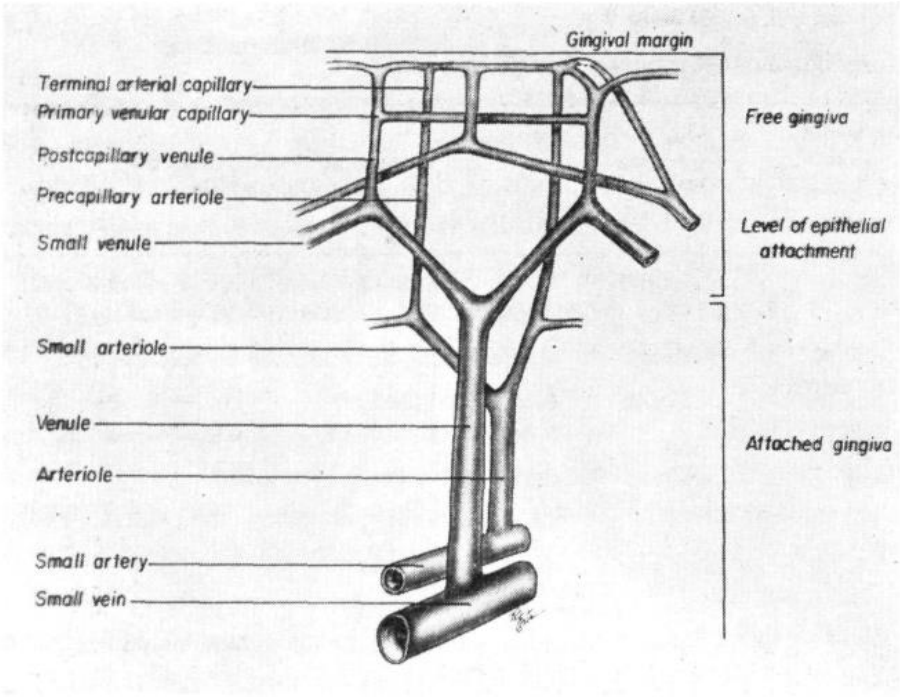

Figure 3: Drawing of the framework of the microvasculature.
Data were obtained from microfilm perfusion and serial tissue sections (64) (Nuki and Hock 1974).

Morphologic changes in the capillary units of the network were seen as plaque increased, but prior to clinical signs of inflammation. The width and the length of the vessels changed and also their morphology has become different. Loop formation was observed on the vessels (Figure 4). With continuing inflammation, certain connecting vessels were lost while other vessels became spatially rearranged (64).

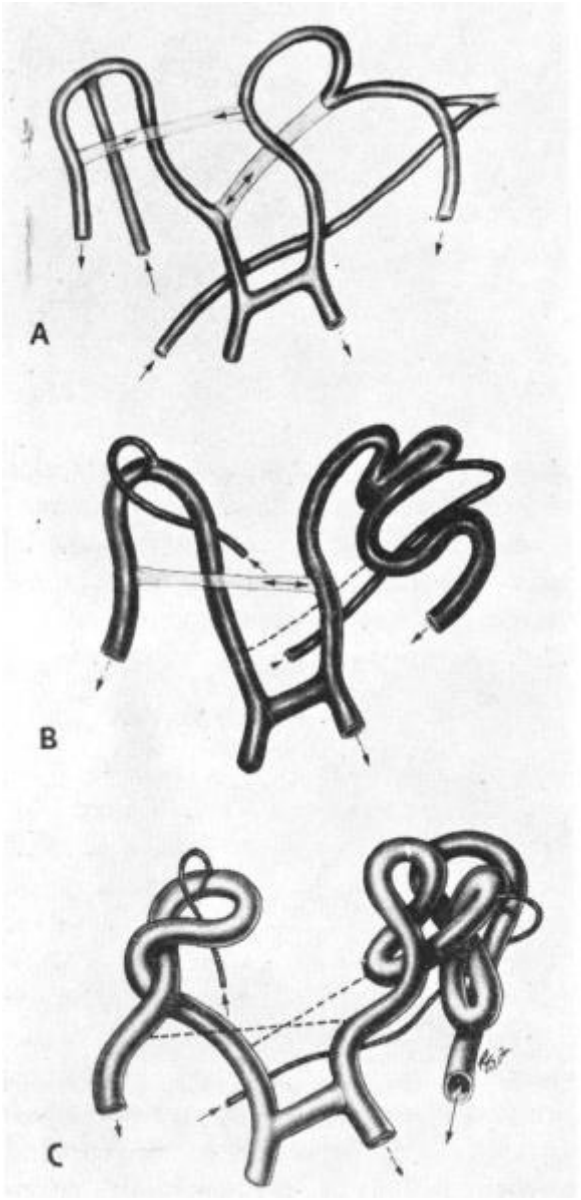

Figure 4: Illustration of the alteration of the vessels from a regular network to loops. A: regular network, B: intermediate state, C: loops (64).

Also, in 1974, Kennedy examined the effect of inflammation on the collateral circulation of the gingiva and the periodontal ligament on monkeys. In clinically healthy tissues, a vascular connection between gingival and periodontal vessels was seldom observed. Nevertheless, when inflammation occurred, vascularity and the number of connecting vessels increased (65).

The number of vessels connecting vessels of the periodontal ligament with supracrestal vessels was also significantly higher. There was a tendency for the number of vessels

perforating either the alveolar bone proper or the alveolar crest to increase when inflammation was induced. The results of this study demonstrate the development of collateral circulation from the periodontal ligament to the gingiva in response to inflammation and help to explain the extension of inflammation into the deeper structures of the periodontium, including the periodontal ligament. Vascular changes and remodeling in periodontitis were reported to affect the progression of the disease (12, 13). Some vascular changes including the dilation of vessels, the formation of tortuous looping structures and the development of columnar endothelial cells may promote the defense mechanism against bacteria, however, the formation of perivascular hyaline material and the accumulation of basement membrane rests may assist the progression of periodontitis (12).

Interestingly, the thickening of the basal lamina around microvessels occurs just as in the case of diabetes mellitus (DM) (66, 67). In DM patients, microvascular complications in other organs, e.g. neuropathy, nephropathy and retinopathy, were found to be associated with the presence of a more severe inflammatory pathology of periodontal tissues (68). A recent study (20) found decreased post-occlusive reactive hyperemia in the gingiva of a diabetic rat compared to a healthy one and this response was further reduced by experimentally induced periodontitis. However, there is no direct evidence for the impairment of vascular reactivity in the human gingiva in periodontitis and/or DM.

3.5.2 Change in blood flow

Studies investigating the effect of periodontal inflammation on basal gingival blood flow found conflicting results (Table 1).

Table 1: Studies investigating the effect of periodontal inflammation on basal gingival blood flow.

Author	Year	Title	Method	Tendency	Description
Hock JM, Kim S.	1987	Blood flow in healed and inflamed periodontal tissues of dogs	• radioisotope-labelled, plastic microspheres • teeth were ligated	I	Control teeth ✓ Spontaneous: decrease after treatment, induced Gingvititis: increase
Kaplan ML, et. al.	1982	Blood flow in gingiva and alveolar bone in beagles with periodontal disease	• radiolabeled microspheres • dental-plaque-inducing diet	I	„Spontaneous", pos. correlation with the severity
Baab DA, Oberg PA	1987	Laser Doppler measurement of gingival blood flow in dogs with increasing and decreasing inflammation	• LDF • dental-plaque-inducing diet	NS	Control teeth ✓ No decrease after treatment

Dog studies (69, 70) demonstrated **increased** blood flow in the inflamed gingiva, involving bone loss (a combination of gingivitis and chronic periodontitis). However, in the same species, Baab and Öberg (71) found **no significant** correlation between the gingival index, GCF and blood flow, and the elimination of the inflammation did not result in a decrease in blood flow either. In humans, experimentally induced gingivitis resulted in **decreased** blood flow to the gingiva (72, 73) whereas naturally occurring gingivitis resulted in **increased** blood flow (72). GBF at rest was found to be smaller in periodontitis patients compared to the reference subjects (74) and the treatment of gingivitis (75) or periodontitis (76) reduced blood flow. One possible explanation for conflicting results is variations in gingival blood flow as a function of time and the location of the laser Doppler probe. Temporal variation related to biological variation may be influenced by many physiological factors in addition to the inflammation, such as circadian rhythm (77), blood pressure (78), temperature (79) or tooth brushing (72, 80, 81). Furthermore, although no data are available about the effects of disinfectant mouth rinses, eating and drinking on GBF, they may also influence the recordings. That is why it is important to standardize these factors before and during the measurements. To better control the temporal and spatial variation of blood flow, it is useful to implement a provocation test on the gingiva, which is a relative functional measurement, instead of an absolute measurement.

3.6 Vascular changes in the periodontal flap

3.6.1 Changes in morphology and the number of vessels

Histological observations suggested that tissue revascularization begins in mucosal flaps as early as **2–3 days** postoperatively (82, 83).

Mormann et al. (84) demonstrated by fluorescence angiography that mucoperiosteal flap circulation dropped by 50% 24 hours after elevation. It was also observed that various incisions influence the circulation differently.

A study on four dogs (23) showed that in the case of simple elevation of mucoperiosteal flaps with immediate repositioning, the number of vessels dropped significantly, as assessed by fluorescence angiography, and it returned to the baseline level after **3 days** at the interproximal site and after **10 days** at the mid-buccal site. The application of either horizontal mattress or direct interrupted sutures did not influence revascularization.

Using orthogonal polarization spectral (OPS) imaging to capture and count the gingival vessels, Lindeboom et al. (85) investigated the revascularization of a mucoperiosteal flap elevated palatal to the top of the alveolar process with two vertical releasing incisions, followed by horizontal osteotomy and sinus elevation. The lateral wall osteotomy was covered with a resorbable collagen membrane. They found that vascularity regained its normal level in **14 days** when only bone chips were applied but it took only **7 days** when bone chips were combined with platelet-rich plasma (PRP).

The vascularity of a palatal flap, measured with a similar technique as OPS (sidestream dark field method), regained its normal level in **11 days** after surgery in rabbits if there was no significant interface (i.e. any grafting material) between the bone and the flap and the flap was repositioned after 30 min suspension (86).

Another study (87) with OPS investigated the revascularization of a mucoperiosteal flap in patients receiving immediate dental implants, elevated by intraoral sulcular incision with two buccal releasing incisions. The bone defect at the buccal aspect was subsequently grafted with autogenous bone chips and covered with a native collagen membrane. This resulted in the separation of the flap from the bone. Immediately after surgery, the number of vessels dropped to 36% of the preoperative level. It took **3 weeks** to be normalized, which highlights the role of re-uniting the alveolar and periodontal plexi to the mucosal one.

In conclusion, vascularity regains its normal level between 3 days to 3 weeks depending on flap and incision design, graft application and localization. However, this range seems to be considerable wide. Possible reasons for this may be the limited number of time points and the inaccuracy of some of the abovementioned methods (see later in Section 7.3). Furthermore, most of these methods are lacking in spatial resolution. Consequently, this wide range may also be explained by the various localization and different spanning area of the measurement points in the various studies.

3.6.2 Changes in blood flow

Only a few studies measured the blood flow of the healing flap directly in the oral mucosa. In cats, a 4.5-fold elevation of blood flow, measured by radiolabeled microspheres, was observed 2 hours after elevation of a mucoperiosteal flap, prepared by sulcular and vertical incisions (88). This suggests that the drop in the number of vessels after flap elevation mentioned in the previous section could be compensated by vasodilation.

In a human study (89), blood flow was measured by non-invasive Laser Doppler Flowmetry (LDF) after periodontal surgery. Blood flow was measured at the alveolar mucosa, and the buccal and palatal papillae of the flap on postoperative days 1, 2, 3, 4, 7, 15, 30 and 60. A full-thickness flap was formed by intrasulcular incision without vertical releasing incisions. No ischemia was observed, but hyperemia was detected from day 1 to day 7 at the alveolar mucosa and the palatal papillae (Figure 5). Blood perfusion returned to the baseline on **day 15**. No change was observed in the buccal papillae.

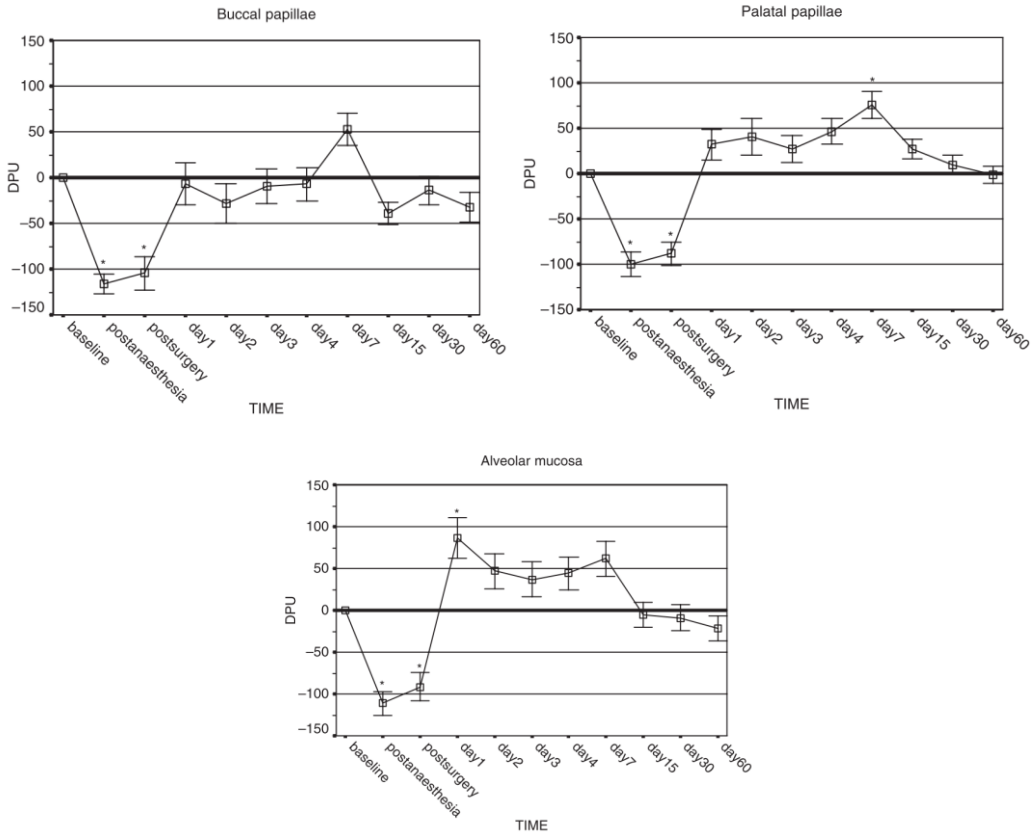

Figure 5: Blood flow change values (Doppler Perfusion Units =DPUs) using the Laser Doppler method, in the alveolar mucosa and the flap over time.
Axis x shows measurement times; blood flow values on axis y are expressed in artificial DPU units (90).

After socket preservation, the flap – measured by LDF – was hyperemic for one month and returned to the control level at **4 months** (91). Blood flow was measured only at one site, 1 mm buccally from the gingival margin.

Further evidence suggests that surgical factors, such as flap design or the application of graft material, significantly influence the blood flow.

In a study, blood flow was measured by fluorescent angiograms after the coverage of localized gingival recessions with two different surgical techniques (92). The microsurgical approach applied resulted in a higher rate of vascularization on day 3 (53%) and day 7 (85%) than the macrosurgical technique.

A clinical trial using LDF showed that the simplified papilla preservation flap may be associated with faster recovery of the gingival blood flow post-operatively compared with the modified Widman flap after pocket surgery (89).

In another study (93), the blood flow of the healing flap was measured at the buccal papillary base by LDF. In the control group, a mucoperiosteal flap was reflected by beveled internal and sulcular incisions for surgical crown lengthening and the bone was exposed without vertical releasing incisions. In the test group, the simplified papilla preservation flap or the modified papilla preservation flap techniques were used without vertical releasing incisions for regenerative periodontal therapy, combined with Emdogain and a granular bone substitute. In all groups, blood flow reached **an ischemic level on day 1**. On day 3, 7 and 14, it was not different from the baseline or the non-treated site, i.e. no hyperemic response was observed. No significant difference with regard to reduction in blood flow was found between the surgical groups.

Blood flow changes in a trapezoid full-thickness flap used for root coverage procedures were measured by LDF in a recent study (94). The flap was mobilized by two vertical incisions and a periosteal releasing incision. A xenogenic collagen matrix or an autogenous subepithelial connective tissue graft was applied on the exposed root surface before repositioning of the flap on the root surface. Blood flow was measured at two sites, at the gingiva and at the alveolar mucosa of the flap. **No ischemia** was observed during the healing period. At the gingival site, the microcirculation of the flap combined with the autogenous graft showed a more homogeneous curve and overall lower mean values – with the exception of the perioperative period and day 3 – compared with the flap with xenogenic matrix. At the alveolar mucosa, the autogenous graft had consistently lower mean blood flow values than the ginigva.

In conclusion, it seems that despite the fact that angiogenesis begins only 2–3 days after injury and the number of vessels returns to normality after 1–3 weeks, blood flow could be compensated in very early stages in most surgical conditions due to vasodilation.

To fully reveal the regulatory mechanism of flap circulation, we definitely need a non-invasive method with high spatial resolution.

3.7 Examination methods of gingival microcirculation

Blood flow can be tested from a morphological and also from a functional point of view. The methods of measurement can be divided into invasive and non-invasive methods.

3.7.1 Invasive methods

Several methods can be used in small tissue quantities with exact results in ml, min or 100 g. However, their disadvantage is that they are invasive and cannot be used in human studies as they are harmful to health (e.g. radioactive materials are used in some methods) or lead to the death of the experimental animal, so it is difficult to perform reproducible measurements.

1. Fluorescence measurement:
    a. The fluorescence principle is based on the fact that a material absorbs electromagnetic radiation of a certain wavelength and as a result emits light of a wavelength that is different from incoming radiation. Ohba et al. (95) used this method for mapping the arteries supplying head-to-neck tumors with indocyanine green.
    b. Fluorescein angiography is a direct measurement of the number of vessels, however, its non-invasiveness strongly limits the amount of measurement time points. These measurements have no absolute value, but because of the distribution of fluorescein some spatial information is provided. The high reliability of these measurements allows us to use only a few animals per group in animal studies. The use of fluorescein angiography to observe blood circulation in the healthy and inflamed gingiva in man was described by Mormann & Lutz in 1974 (96). Employing antecubital venipuncture, 2 ml of a 20% sodium fluorescein solution was administered in these studies. After 15–20 s, the Na-fluorescein entered the gingival capillary system, and a photographic sequence showed the intracapillary phase of fluorescein labeling. The studies covered free gingival autografts (97), surgical procedures (84) and experimental wounds in man (98).

2. Radioactive microspheres: Kaplan et al. used microspheres to measure blood flow in beagle dogs with gingivitis and periodontal lesions. Microspheres were labeled with cobalt 57 isotopes. From the $9x10^6$ microspheres suspension, 3 ml was injected into the left ventricle of the dogs. Isotope activity was measured using a gamma scintillation counter in the removed gingival set and the removed piece of alveolar bone. Blood flow rates were set for 100 grams / tissue (70).

3. Fluorescence microspheres: The microspheres can be tested after fluorescence labeling. In Söderhol and Attström's experiment, blood flow was examined in 4 beagle dogs suffering from gingivitis caused by a modified diet. Microspheres made of methyl methacrylate were used to which Fluorescent green HW 185 was added. After the study, the biopsy samples were evaluated by microscopy (99).

4. ^{86}RB Isotope Assay: Fazekas et al. studied the blood flow of the lower jaw's gingiva in rats with the ^{86}RB isotope. Based on its physico-chemical properties, ^{86}RB is an analogue of K. However, while the half-life of potassium is 12 hours, the rubidium isotope has a half-life of 18 days. The more increased the circulation is, the more isotopes the tissue can absorb (100).

5. H_2 clearance: This method, which is based on the inhalation of H gas, was used for measuring the blood flow of the submandibular salivary gland. It provides accurate, absolute value measurements in ml/100 g/min. During the measurement, a platinum electrode and a reference electrode are used. The concentration of inhaled H_2 gas is determined by a polarographic method. The development of the polarographic method is attributed to Aukland (101). Fazekas et al. used it on rabbit salivary glands (102), while Sasano et al. (103) employed it to study gingival blood flow in cats.

6. ^{133}Xe clearance: It is a highly invasive method. Therefore, limited case numbers and time points are available. It does not provide regional information, but gives an absolute value. High variance requires a relatively larger sample size; 11–34 patients were involved in previous studies with ^{133}Xe clearance (104, 105).

7. Histology: Invasive methods also include histology. It allows for the in-depth study of morphology during microscopic tissue examination. Histology is non-quantitative and does not furnish information on the blood flow. It only serves to make indirect inferences regarding angiogenesis and limited time points can be used for sampling. Studies often include 4–8 animals per group (106, 82, 107-111).

3.7.2 Non-invasive methods

For human studies, the most ideal method is a non-invasive, quick, easy to carry out and reproducible test. Many methods of analysis are known in the literature. The most appropriate methods in terms of the listed parameters, which are also used in human

studies are the Laser Doppler Flowmetry, Laser Doppler Imager and Laser Speckle Contrast Imager techniques.

1. Laser Doppler Flowmetry (LDF): It allows for the measurement of microcirculation in tissues of humans and animals, and measures blood flow in about 1 mm^3 of tissue. Direct measurements of microcirculation can be made with low reliability, no spatial information and a bit larger sample size is required. This approach was first used in the 1980s and continues to be applied, because it is non-invasive, easy to use after training and provides a continuous or near-continuous record (112). The theory is based on the Doppler effect (113) (Figure 6). The main disadvantage of LDF is that it does not accurately measure blood flow, so it cannot be used to calculate absolute blood flow (e.g. in units of ml/min/100 g tissue) (114), i.e. LDF produces a relative value of blood flow. Furthermore, it has some additional drawbacks, namely that it only measures a small surface, it is motion-sensitive and it is hard to measure non-homogeneous tissues with it. In case of wound healing, we cannot place the probe on the same point every day. By contrast, it has the advantage of quick sampling, enabling non-invasive, self-controlled comparisons and being applicable in humans. It has been widely used in the field of plastic surgery for monitoring microvascular blood flow in skin transplants and flaps, in order to detect early signs of impaired circulation and thus predict and possibly prevent surgical complications (115). In the field of dentistry, LDF has been used, among other applications, in order to evaluate gingival blood flow variations related to periodontal disease (116) and smoking (117) or following periosteal stimulation (118) and LeFort I osteotomy (119). This technique has been used repeatedly for monitoring blood flow during periodontal surgical interventions. Donos et al. treated five chronic generalized periodontitis patients with the modified Widman flap technique (120). Red or near-infrared light (780 nm) from a low-power solid-state diode laser (1.6 mW) was directed via an optical fiber of 1.5 mm in diameter to the tissue and the laser light scattered back from the tissue. Within the tissue, light which is scattered from moving blood cells undergoes Doppler shifts in frequency, the magnitude of which depends upon the velocity of the cells. Wavelength shifts do not occur in the case of light reflected from non-moving cells. The light is collected by one or more optical fibers and analyzed. All the fibers are arranged in parallel within a single probe. The machine determines the relative amount of light beams affected by Doppler shifts. This provides information about the amount of

red blood cells (RBC) in the tissue unit, while the magnitude of frequency shifts depends upon the velocity of the cells. By multiplying the concentration of RBC and their mean velocity the FLUX unit is obtained, which correlates well with blood flow. This is a relative, arbitrary unit.

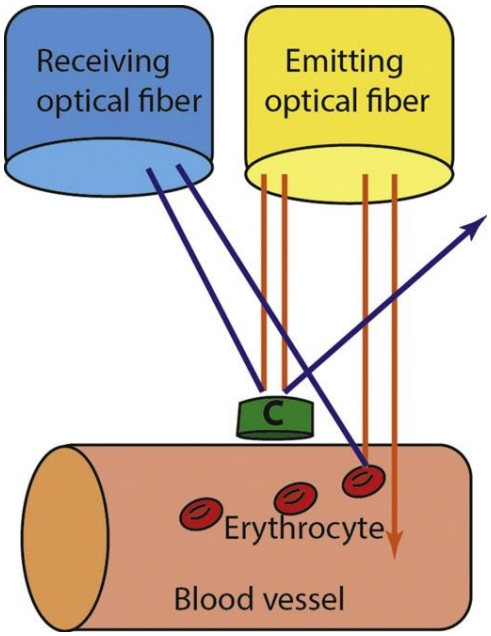

Figure 6: Effect of Doppler applied to laser radiation; c: immobile cell (A.A. Kouadio et. al.).

2. Laser Doppler Imager (LDI): For LDI, the laser beam is moved over a larger surface with a moving mirror. There is no direct contact with the test tissue. It can map the blood stream in large and small areas and assign color-coded images to it. Its advantage is that it provides information on the entire surgical area, but scanning one image takes more than a minute even with the latest devices. Bay et al. studied histamine-sensitized neurons in 13 healthy individuals and in 6 chronic oral disease patients. Blood flow in the palate, tongue and face was measured by the LDI method. Initial scanning was followed by 15 scans after histamine iontophoresis. After histamine administration, blood flow increased in all areas. Significantly higher values were obtained in the skin than in the oral regions. There was no significant difference between the blood flow of the examined groups (121).

3. Laser Speckle Contrast Imager (LSCI): LSCI is characterized by much higher reliability than LDF (122, 123). It has the unique advantage of providing regional

information. A small sample (4–7) is sufficient to distinguish minor differences in gingival blood flow between the groups (124, 125). The principle of the method is that illumination of the tissue surface with coherent monochromatic laser light creates an interference pattern on the surface of the tissue. This speckle pattern is captured by a camera, digitalized and transmitted to a computer, where the information is processed and an image is constructed based on the blood stream. If the illuminated particle is static, the speckle pattern is static. In the case of moving particles, the pattern fluctuates. Spatial resolution is determined by the camera used. The camera has a resolution of 1386x1034 pixels. 3x3 pixels represent a measurement unit for the purpose of contrast analysis. The more static the image – the smaller the red blood cell movement – the more contrast the image of the measurement unit will have. If blood flow increases, the image of the measurement units becomes blurred and contrast decreases. The software assigns a color code to the contrast value of the measurement pixels. The lower the contrast, the cooler the color of the pixel will be, while warmer colors are assigned in the case of higher blood flow. The measured pixels compose encoded, full-frame images. The recommended minimum measurement distance is 10 cm. The size of the measurement area is determined by the distance. For a 10 cm measurement distance it is 5.9x5.9 cm. The LSCI method has many benefits, such as quick sampling and the possibility to measure microcirculation on a large surface. Due to its high resolution, it is also suitable for evaluating functional tests. Furthermore, it has good reproducibility, as there is no contact and no effect on the tissue to be measured. Since the LSCI signal is based on the rate and concentration of red blood cells, the measurement results cannot be expressed in absolute values (ml / min / 100 g) (PeriCam PSI System Extended User Manual), (126) (Figure 7). Clinical studies are suggesting that this technique may be a useful tool for assessing proper circulation during surgical intervention (127, 128) and evaluating wound healing (129, 130).

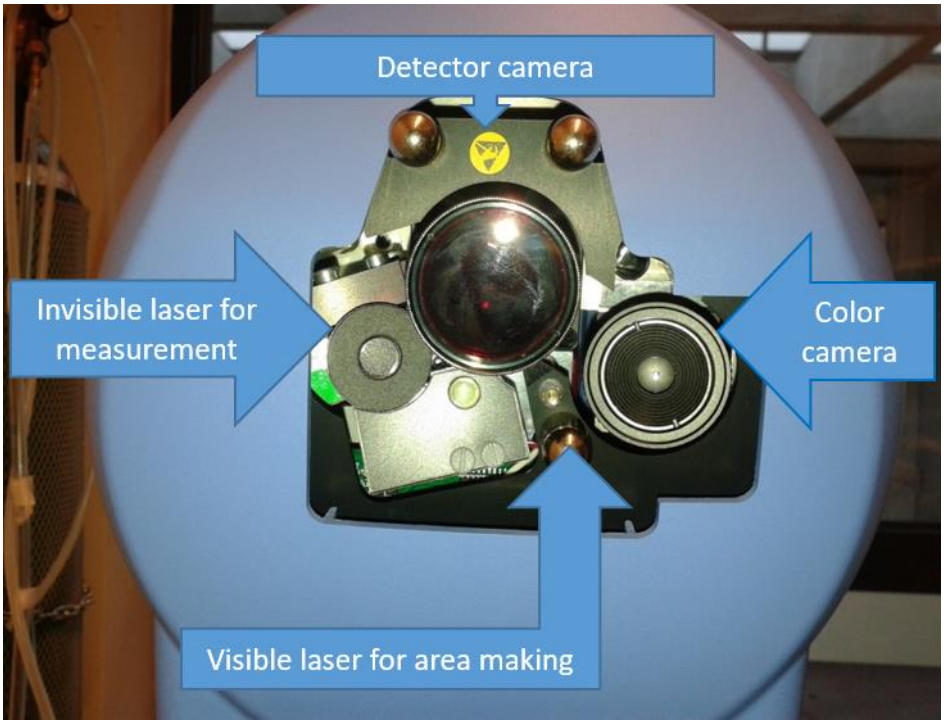

Figure 7: Perimed PeriCam PSI HR System Design.
Visible parts are marked with arrows (own photo).

4. Videomicroscopy: Videomicroscopy techniques are suitable for the direct visualization of microcirculation. In the case of the orthogonal polarized spectral (OPS) technique, the examined tissue is illuminated with polarized light. This method can measure the number of capillaries formed during healing with high reliability, but does not provide information on blood flow and spatial/regional relations. The penetrating light depolarizes in the tissue and the reflected beams enter back to the polarizer. The collected light forms an image of the illuminated area taken by the camera. 548 nm light is used for visualization, which is absorbed by hemoglobin, thereby allowing any structure containing hemoglobin to be seen (131, 132). It is possible to examine vessel density, flow and perfusion. Lindeboom et al. used the OPS method to study the capillary density of mucous membrane during healing after sinus-lift surgery. The maxillary reconstruction was performed with a hip bone by the addition of PRP bioactive material on one side and with placebo on the contralateral side. They found that PRP significantly enhanced mucosal revascularization (85). In another clinical trial, a mucoperiosteal flap was prepared to insert a dental implant (87). The OPS technique has shown that initial capillary density returns about 3 weeks later in the flap. The disadvantage of OPS is that

it needs a very high-intensity light source and visibility is limited due to blurred capillaries. This is why the SDF (sidestream darkfield) method has been developed, where the light source consists of concentrically placed diodes, emitting light around the optics. The diodes emit pulsating green light, synchronized with the camera's frequency, eliminating blurs caused by moving red blood cells (Figure 8). Because of the dark field of vision, red blood cells appear dark. Figure 8 shows the SDF imaging technique. The SDF method was used to monitor blood flow in rabbits after palatal flap formation and it was found that the flap was able to reach initial capillary density by day 11 (86).

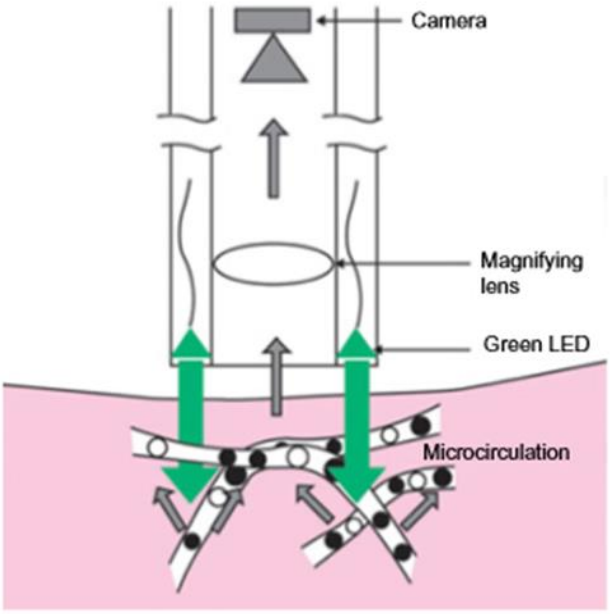

Figure 8: The SDF technique: the light source consists of diodes emitting light, placed concentrically around the optics.
The diodes emit pulsating green light, synchronized with the camera's frequency (126).

5. Photoplethysmography: Photoplethysmography is a non-invasive optical method that is suitable for pulse amplitude testing. RR intervals, i.e. the time elapsed between heart beats, carry a lot of information, among others regarding breathing and the funczion of the autonomic nervous system (133, 134). Using this method, Ikawa et al. compared blood flow changes in gingivitis and in the healthy gingiva after thermal (cold water, warm water) and mechanical (brushing) stimulation (Figure 9). It has been found that in

inflamed tissues, the pulse amplitude rise caused by warmth and mechanical stimuli decreases significantly (79).

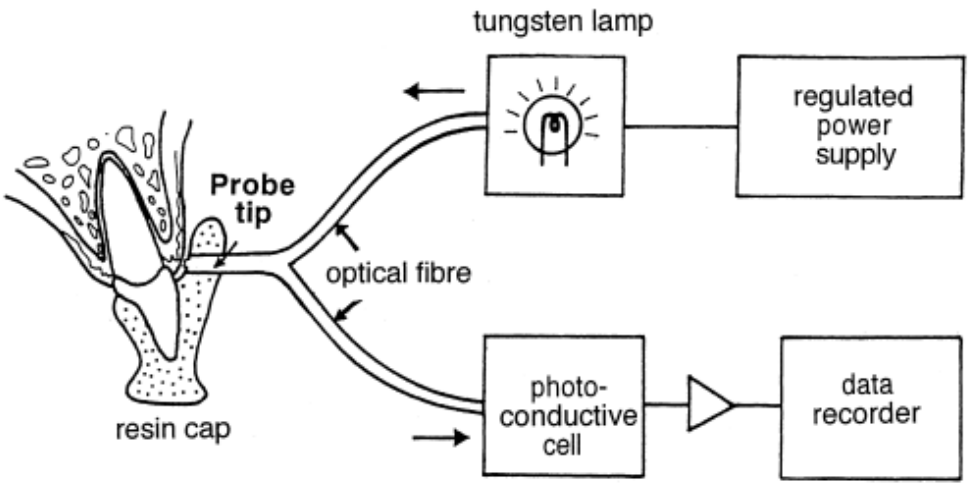

Figure 9: Schematic drawing of photoplethysmography on the labial gingiva of an upper incisor (79).

3.7.3 Other factors that may affect oral mucosal blood flow

Spatial variation

Blood circulation in the gingiva is unique compared to the skin. The speckle image of the gums is more heterogeneous (Figure 10). Due to this, as the laser Doppler probe is too small, it may give confusing results measured on a small surface. It is accepted in the literature that the papilla is vascularized also by vessels originating in the crest of the interdental septa and in the palatal collaterals crossing the alveolar crest. These pathways of gingival blood supply are densely interconnected. The supraperiosteal plexus issues communicating branches to the plexus of the lamina propria and the periosteum. The multiple interconnections between the different plexuses through numerous anastomoses and native collateral pathways of circulation establish an adequate blood supply in the gingiva; however, the extent of the contribution of the various collaterals in maintaining resting blood flow has not been evaluated yet (124).

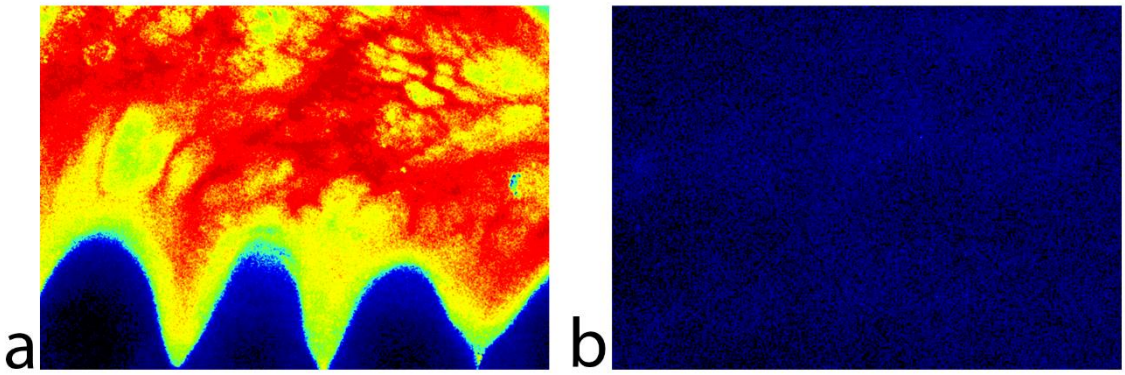

Figure 10: Color-coded LSCI images of healthy untreated gums (a) and forearm skin (b) with the same setup parameters.

Blood circulation is highly inhomogeneous in the human mucosa compared to the skin.

Temporal variation

Gingival blood flow has a high temporal variation as well. This could be related to many physiological factors which continuously occur during everyday life, such as gingival inflammation (72, 76, 135), circadian rhythm (77), blood pressure (78), temperature (79, 135), mechanical pressure (116, 136, 137, 124), tooth brushing (72, 80, 81) or orthodontic force (138). Therefore, the standardization and stabilization of these factors are obligatory for successful follow-up measurements.

3.8 Significance of the measurement of gingival crevicular fluid (GCF)

The junctional epithelium (JE) is part of the dento-gingival unit in relation with the teeth surfaces. GCF reaches the sulcus gingivalis between junctional epithelial cells through intracellular gaps, such as desmosomes and gap junctions (139-142). GCF plays an important role in the homeostasis of periodontal tissues, flushing the pocket, and gives passage for immune proteins (143), thereby contributing to microbial defense (144). Its antibacterial role is manifest both directly (physical barrier) and indirectly (e.g. differentiation of molecules that promote the migration of polymorphonuclear cells) (145). There are a large number of lysosomes in JE cells that contain enzymes fighting various bacteria (146). Moreover, many other factors can increase the production of GCF,

such as smoking (147), oral contraceptives and pregnancy (148), or orthodontic treatment (149).

The initial fluid represents interstitial fluid which is a result of an osmotic gradient (150, 151). The pre-inflammatory fluid is considered to be a transudate, which changes to become an inflammatory exudate upon chemical or mechanical irritation. In the case of inflamed periodontal tissues, GCF is similar to serum. GCF flow appears to be directly related to the severity of the periodontal inflammation, and flow increase depends on greater vascular permeability and ulceration of the epithelium at inflamed sites (152).

The diagnostic assessment of periodontal disease by clinical methods is essential, but the currently available instruments and techniques are not always sufficient to locate the sites where the inflammation is progressing (153). In the 1960s, it was suggested that gingival crevicular fluid could be analyzed to assess quantitatively the site-specific inflammatory status of the periodontal tissues (152). Periotron (OraFlow Inc., NY, USA), a digital device, is suitable for the accurate determination of GCF volume and also for sample collecting to determine the composition of the fluid by laboratory tests. The instrument measures the effect on the electrical current flow of the wetted paper strips. The technique is rapid, very sensitive (can measure as low a volume as 0.05 μl) and has no discernible effect on the GCF sample (143). Therefore, it is also suitable for measuring postoperative wound fluid through several sessions in human subjects.

The following table (Table 2) shows the degree of gingivitis and the corresponding gingival index value that can be assigned to the values measured by the device. The values increase from 0 (calibration), indicating the severity of the inflammation. A value of 0 to 20 indicates that there is no or very slight inflammation. A value of 21 to 40 refers to mild inflammation. Values between 41 and 80 represent a moderate state, while values over 81 indicate severe inflammation. The sampling time was 5 s (143).

Table 2: Translation of Periotron values into clinical conditions and the Gingival Index with which they may be associated (143).

Periotron reading	***Level of gingival inflammation***	***Gingival Index***
0-20	healthy	0
21-40	mild	1
41-80	moderate	2
81-200	severe	3

The inflammatory phase is an important part of wound healing and during inflammation vascular leakage increases. The measurement of crevicular fluid production to indirectly assess vascular permeability could be a useful tool for evaluating wound healing (91, 154, 93).

4 OBJECTIVES

The goal of my PhD dissertation was to develop a practical method for investigating the microcirculation of the human gingiva. This was not so easy to implement, because standardization required great attention due to the varied tissue structure and function of the oral cavity. Wide variations take place in the blood flow of gingival tissues to adapt to the physiological stimuli. For a reliable method, resting and stimulated blood flow data had to be collected. Local tests had to be developed and adapted to the oral cavity. For the investigation of the marginal gingiva, laser Doppler was a more appropriate method, because it was easier to apply and fix the position of the probe. After the laser Doppler experiments, LSCI seemed more suitable for observing the healing of surgical flaps where two dimensional measurements of blood flow are indispensable. However, little data is available in the literature on the oral application of LSCI and there is a lack of relevant experience. My focus was on establishing a methodological basis for the development of future imaging applications for clinical use. During my PhD research, I have conducted clinical studies to find answers to the following main question: how non-invasive microcirculation measurements can be used in dentistry beyond scientific experimental approaches?

The aim of our studies were as follows:

I. Develop a heat provocation test in clinical practice. Test the effect of warm saline on GBF as a function of time in the healthy gingiva with LDF.
II. To investigate the effect of light-induced heat on GBF in the healthy gingiva with LDF.
III. To compare the effect of periodontal inflammation on heat-induced hyperemia between non-smokers and smokers.
IV. To evaluate the intraday reliability of LSCI in oral mucosa measurement and investigate the effect of a change of the incidence angle.
V. To evaluate the effect of retraction on intraday reliability and the assessment of inter-day reproducibility.
VI. To investigate the effect of measurement based on reflected images on reliability.

VII. To evaluate the test-retest reliability of repeated LSCI measurements at the contralateral side of the oral cavity of patients involved in a surgical clinical trial described in experiment (exp.) VIII.

VIII. To evaluate the capacity of LSCI to characterize the kinetics of blood flow after periodontal plastic surgery. As a further objective, comparison was made between the blood flow of Modified Coronally Advanced Tunnel (MCAT) flaps combined either with xenogenic (Geistlich Mucograft®) collagen graft material or the gold standard autogenic collagen tissue graft (CTG) harvested from the palate.

5 METHODS

5.1 Applied experimental methods

5.1.1 Laser Doppler blood flow measurement

Blood flow to the gingival margin (GBF) was measured by LDF (780 nm; MoorLAB; Moor Instruments Ltd, Devon, UK). Laser Doppler instruments measure net red blood cell flux (Flux) as the product of the average speed of the blood cells (Speed) and the concentration of moving red blood cells (CMBC). Blood perfusion readings were made at rest in a room of a steady ambient temperature (26 °C). Subjects were forbidden to brush their teeth, gargle or eat and drink anything for 30 minutes prior to the measurements. Each patient was placed comfortably in supine position in a dental chair and was left undisturbed for a minimum of 15 minutes before any measurements were taken. The lips were retracted with a set of cheek retractors. Care was taken to ensure that the mucosal surface adjacent to the site of recording remained unstrained. A straight laser Doppler probe (outer diameter: 1.5 mm; Moor Instruments Ltd, UK) was attached to the flowmeter and directed 1 mm apical to the mid-buccal gingival margin at a perpendicular angle, without touching the gingiva. The probe was positioned using a steel manipulator anchored in a custom-made silicone occlusion block (Figure 11). The laser Doppler flowmeter was connected to a computer and the readings were recorded by a data acquisition software (MoorSoftMoorLab v2.01, Moor Instruments Ltd, Devon, UK). Blood perfusion was recorded with a sampling rate of 40 measurements per second and averaged by seconds. LDF was used in exp. I, II, III.

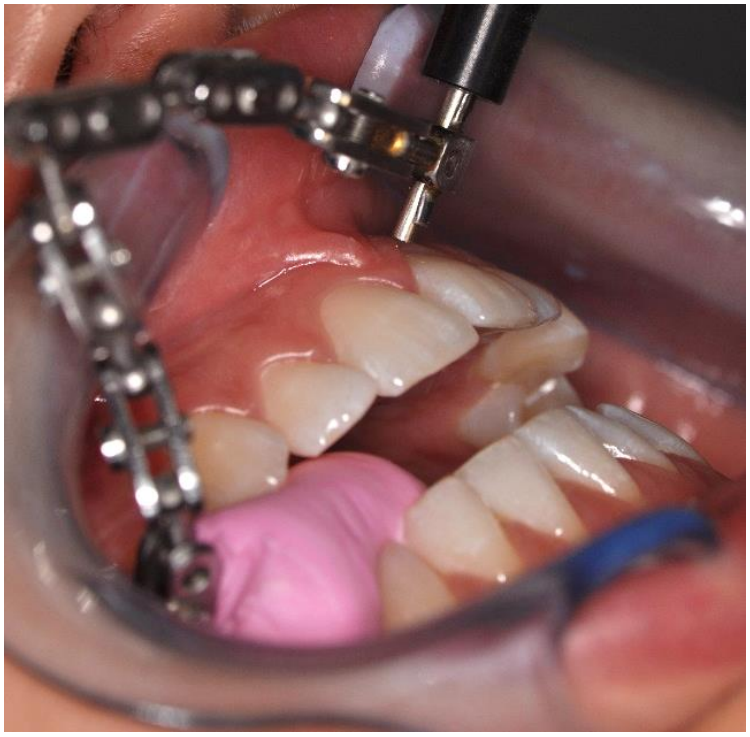

Figure 11: Laser Doppler probe, lip retractor, silicone block in situ.
The probe is perpendicular to the gingival surface, and it is positioned as close as possible, but avoiding contact (own photo).

5.1.2 Laser Speckle Contrast Imaging

Blood flow was measured by a LSCI device (785 nm PeriCam PSI HR System, Perimed AB, Stockholm, Sweden) with a focal distance of 10 cm. The resolution was set to 60 µm/pixel. The measured values were displayed and recorded by a software (PimSoft, Perimed AB, Stockholm, Sweden). Non-smoker, healthy subjects were forbidden to brush their teeth, gargle and rinse, or eat and drink anything for 30 minutes prior to the measurements. Each patient was placed comfortably in supine position in a dental chair and a vacuum pillow was used for fixing their head. The patient was left undisturbed for 15 minutes before any measurements were taken. All measurements were carried out at a constant room temperature of 26 °C.

Our preliminary observation of GBF with rapid sampling (20 image/sec) revealed a clear and significant microcirculatory pulse (Figure 12). Therefore, to average out pulsatile variation, one snapshot included 20 consecutive images within a two-second time interval. Such rapid imaging facilitated multiple measurement at each session and reduced

the risk of movement artefacts (155). GBF was expressed in Laser Speckle Perfusion Units (LSPUs).

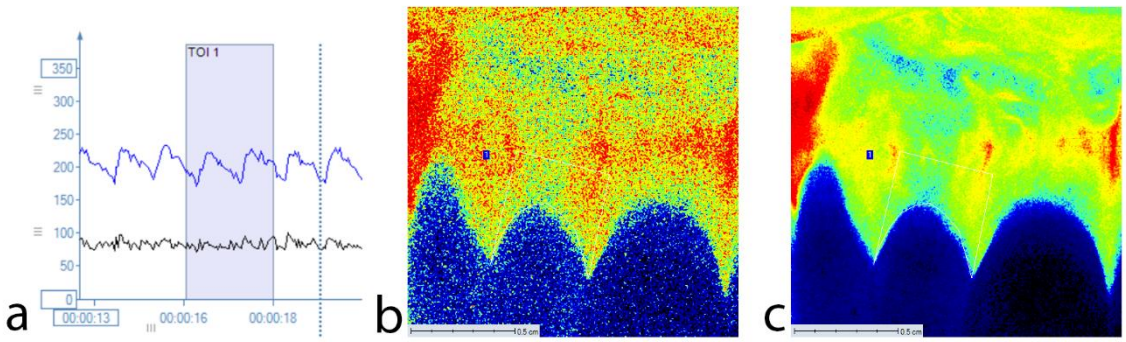

Figure 12: A representative recording of GBF in the selected region (blue curve) shows that gingival microcirculation has a pulsatile feature (a).
A single image is shown in panel (b). Twenty such images were averaged and the resulting smoothed image is shown in panel (c).

Regions of interest (ROIs) were defined on all snapshots using the rulers in the PimSoft software. Zone A is a 2 mm high ROI with a width equal to the distance between the tip of the two interdental papillae next to the selected tooth. The coronal margin of Zone A was determined by the contour of the marginal gingiva. Two more rectangular ROIs, Zone B and Zone C of the same height and width were drawn above Zone A (Figure 13). LSCI was used in exp. IV, V, VI, VII, VIII.

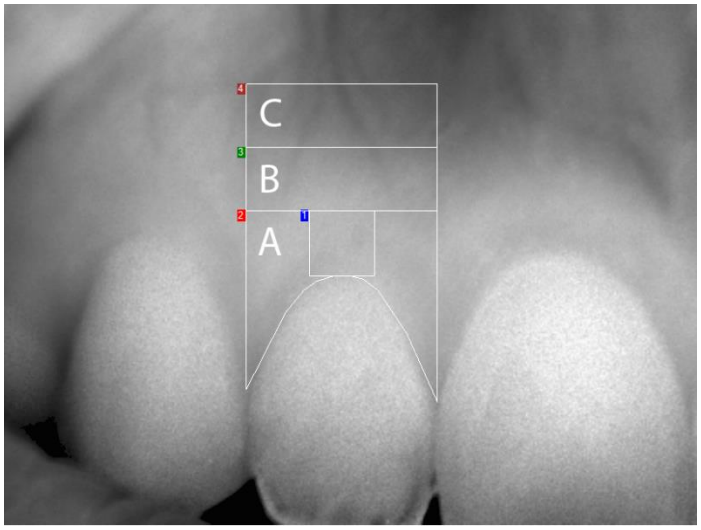

Figure 13: Regions of interest (ROIs) around the teeth investigated.

5.1.3 Crevicular fluid measurement

Gingival crevice fluid (GCF) emerges between the epithelium and the surface of the tooth (143). As the MCAT involves an intrasulcular incision just at this site, we can regard GCF as a wound fluid (WF) after surgery. The relative volume of GCF and WF were assessed by Periotron 8000 (OraFlow Inc., NY, USA) with a filter paper (Periopaper, OraFlow Inc., NY, USA). Periopapers are 1.4 cm long paper strips that can be soaked to measure up to 1.2 µl fluid. They are composed of an absorbent part that has to be inserted into the measured area and the other extremity is coated with plastic for better handling with forceps. The Periotron device allows for the measurement of fluid volume by detecting conductivity changes between a dry control Periopaper and a test strip that has been dipped in fluid. The area was isolated with cotton rolls and the teeth were gently air-dried from saliva. The tip of the Periopaper was placed close to the orifice of the gingival sulcus of the teeth investigated for 10 s (156). Trauma was carefully avoided during the insertion of the strip in order to minimize the mechanical irritation of the healing sulcus. The Periotron instrument was operating at a room temperature of 26 °C and was 'zeroed' before each measurement. The samples were valued as fast as possible to avoid evaporation. The values are shown in Periotron Scores (PS) (157).

5.1.4 Systemic Blood Pressure measurement

Systemic blood pressure (systolic and diastolic) and the heart rate were recorded by an automated blood pressure monitor (Omron M4; Omron Healthcare Inc., Kyoto, Japan) on the left upper arm. In all the experiments, blood pressure was measured before and after the blood flow recordings and mean arterial blood pressure (MAP) was calculated.

5.2 Subjects

All participants were systemically healthy. The exclusion criteria were pregnancy, smoking (except in exp. III), general diseases; furthermore, the subjects were not allowed to take any antibiotics before the investigation, anti-inflammatory drugs, systemic

steroids, bisphosphonates and any other medicine possibly influencing mucosal wound healing, or any other products (except for contraceptives) in the preceding three months. The studies were carried out in accordance with the Declaration of Helsinki. Ethical approval was granted on October 29, 2014 by the Hungarian authority called Committee of the Health Registration and Training Center (approval number: 034310/2014/OTIG).

5.3 Studies

5.3.1 I. The effect of warm saline on GBF in the healthy gingiva

This experiment was performed on nine non-smoking volunteers with a healthy gingiva. Blood flow was recorded before, during (30 s) and after dropping 2 ml of pre-warmed (44 °C) sterile saline solution on the marginal gingiva right next to the laser Doppler probe. Baseline GBF was recorded for 1 minute. The recording of gingival perfusion was continued for an additional 5 minutes after carrying out the test. General measurement procedure of exp. I, II, III are shown in Figure 14.

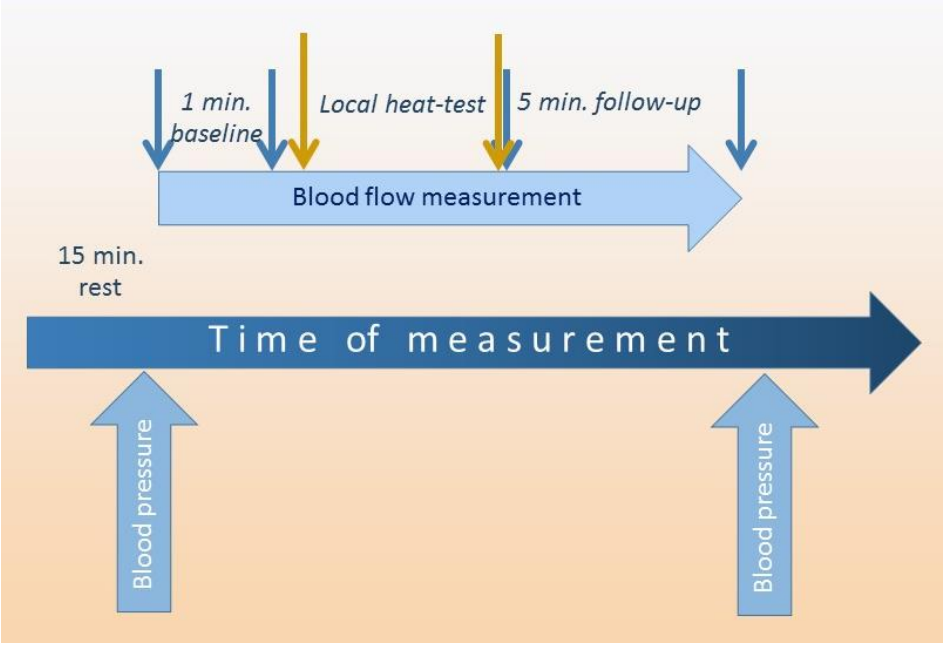

Figure 14: General measurement procedure of exp. I, II, III.

5.3.2 II. The effect of light-induced heat on GBF in the healthy gingiva

This experiment was performed on twelve non-smoking volunteers with a healthy gingiva. Heat was generated on the gingiva using a dental curing light (Ivoclar Vivadent AG, Liechtenstein, 35W) from which the light filter was removed. The light guide was directed to the marginal gingiva at a distance of 1.5 cm. GBF was recorded before and 5 minutes after heat provocation, which was applied for 80 s on the marginal gingiva around the Laser Doppler Flowmeter probe. GBF and blood pressure were recorded in a similar way as in the first set of experiments, with the exception that GBF could not be recorded during the application of light as the high-intensity light characterized by a broad spectral feature interfered with the LDF signal.

5.3.3 III. The effect of periodontal inflammation on heat-induced hyperemia in non-smokers and smokers

This group was composed of twenty-nine volunteers with a periodontal condition of varying severity, from healthy to suffering from a moderately severe inflammation, assessed by a GCF reading (0–71). These patients were also systemically healthy based on the same exclusion criteria as above and were separated into two groups: smokers (n = 11) and non-smokers (n = 18). Prior to blood flow measurements, GCF production and blood pressure were measured as described above. As in the previous set of experiments, GBF was recorded for at least 1 minute before and 5 minutes after the application of heat induced by light. The following circulatory parameters were calculated to characterize the individual heat response curve: maximum absolute change (MAX), maximum percentage change from the baseline (MAX%), the time to decrease to one third of the MAX% corresponding to the speed of recovery after hyperemia (RT, chosen due to differences in the point where the end of the curve returns to the baseline) and the area under the curve from the start of recording after heat stimulation to the point of RT (Area). Average gingival flux pulse amplitude (GFPA) was also calculated at baseline (GFPA-bsl) and the first 15 seconds after heat provocation had been completed (GFPA-heat).

5.3.4 IV. The effect of the incidence angle on reliability

Twenty-two participants (6 males, 16 females) were involved in this series. Their mean age was 30 years (23–58). The lips were retracted by a lip retractor (Spandex®, Hager & Werken, Germany) (Figure 15a). The LSCI device was centered perpendicular to the keratinized gingiva above tooth 12 for the first snapshot. Then the subject's head was turned right as much as possible for tooth 12 to be seen on the side of the 2x3 cm wide snapshot picture. The incidence angle was recorded by a protractor. After GBF measurement, the same procedure was performed with a turn to the left and then all three types of measurements were repeated.

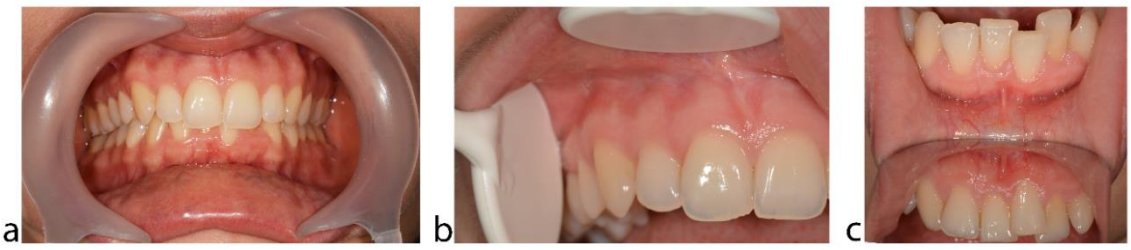

Figure 15: Three different methods of retraction.
The lip retractor (a) was not removed during the consecutive measurements. The dental mirror (b) and the photographic mirror (c) were removed between two readings.

5.3.5 V. The effect of retraction on reliability and the assessment of inter-day reproducibility

Twenty-two participants (6 males, 16 females) were involved in this series. Their mean age was 28 years (21–48). Participants' upper lips were carefully retracted by two dental mirrors (Figure 15b). The LSCI device was centered perpendicular to the keratinized gingiva above tooth 12 for the first snapshot. The procedure was repeated twice more. In-between, the patients closed their mouth. This protocol was suitable to assess intra-day reliability, i.e. repeatability within one session. After one week, the whole experiment was repeated in order to assess inter-day reliability, i.e. reproducibility (158).

5.3.6 VI. The effect of mirrors on reliability

Twenty-five patients (11 males and 14 females) were recruited. Their mean age was 31 years (21–41). The LSCI device was centered perpendicular to the keratinized gingiva below the mandibular central incisors. Six snapshots of GBF were alternately taken either directly using a dental mirror for retraction of the lips or a silhouette-free dental photographic mirror, placed in the mandibular vestibulum to reflect the same region of interest (Figure 15c). The distance measurement of the LSCI was set to manual in PimSoft. ROIs were defined around tooth 31. Since the mirror interfered with the visibility of the other two zones, the data were evaluated in Zone A only.

5.3.7 VII. The long-term reliability of repeated measurements

This analysis was done on data from exp. VIII. Eight subjects (4 women and 4 men) exhibiting multiple Miller Class I and II gingival recessions had undergone periodontal plastic surgery in order to cover the exposed tooth surface. During this trial, the gingiva of 2–4 teeth in the non-operated area were selected as reference sites in each subject in order to control the possible systemic variation of GBF during the six-month follow-up (Figure 16).

Measurements were taken twice preoperatively and on the following days postoperatively: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 17, 30, 60, 90, 120, 150 and 180 by LSCI. On each day, the measurements on each site were repeated 2–4 times in a randomized manner by retracting the lips carefully by dental mirrors. Zone A, B and C were defined on the keratinized gingiva at each reference site.

Patients									
Teeth	P1	P2	P3	P4	P5	P6	P7	P8
17								treated, mucograft
16							treated, mucograft	treated, mucograft
15						treated, measured, CTG	treated, mucograft	treated, mucograft
14						treated, measured, CTG	treated, measured, mucograft	treated, measured, mucograft
13	control		control		control	treated, CTG	treated, measured, mucograft	treated, measured, mucograft
12		control	control		control			
11		control		control				
21			treated, measured, mucograft	control	treated, measured, mucograft		treated, CTG	
22		treated, measured, mucograft	treated, measured, mucograft		treated, measured, mucograft		treated, CTG	treated, CTG
23	treated, measured, CTG	treated, measured, mucograft			treated, mucograft		treated, measured, CTG	treated, measured, CTG
24	treated, CTG				treated, mucograft	treated, measured, mucograft	treated, measured, CTG	treated, measured, CTG
25					treated, mucograft	treated, measured, mucograft	*missing closed*	treated, CTG
26					treated, mucograft	treated, mucograft	treated, CTG	treated, CTG
27								treated, CTG
37								
36								
35						control		
34		control				control		control
33	treated, measured, CTG	control		treated, measured, mucograft			control	control
32	treated, measured, CTG			treated, measured, mucograft			control	
31		treated, CTG		treated, mucograft				
41		treated, measured, CTG						
42	control	treated, measured, CTG		treated, measured, CTG			control	
43	control	treated, CTG		treated, measured, CTG			control	control
44				treated, CTG		control		control
45				treated, CTG		control		
46				treated, CTG				
47								

		Mucograft	CTG	Total	
Treated	unmeasured		11	14	25
	measured		14	13	27
	total		25	27	52
Control	measured				25
Total	measured				52

Number of teeth in one Tunnel	Mucograft	CTG	
More than 2 teeth		5	5
2 teeth		2	2

Figure 16: Treated, measured, control teeth in experiment VII, VIII.

5.3.8 VIII. Periodontal plastic surgery for root coverage

Eight subjects (4 women and 4 men) exhibiting multiple Miller Class I and II gingival recessions (Multiple Adjacent Recession Type Defects, MARTD) were recruited. All subjects had a thin gingival biotype assessed by thickness which had been measured preoperatively with a Kerr file. They were in good general health, and their mean age was 35 (their age ranged between 26 and 46). The patients had good oral hygiene, PSRs (Periodontal Screening and Recording) were zero at each sextant, and full mouth plaque and bleeding scores were maintained below 20% throughout the study. Each subject received written information about the surgery and the subsequent measurements, enabling them to give a written informed consent.

MARTDs were treated with MCAT (reported elsewhere: (159) by an experienced periodontist. Two types of grafts were used during the surgeries: either a subepithelial connective tissue graft (CTG) removed from the palate or a xenogenic collagen matrix (Geistlich Mucograft®). Five patients received both grafts in a split-mouth design. Three patients were treated only at one surgical site (two of them received Geistlich Mucograft®, one received CTG). Immediately before surgery, root scaling was performed with hand instruments, and a flow composite was applied coronally at the contact points for later suture suspension. Patients were instructed to follow postoperative regimes. In the control period, patients had to rinse with a mouthwash containing 0.2% chlorhexidine (Curasept 220, Curaden, Switzerland) until 14 days after the surgery. Manual brushing at the treated sites was prohibited until suture removal. Supragingival debridement was performed at the operation sites using a scaler and chlorhexidine-soaked cotton balls. Patients were administered systemic antibiotics postoperatively for 7 days. The study was registered with ClinicalTrials.gov (identifier: NCT02540590).

Clinical data collection was carried out at baseline (bsl.) and six months postoperatively. Photo documentation was prepared at all visits. Blood flow, blood pressure and wound fluid measurements were performed before the operation (baseline) and postoperatively on the following days: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 17, 30, 60, 90, 120, 150 and 180.

Clinical parameters

The following clinical parameters were recorded by means of a periodontal probe at baseline and after six months: gingival recession depth (GRD0, GRD6), gingival recession width (GRW0, GRW6) and the width of the keratinized tissue (KT0, KT6). The

change of these parameters was calculated as follows: recession depth reduction (REC), recession width reduction (RW) and increase of the keratinized tissue in width (KT).
Blood flow was measured at the gingiva of 52 teeth in total: at 27 sites of operated teeth (test sites) and at 25 control teeth (reference sites, 13 in female and 12 in male). Of the measured test sites, 14 were Geistlich Mucograft®-treated (7 in female and 7 in male) and 13 were CTG-treated (6 in female and 7 in male).

Blood flow measurements by LSCI

The lips were retracted carefully and tension-free with dental mirrors. Care was taken to ensure that the mucosal surface adjacent to the site of recording remained unstrained. The measurements were always obtained between 7 and 10 o'clock in the morning.
Three regions of interest (ROI) were defined at each tooth as shown in Figure 17 (Zone A, Zone B and Zone C, moving away from the crown). The selection of regions and further steps of data processing were accomplished by blind analysis. The blood flow value of a ROI was defined as the average of all the pixel perfusion values in that ROI. The approximate pixel number was 7000 for Zone A and 3500 for Zone B and C. According to point density, they spanned 20 mm^2 and 14.5–14.5 mm^2, respectively.

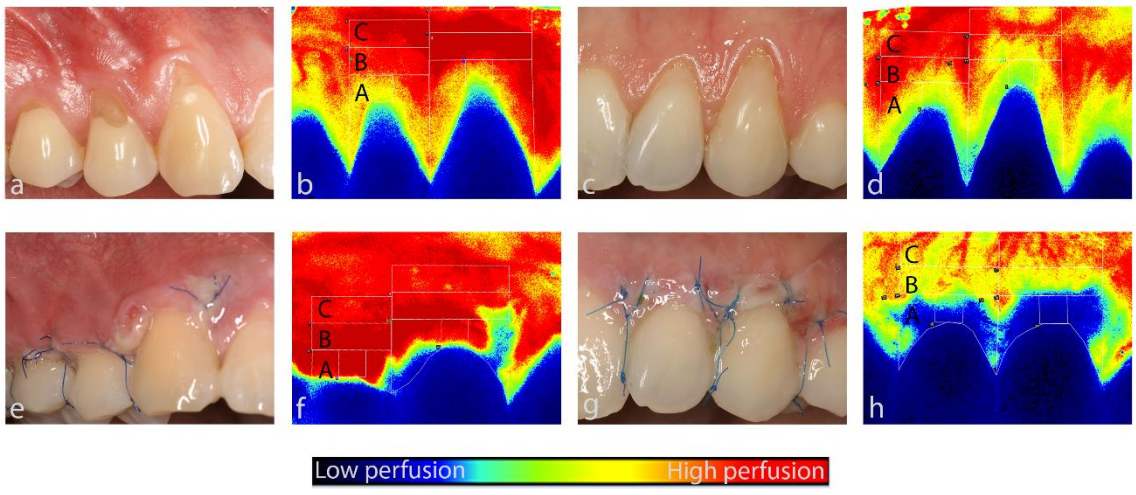

Figure 17: Representative photographs and LSCI images of a male (a, b, e, f) and a female (c, d, g, h) subject.
A combination of a modified coronally advanced tunnel and Geistlich Mucograft® in both cases. (a), (b), (c) and (d) are images representing preoperative perfusion. (e), (f),

(g) and (h) show wound healing and perfusion 3 days postoperatively. Capital letters A, B and C indicate the regions of interest for blood flow evaluation.

5.4 Statistics

In the case of LDF studies (exp. I, II, III) for data with normal distribution (MAP, baseline GBF, MAX, MAX%, GFPA) the mean ± standard error of the mean (SEM), the median and the interquartile range for non-normal distribution (age, GCF, RT, Area) were calculated. Alterations in the circulatory parameters as a function of time (repeated measurement factor) were statistically evaluated by analysis of variance (ANOVA). The circulatory values of various periods were compared to the baseline period by Dunnett's test. The parameters of smoking and non-smoking groups were compared either by parametric T-test (MAP, MAX, MAX%, GFPA) or by non-parametric Kruskall–Wallis test (age, GCF, baseline GBF, RT, Area), depending on the distribution of the data and homogeneity of variances (Levene's test). The relationship between MAP and GBF was examined calculating the Pearson correlation coefficient. For the correlation between age, GCF, MAX, MAX%, RT, Area and GFPA the Spearman test was used. An alpha value of $p<0.05$ was used for all statistical analyses. Statistical evaluation was carried out using a statistics software (Statistica; StatSoft Inc., Tulsa, OK, USA).

GBF values in the text and the figures are presented as mean ± standard error of the mean in experiments where LSCI was used (exp. IV, V, VI, VII, VIII).

Plotting absolute subject difference in GBF between repeated measurements against subject mean GBF (160) clearly showed (Figure 18) that variability increased with the magnitude of GBF (heteroscedasticity). There was a statistically significant correlation between the two parameters (Kendall's tau: 0.257, $p<0.001$). Therefore, as recommended (161, 160, 162) log-transformation was performed due to heteroscedasticity in exp. IV, V, VI, VII. Statistical evaluation was carried out by SPSS 24 (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

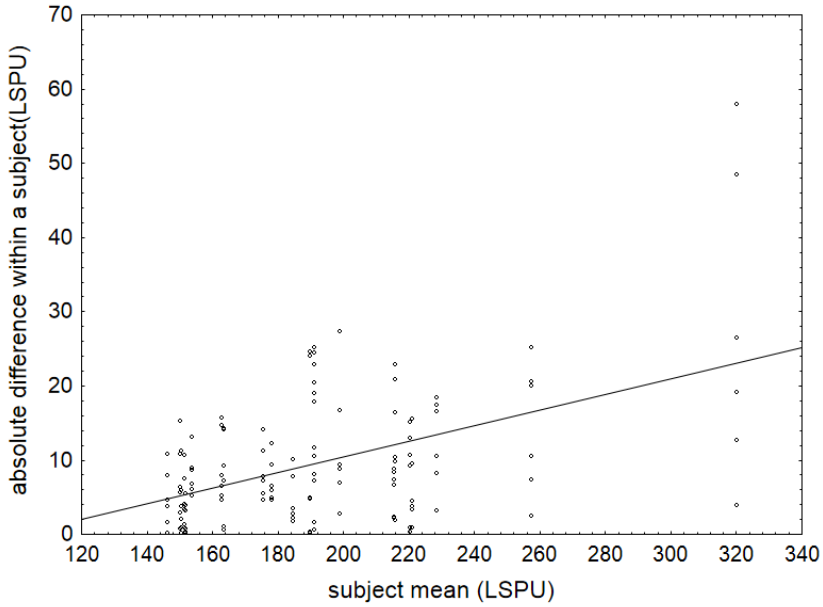

Figure 18: The relationship between the mean GBF values and the differences between repeated measurements within subjects.
The positive correlation suggests increasing error with increasing blood flow.

For the assessment of test-retest reliability (exp. IV, V, VI, VII), three parameters were calculated. The intraclass correlation coefficient (ICC) was calculated by the ratio of the variance between the subjects to the total variance and it represents the 'relative' reliability of a zone within a site within a patient (161). ICC values of <0.40, 0.40–0.75 and >0.75 were considered as poor, fair-to-good and excellent reproducibility, respectively (123). The coefficient of variation (CV) was calculated from the variance component (VC) of the log-transformed variable using the following formula $\mathrm{CV} = 100 \times \ln(10) \times \sqrt{VC}$ (162), with a confidence interval of 95%. CV values of ≤10%, 10–25% and ≥25% were considered as good, moderate and poor reliability, respectively (123). The repeatability coefficient (r) was used to determine the smallest difference indicating a real change (with 95% confidence) at the individual level (160, 163). The repeatability coefficient was calculated according to the formula $r = (10^{SDw})^{2.77}$, where SDw is within-subject standard deviation. The factors affecting GBF changes were analyzed by a mixed-model approach using restricted maximum likelihood estimation. Pairwise comparison was made by a Least Significant Difference post-hoc test. The p values were adjusted by the Bonferroni method. No outliers were excluded from the analysis, except where it is otherwise indicated.

In exp. VIII, the blood flow changes were analyzed by a mixed-model approach. For pairwise comparison, the p values were adjusted by the Benjamini and Hochberg method in order to control the false discovery rate in multiple testing. To determine the association between the clinical outcome and baseline clinical parameters, and between the blood flow and WF data, Spearman's correlation coefficients were calculated. The differences in clinical parameters between grafts and between genders were tested using the non-parametric Mann–Whitney U test. Statistical evaluation was carried out by IBM SPSS Statistics for Windows (Armonk, NY: IBM Corp., USA).

6 RESULTS

6.1 The effect of warm saline on GBF in the healthy gingiva (exp. I)

The average MAP of this group of patients was 107 ± 4 mm Hg. The Flux value represents GBF and in our experiments we recorded the components of the flux (CMBC and Speed) separately as well in order to better characterize the vascular changes in the gingiva after the heat challenge. The application of warm saline to the buccal gingiva resulted in a quick increase in CMBC for periods of 20 s (Figure 19). The values within these periods were very noisy; therefore, we excluded them from the statistical analysis as they suggest an artefact due to the reflection of the laser light from the surface of the saline flow and/or mechanical irritation caused by the impact of saline drops. At the end of heat provocation, CMBC dropped to the baseline level and remained there while Speed and Flux increased rapidly in parallel. Flux reached its peak response (76 ± 6.0%) 21 seconds after completing the application of heat. Mean percentage changes during saline application and the mean values for every minute after application are shown in Table 3. Increased GBF after heat application was due solely to the increase in the average speed of blood cells, without any change in CMBC. In further experiments, only the Flux was used to estimate GBF.

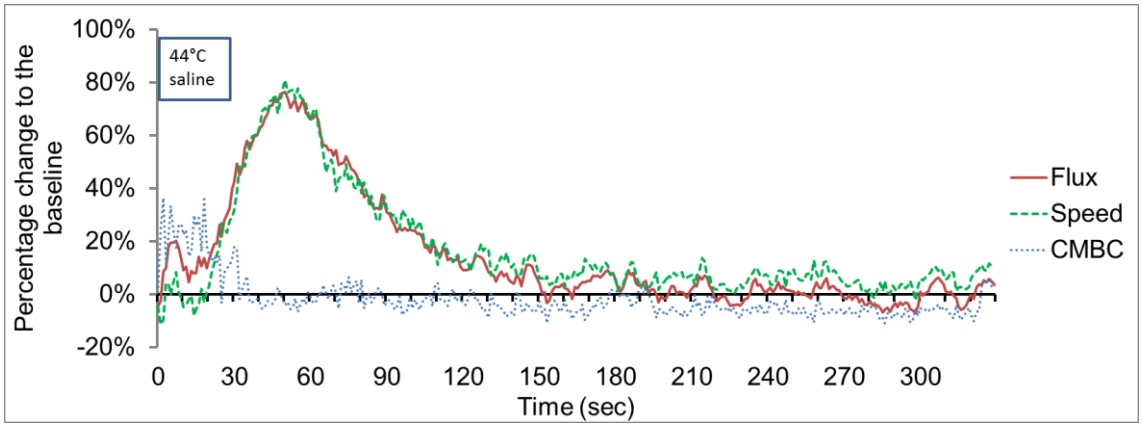

Figure 19: The percentage change of the flux (gingival blood flow), the speed of the moving blood cells and the concentration of moving blood cells (CMBC) compared to the baseline during and after local application of warm saline.

The Flux value represents blood flow in the gingival margin, calculated by multiplying CMBC in the measured tissue volume and the average velocity of the cells (Speed). The mean values indicated on the graph were calculated from nine individual experiments where 40 values were recorded per second and were averaged for 1 s. The period of saline application corresponds to the width of the framed text (44 °C saline). Note that the CMBC values within this period are very noisy, possibly due to the reflection of the laser light from the surface of the saline flow and/or mechanical irritation caused by the impact of saline drops.

Table 3: Mean percentage changes of blood perfusion parameters compared to the baseline.

*Measured by a laser Doppler instrument after warm saline treatment (mean ± SEM). GBF(Flux): gingival blood flow as measured by net red blood cell flux (Flux); Speed: average speed of the blood cells; CMBC: the concentration of moving red blood cells; *** p<0.001 from the respective baseline values.*

	Time of measurement				
n=9	Min 1	Min 2	Min 3	Min 4	Min 5
GBF(Flux)	56±4.8% ***	14±8.5%	3±4.3%	1±2.9%	-1±3.6%
CMBC	0±3.8%	-3±2.3%	-4±1.5%	-6±3.5%	-6±3.4%
Speed	55±4.7% ***	17±7.1%	7±3.3%	6±2.5%	5±2.5%

6.2 The effect of light-induced heat on GBF in healthy gingiva (exp. II)

The effect of halogen lamp on blood flow was plotted on a dose-response curve (Figure 20). We choose the biggest response with no discomfort for the patient, which meant 80 s illumination, total time: 200 s. Further illumination caused pain for some participants.

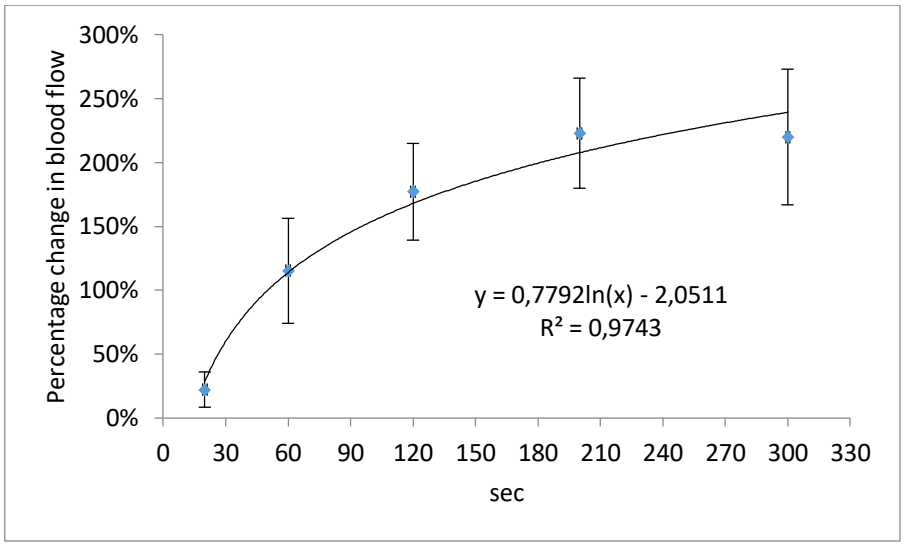

Figure 20: The effect of halogen lamp on blood flow (DPU).

The average MAP of this group of patients was 109 ± 5 mm Hg, and it did not differ statistically from the warm saline group. The recording of GBF (Flux values) was started just after the halogen light had been switched off as it interfered with laser Doppler measurements. The changes in GBF after the application of light were expressed as a percentage of the baseline, and the mean change is shown in Figure 21. The GBF values for each minute of recording were averaged and tested for statistical differences to the baseline values. The value of averaged GBF was significantly elevated at minutes 1 and 2 (80 ± 12%, $p<0.001$ and 44 ± 10, $p<0.001$, respectively). After 2 minutes, GBF returned to baseline values (min 3: 15 ± 5%, NS; min 4: 8 ± 4%, NS; min 5: 7 ± 5%, NS). The mean peak GBF value was 89 ± 15% at 30 s after heat application had been finished, which is very close to the value obtained in the case of warm saline. The average RT time was 110 s. Both methods were effective in inducing a rapid increase in GBF and even after provocation was finished GBF remained at an increased level long enough for data acquisition. This was an important criterion as both methods interfered with laser Doppler measurements, hindering recording during the provocation test. The application of warm

saline was technically more demanding and sometimes run-off fluid caused discomfort to the patient, resulting in movement artefacts. Therefore, we opted for heat-induced light for the heat test in further experiments.

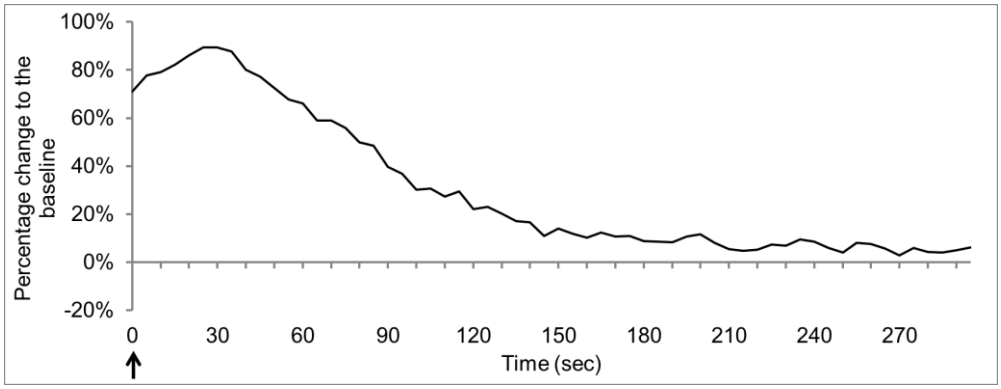

Figure 21: The effect of light-induced heat on the mean Flux (gingival blood flow) in the gingiva of healthy subjects.

The mean values from twelve individual experiments shown on the graph were calculated after the 40 values recorded per seconds were averaged for 1 s. The arrow at 0 indicates the end of light application. The recording of the Flux started just after the halogen light was switched off as it interfered with laser Doppler measurements.

6.3 The effect of periodontal inflammation and smoking on heat-induced hyperemia (exp. III)

There was no correlation observed between the baseline GBF values and the MAP of patients in either the non-smoking or the smoking group (r = 0.13, NS and r = –0.44, NS, respectively). As there was no change in MAP before and after heat provocation, the GBF values were used for further comparison instead of vascular resistance or conductance.

There were no significant differences observed between the non-smoking and the smoking group in terms of most of the baseline values such as age (26 (24–41) years vs. 25 (24–28) years), MAP (116 ± 4.2 vs. 113 ± 3.8 mm Hg), GCF (10 (5–24) PU vs. 3 (2–10) PU), GBF-bsl (172 (143–312) Flux vs. 213 (166–238) Flux), MAX (415 ± 41 Flux vs. 450 ± 52 Flux), MAX% (101 ± 12% vs. 112 ± 15%) and Area (10 (6–17) vs. 14 (11–18)).

On the other hand, there was a significant difference in RT values between the two groups (85 s (55–105) vs. 115 s (75–155), $p<0.05$).

GFPA was 68 ± 7 Flux at baseline and increased to 114 ± 10 Flux ($p<0.001$) after heat provocation in non-smokers and it similarly changed from 79 ± 9 Flux to 117 ± 12 Flux ($p<0.001$) in smokers. Smoking itself did not influence absolute GFPA values. No change was observed in relative GFPA values (GFPA/GBF) after the application of heat in the non-smoking group (40 ± 3% vs. 40 ± 3%), but in the smoking group, relative GFPA decreased significantly after the heat test (44 ± 5% vs. 37 ± 4%, $p<0.05$), indicating a significant ($p<0.05$) interaction between the effect of smoking and the heat test.

The correlations of circulatory parameters to age or GCF are shown both for the non-smoking and the smoking group in Table 4. No significant correlation was observed to age in any groups. No correlation was found between GCF and baseline GBF; however, a moderate positive correlation was found between GCF and the MAX value in the non-smoking group, but not in the smoking group. Similarly, both GFPA-bsl and GFPA-heat were highly correlated to GCF values, but only in the non-smoking group. No correlation was observed between GCF and relative GFPA in either period or group (data not shown). A strong negative correlation was found between GCF and RT ($r = –0.64$, $p<0.01$) in non-smokers, but not in the smoking group.

Table 4: Correlation coefficients of age and GCF to the GBF parameters in non-smokers and smokers.

***MAX**: the maximal absolute change of gingival blood flow (GBF) after the application of light-induced heat; **MAX%**: maximal percentage change relative to the baseline; **RT**: the time needed to decrease to one third of MAX%, representing the speed of recovery after hyperemia; **Area**: the area under the curve from the start of recording after heat stimulation to the point of RT; **GFPA**: the average gingival Flux pulse amplitude was calculated at baseline (GFPA-**bsl**) and the first 15 seconds after heat provocation had been completed (GFPA-**heat**); # p<0.05; ## p<0.01; ### p<0.001.*

		bsl	**MAX**	**MAX %**	**RT**	**Area**	**GFPA-bsl**	**GFPA-heat**
non-smokers	**age**	0.13	-0.02	-0.27	-0.28	-0.43	0.32	0.04
	GCF	0.44	0.48#	-0.05	-0.64##	-0.43	0.65##	0.69##
smokers	**age**	-0.32	-0.43	-0.56	0.06	-0.15	-0.09	-0.20
	GCF	-0.14	-0.04	0.39	-0.18	-0.24	-0.31	-0.14

6.4 The effect of the incidence angle on reliability (exp. IV)

Neither the mean blood pressure (89.1±2.14 vs. 88.2±2.07 mm Hg) nor the heart rate (68.8±2.51 vs. 71.1±3.06 1/min) changed significantly during of the experiment.

6.4.1 The effect of the incidence angle on reliability in Zone A

The effect of turning was found to be significant ($p<0.05$). Pairwise comparison showed that GBF in Zone A was slightly but significantly higher (3.8%) during the turn to the left (196±7.0) than in the central position (189±6.3, $p<0.05$). When turned to the right (190±6.4, $p=1.000$), there was no statistical difference in GBF from the central position and the turn to the left ($p=0.116$). No significant change was observed in GBF means between the two repeats (190±5.0 vs. 193±5.7, $p=0.287$).

Repeatability was good with and without turning (Table 5, Figure 22a). After removing the outlier, statistical difference between the left and the central position disappeared

(p=0.118) and the divergence in means decreased (2.8%) as well as the CV values (Table 5).

The between-subject CV was 20.0% [14.7%–27.2%] with the outlier and 16.6% [12.1%–22.8%] without the outlier. The ICC was found to be excellent with and without turning (0.93 and 0.91). Removing the outlier influenced these values only slightly (0.88 and 0.91).

Table 5: Repeatability of the repetitions with or without changing the incidence angle

		With outlier		Without outlier	
		CV	CR	CV	CR
Zone A	repeat	5.5% [4.6% - 6.7%]	1.17	5.4% [4.4% - 6.7%]	1.16
	turn	3.2% [1.8%-5.8%]	1.09	2.9% [1.4%-6.1%]	1.08
	turn+repeat	6.4% [4.9% - 8.9%]	1.19	6.2% [4.6% - 9.0%]	1.19
Zone B	repeat	6.0% [5.0% - 7.1%]	1.18	5.7% [4.7% - 6.9%]	1.17
	turn	4.5% [3.2%-6.5%]	1.13	3.3% [1.9% - 5.8%]	1.10
	turn+repeat	7.5% [5.9% - 9.6%]	1.23	6.6% [5.1% - 9.0%]	1.20
Zone C	repeat	6.3% [5.2% - 7.7%]	1.19	6.0% [4.9% - 7.3%]	1.18
	turn	4.0% [2.4%-6.8%]	1.12	3.2% [1.6% - 6.3%]	1.09
	turn+repeat	7.4% [5.7% - 10.2%]	1.23	6.8% [5.2% - 9.7%]	1.21

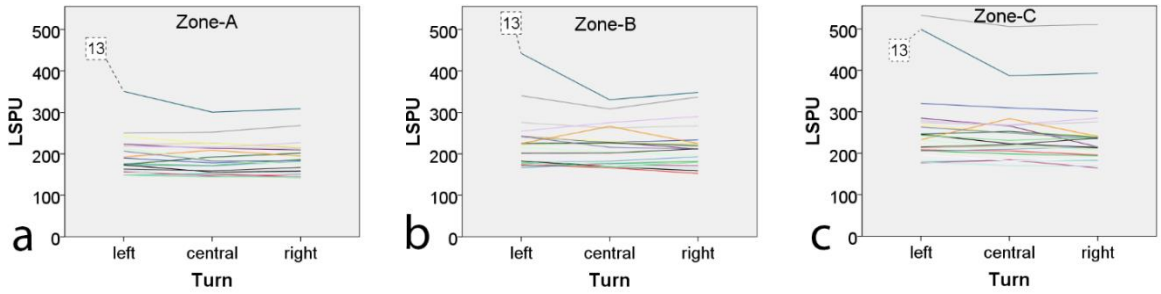

Figure 22: The variation of GBF in Zone A, B and C within subjects due to different angulation.

The lines of different colors represent the mean of two repeats on different subjects measured from the left, central and right aspect. Subject 13 had an extremely high GBF value with the head turned to the left, therefore, this case was considered an outlier.

6.4.2 The effect of the incidence angle on reliability in Zone B

The effect of turning was found to be significant ($p<0.05$) but pairwise comparison did not reveal any statistical difference among the three values (left: 226±9.7, right: 216±8.1, central: 216±7.6) and between the two repeats (217±6.7 vs. 221±7.2).

Repeatability was good with and without turning (Table 5, Figure 22b). After removing the same outlier as in Zone A from the analysis, the effect of turning became non-significant ($p=0.108$) and CV values decreased.

The between-subject CV was 21.8% [16.0–29.6%] with the outlier and 18.7% [13.6%–25.6%] without the outlier. The ICC was found to be excellent with and without turning (0.93 and 0.89). Removing the outlier influenced these values only slightly (0.92 and 0.89).

6.4.3 The effect of the incidence angle on reliability in Zone C

The effect of turning was found to be significant ($p<0.01$). Pairwise comparison showed no statistical difference between the central position (249±11.9) and the turn to the left (259±14.0, $p=0.236$), and between the central position and the turn to the right (242±12.1, $p=0.303$). The value for the turn to the left was significantly (by 6.7%) higher than that recorded for the turn to the right ($p<0.01$). No statistical difference was observed between the two repeats (246±10.6 vs. 253±10.1, $p=0.110$).

Repeatability was good with and without turning (Table 5, Figure 22c). After removing the outlier, the effect of turning was still significant ($p<0.01$) as well as the differences in GBF between the turn to the right and the turn to the left ($p<0.01$). Moreover, CV values decreased.

The between-subject CV was 26.2% [19.3%–35.6%] with the outlier and 23.7% [17.3%–32.5%] without the outlier. The ICC was found to be excellent with and without turning (0.95 and 0.93). Removing the outlier influenced these values only slightly (0.94 and 0.92).

6.5 The effect of retraction on intra- and inter-day reliability (exp. V)

The mean blood pressure (85.2±1.10 vs. 80.9±1.28 mm Hg, $p<0.001$) changed significantly, but the heart rate (66.6±1.56 vs. 66.4±1.53 1/min) remained unchanged within a measurement session.

Neither the mean blood pressure (82.4±1.30 vs. 83.7±1.17 mm Hg) nor the heart rate (66.0±1.46 vs. 67.0±1.62 1/min) changed significantly between two sessions.

6.5.1 The effect of retraction on intra- and inter-day reliability in Zone A

No significant differences were found between the means of the three repeated retractions (228±5.6, 230±5.3, 231±5.4, $p=0.595$) and between the means of the two measurement weeks (227±4.1, 233±4.7, $p=0.375$). Intra-day repeatability with retraction was good and inter-day reproducibility including the intra-day repetitions was moderate (Table 6). Individual variation is shown in Figure 23a and d. Up to four intra-day repeats, the calculated standard error (164, 165) between two different days decreased noticeably (Figure 24).

The between-subject CV was 11.0% [7.2%–17.0%]. The intra-day ICC was 0.70. Using only a single measurement within a day, the inter-day ICC was 0.56, but increased to 0.66 by averaging the three repeats.

Table 6: Repeatability with retraction and reproducibility between the measurement weeks

		CV	CR
Zone A	retraction	8.3% [5.9% - 11.5%]	1.26
	week	6.4% [3.8%-10.8%]	1.19
	retraction+week	10.5% [7.1% - 15.8%]	1.34
Zone B	retraction	9.6% [7.1% - 12.9%]	1.31
	week	6.4% [3.4%-11.9%]	1.19
	retraction+week	11.5% [7.9% - 17.6%]	1.38
Zone C	retraction	11.2% [7.7% - 16.3%]	1.36
	week	8.0% [4.7%-13.7%]	1.25
	retraction+week	13.8% [9.0% -21.3%]	1.46

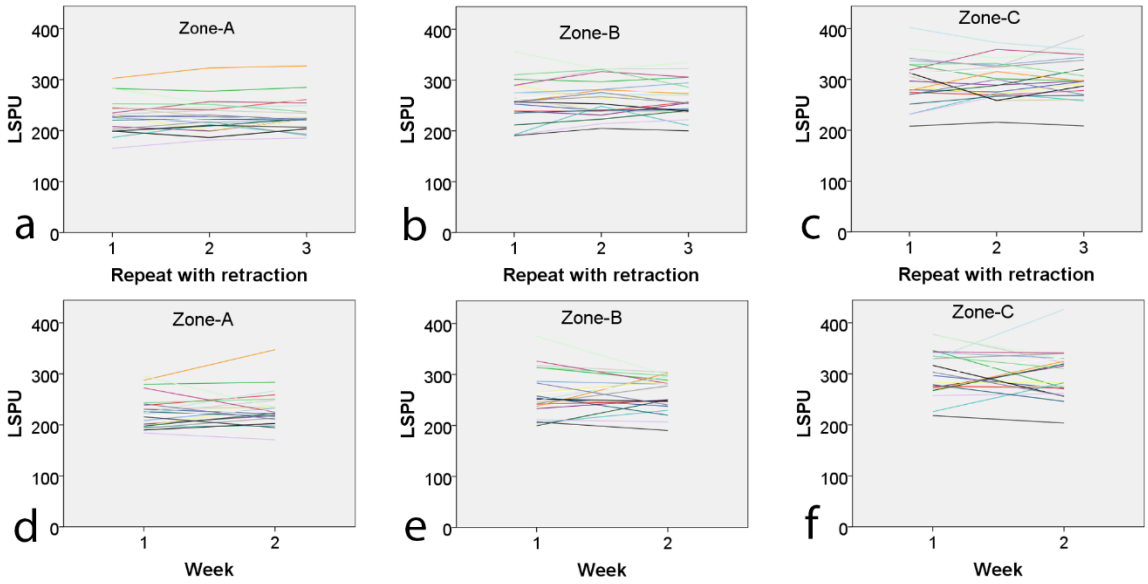

Figure 23: The variation of GBF in Zone A, B and C within subjects. The lines of different colors represent the mean GBF of different subjects during the three repetitions with retraction (intra-day, upper panel) and on two different days (inter-day, lower panel).

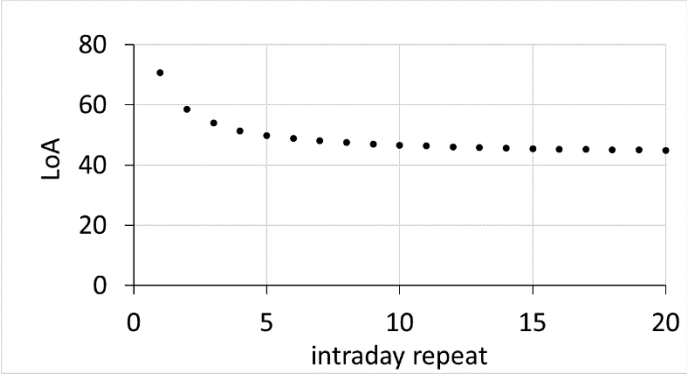

Figure 24: The effect of intra-day repetitions on the standard error (SE) between two measurements performed on two different days (t1 and t2). LoA: Limits of agreement.

6.5.2 The effect of retraction on intra- and inter-day reliability in Zone B

No significant differences were found either between the means of the three repeated retractions (259±6.9, 265±6.1, 264±6.2, p=0.201) or between the GBF means measured on two different weeks (263±5.8, 262±4.6, p=0.944). Intra-day repeatability with retraction was good and inter-day reproducibility including the intra-day repetitions was moderate (Table 6). Individual variation is shown in Figure 23b and e.

The between-subject CV was 11.6% [7.6%–17.8%]. The intra-day ICC was 0.66. Using only a single measurement within a day, the inter-day ICC was 0.51, but increased to 0.64 by averaging the three repeats.

6.5.3 The effect of retraction on intra- and inter-day reliability in Zone C

No significant differences were observed either between the means of the three repeated retractions (297±8.1, 297±6.5, 303±7.7, p=0.528) or between the GBF means measured on two different weeks (263±5.8, 262±4.6, p=0.834). Intra-day repeatability with retraction and inter-day reproducibility including the intra-day repetitions were both moderate (Table 6). Individual variation is shown in Figure 23.

The between-subject CV was 9.5% [4.7%–19.2%]. The intra-day ICC was 0.55. Using only a single measurement within a day, the inter-day ICC was 0.32, but increased to 0.56 by averaging the three repeats.

6.6 The effect of using a mirror on reliability (exp. VI)

The mean blood pressure (88.4±1.45 vs. 84.3±1.2 mm Hg, p<0.001) and the heart rate (70.0±3.44 vs. 66.3±2.93 1/min, p<0.001) changed slightly but significantly within a measurement session.

No significant differences were found either between the means of the three repeats (234±6.4, 241±7.3, 243±7.5, p=0.425) or between the GBF means measured by a mirror versus directly (237±5.7, 242±5.9, p=0.171). Individual variation is shown in Figure 25a. The CV values calculated from the three repeats carried out with the same method (repeatability) and the CV value of the method were both good (Table 7).The CV values of the three repeated measurements using the mirror method (9.5% [7.0–12.8%]) were similar to the directly measured values (10.3% [7.2%–14.8%]). Overall reproducibility without distinguishing between the capturing methods was moderate. The between-subject CV was 16.2% [11.3%–23.1%]. The intra-day ICC was 0.55. The overall ICC was 0.65 and increased to 0.73 if the same method was used for the repetitions.

Table 7: Repeatability of the repetitions with or without changing the capturing method

		CV	CR
Zone A	repeat	10.4% [8.3% - 13.0%]	1.33
	method	5.9% [3.8%-9.2%]	1.18
	repeat + method	11.9% [9.1% - 15.9%]	1.39

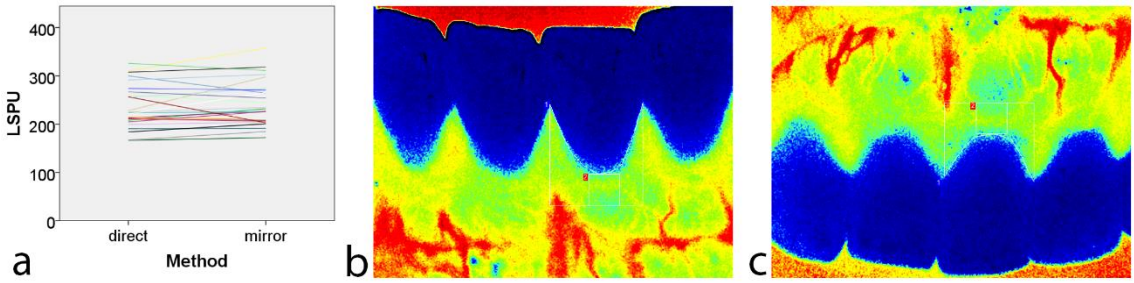

Figure 25: The variation of GBF within subjects due to the method used (direct or indirect).

The lines of different colors represent the mean GBF values of the three repeated measurements using the same method in different subjects (a). A representative LSCI

image was taken of the mandibular front region directly (b) or using a mirror (c). The pattern of the vessels is identical in both images.

6.7 The reliability of repeated measurements in a clinical surgical trial (exp. VII)

No significant change was found in MAP throughout the observation period (90±2.6 mm Hg at bsl. versus 85±2.9 mm Hg on day 180). There was no correlation between MAP and blood flow in either zone (Zone A: r=-0.115, p=0.140; Zone B: r=-0.130, p=0.096; Zone C: r=-0.057, p=0.471). Therefore, the blood flow values were used instead of vascular resistance in the following analysis.

Mean GBF at the non-treated sites was typically under the baseline values during the six-month healing period in all zones (Figure 26); however, this did not reach the conventional significance level (time factor: p=0.097 for Zone A, p=0.182 for Zone B and p=0.250 for Zone C).

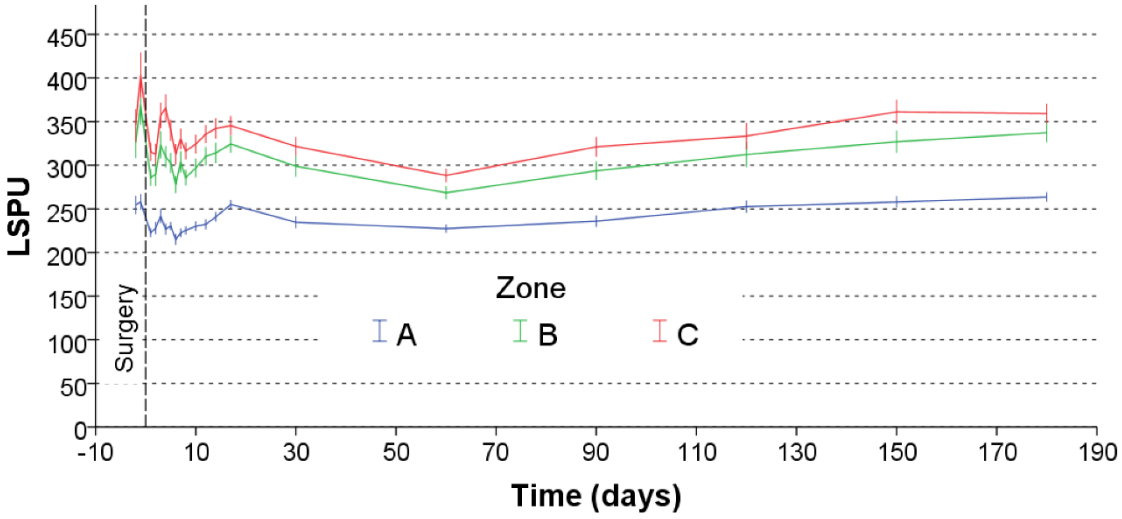

Figure 26: The alteration of GBF in Zone A, B and C during the six-month follow-up at the reference teeth.

In Zone A, the between-subject CV was 12.8% [6.9%–23.8%] and inter-site variation within subjects was 7.9% [5.6%–11.2%]. The intra-day CV and the inter-day CV of a site

were both moderate in all zones (Table 8) with fair to good ICC values. The inter-day ICC of a site increased from 0.47 to 0.52 by averaging the intra-day repeats.

In Zone B, the between-subject CV was 19.4% [10.4%–36.1%] and inter-site variation within subjects was 13.1% [9.2%–18.6%]. The inter-day ICC of a site increased from 0.54 to 0.61 by averaging the intra-day repeats.

In Zone C, the between-subject CV was 11.8% [4.9%–28.6%] and inter-site variation within subjects was 15.7% [11.1%–22.1%]. The inter-day ICC of a site increased from 0.45 to 0.51 by averaging the intra-day repeats.

Table 8: Intra-day repeatability with retraction and inter-day reproducibility of the 20 measurement sessions at a reference site

		CV	CR	ICC -tooth
Zone A	intra-day	11.0% [10.5% - 12.0%]	1.36	0.75
	inter-day	16.2% [14.7%-17.9%]	1.57	0.47
	together	16.3% [14.7% - 18.3%]	1.57	
Zone B	intra-day	16.7% [15.9% - 17.9%]	1.59	0.72
	inter-day	21.5% [19.7%-23.5%]	1.81	0.54
	together	21.6% [19.7% - 23.9%]	1.82	
Zone C	intra-day	17.0% [16.2% - 18.2%]	1.60	0.66
	inter-day	21.7% [20.0%-23.7%]	1.82	0.45
	together	21.8% [20.0% -24.2%]	1.83	

6.8 Assessment of oral mucosal blood flow following periodontal plastic surgery (exp. VIII)

6.8.1 Blood flow at the treated sites on the days following the surgery in Zone A

The statistical analysis showed that not only the graft (graft x time: $p<0.001$) but also gender has a strong influence (gender x time: $p<0.001$) on the blood flow of the healing mucosa. Furthermore, a significant interaction was observed between gender, graft type

and time ($p<0.001$). The data were therefore split into two subgroups based on gender in addition to the two graft types (Figure 27).

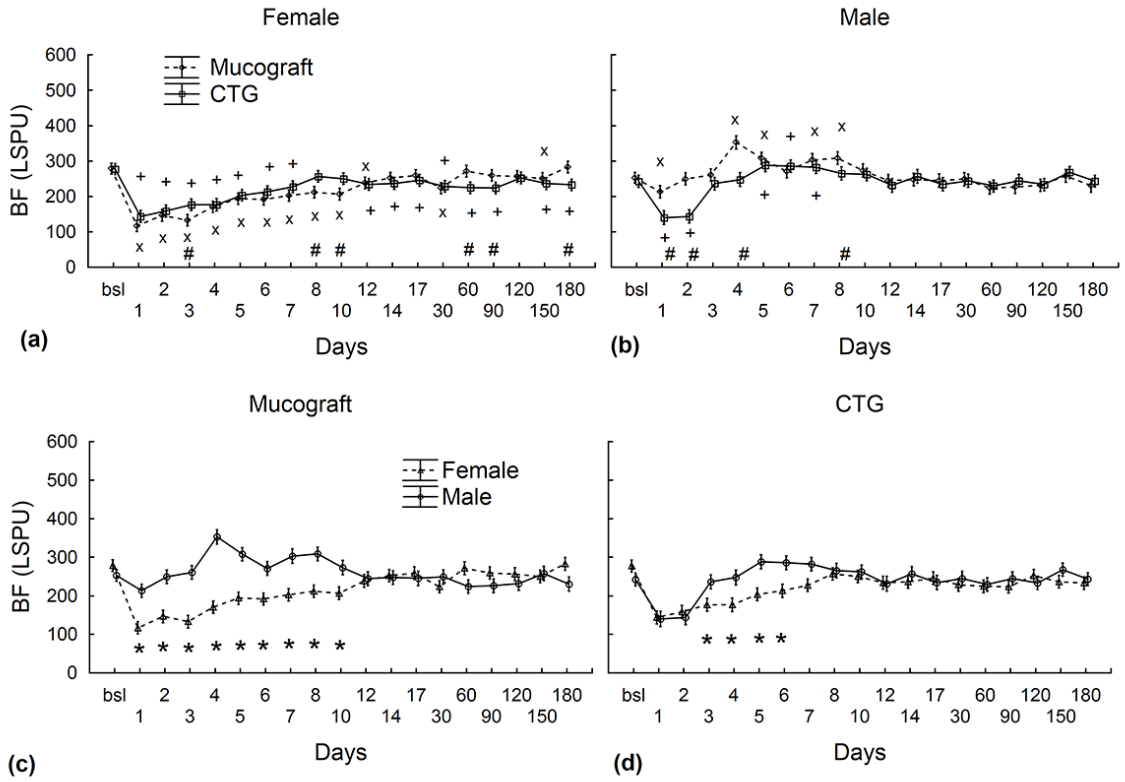

Figure 27: Time-course of the changes of gingival blood flow (BF) in Zone A, expressed in Laser Speckle Perfusion Units (LSPUs).

Time points include preoperative data (bsl.) and postoperative days (1 to 180). Data are presented as means ± SE. In (a) and (b), statistically significant differences in the postoperative values versus bsl. are indicated by × for Geistlich Mucograft® ($n = 14$) and by + for CTG ($n = 13$). Differences between the grafts at the respective time points are indicated by #. In (c) and (d), the same data are shown in a different grouping as gender differences are depicted separately for Geistlich Mucograft® and for CTG. ∗ indicates significantly different time points between the genders. ×, +, # and ∗ mark significance levels of $p < 0.05$ after being adjusted by the Benjamini and Hochberg method.

In females, blood flow at the treated teeth dropped significantly, approximately to half of the baseline values in the case of both Geistlich Mucograft® and CTG on the first day after the surgery (Figure 27a). After day 2, blood flow increased towards the baseline but remained below it until day 12 in Geistlich Mucograft® patients and until day 7 in CTG patients. Over the six-month period, there was only a slight difference in flap circulation between the two graft groups.

In males, contrary to females, there were marked differences in blood flow between the two grafted sites (Figure 27b). Blood flow at Geistlich Mucograft®-treated sites returned to the baseline on day 2 and a hyperemic response occurred from day 4 to day 8. At CTG-treated sites, blood flow returned to the baseline on day 3, and a reduced and shorter hyperemic response developed between day 5 and day 7. Perfusion at Geistlich Mucograft®-treated sites significantly exceeded the corresponding values for CTG on day 1, 2, 4 and 8.

On Figure 27c and d, the same data as on the upper panels were reconstructed for comparison between the genders at each time point, separately for each graft. The blood flow values of males significantly exceed those of females between days 1 and 10 in the case of Geistlich Mucograft® and from day 3 to day 6 in the case of CTG.

6.8.2 Blood flow at the treated sites on the days following the surgery in Zone B

Similarly to Zone A, the analysis was done at the level of the gender x graft x time ($p<0.001$) interaction (Figure 28, upper panels). However, contrary to Zone A, the effect of the graft as a main factor was strong and significant ($p<0.001$) while gender x graft was not due to the fact that blood flow at Geistlich Mucograft®-treated sites was always above CTG values regardless of gender.

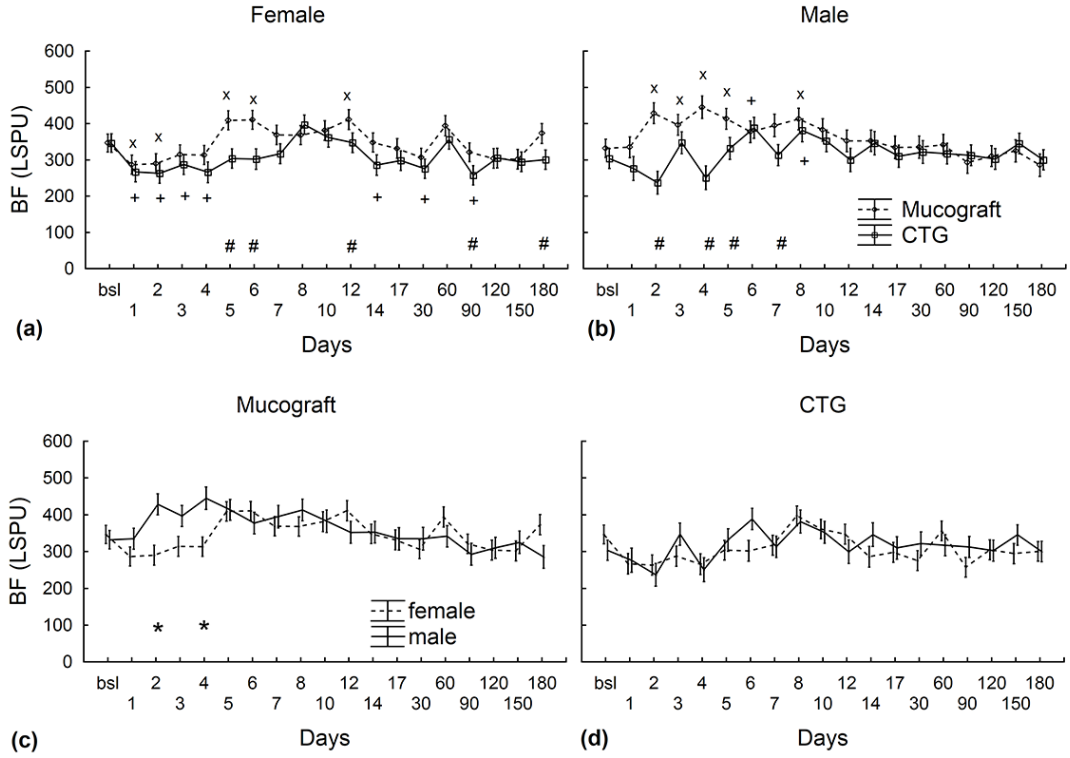

Figure 28: Time-course of the changes of gingival blood flow (BF) in Zone B, expressed in Laser Speckle Perfusion Units (LSPUs).

Time points include preoperative data (bsl.) and postoperative days (1 to 180). Data are presented as means ± SE. In (a) and (b), statistically significant differences of the postoperative values versus bsl. are indicated by × for Geistlich Mucograft® (n = 14) and by + for CTG (n = 13). Differences between the grafts at the respective time points are indicated by #. In (c) and (d), the same data are shown in a different grouping as gender differences are depicted separately for Geistlich Mucograft® and for CTG. ∗ indicates significantly different time points between the genders. ×, +, # and ∗ mark significance levels of $p < 0.05$ after being adjusted by the Benjamini and Hochberg method.

The graphs on the lower panels of Figure 28 demonstrate that, only in the case of Geistlich Mucograft®, blood flow values were significantly higher in males than in females on day 2 and 4 while no difference was detected at CTG-treated sites.

6.8.3 Blood flow at the treated sites on the days following the surgery in Zone C

Blood flow in Zone C was less affected by the surgery, but still, the effect of time was significant ($p<0.001$). Neither the graft nor the graft x gender interaction had an overall effect on blood flow ($p=0.84$; $p=0.89$). As in the case of Zones A and B, the analysis showed that all interactions with the time factor (graft x time: $p<0.001$; gender x time: $p<0.001$; gender x graft x time: $p<0.001$) were significant, indicating some variations in blood flow by time over the observation period (Figure 29). The graphs on the lower panels of Figure 29 demonstrate that there were no differences observed in this zone between the genders.

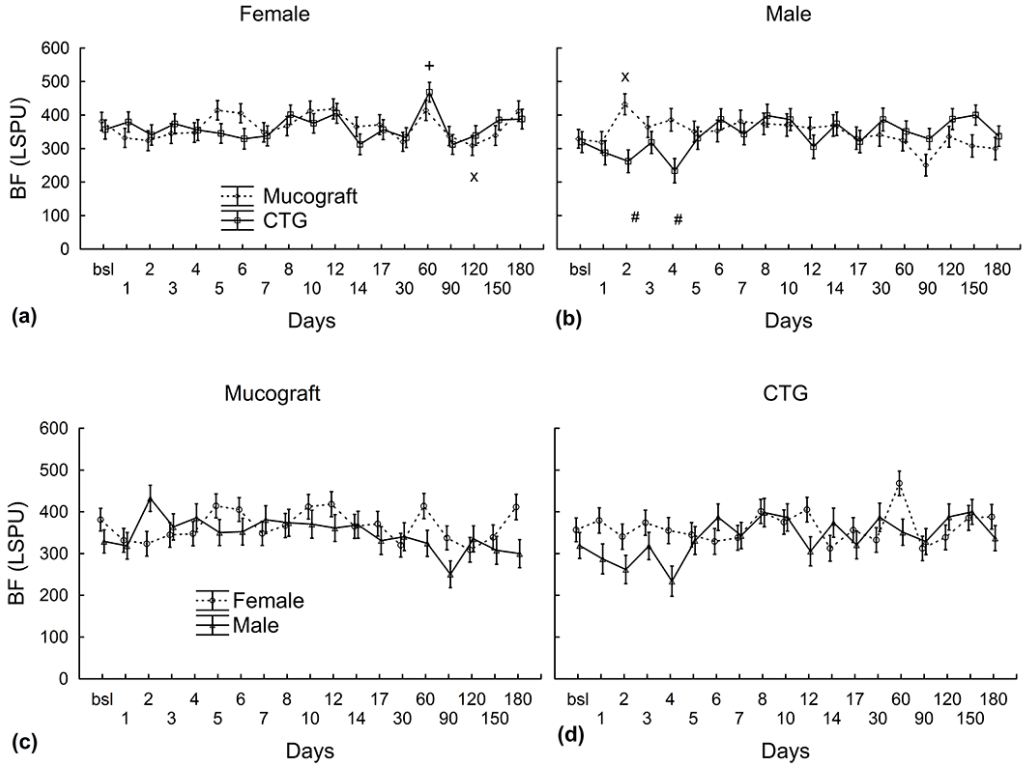

Figure 29: Time-course of the changes of gingival blood flow (BF) in Zone C, expressed in Laser Speckle Perfusion Units (LSPUs).

Time points include preoperative data (bsl.) and postoperative days (1 to 180). Data are presented as means ± SE. In (a) and (b), statistically significant differences of the postoperative values versus bsl. are indicated by × for Geistlich Mucograft® (n = 14) and by + for CTG (n = 13). Differences between the grafts at the respective time points

are indicated by #. In (c) and (d), the same data are shown in a different grouping as gender differences are depicted separately for Geistlich Mucograft® and for CTG. There were no significant differences observed between the genders. ×, + and # indicate significance levels of $p < 0.05$ after being adjusted by the Benjamini and Hochberg method.

6.8.4 The kinetics of wound fluid production

The two main factors, graft type (p=0.85) and gender (p=0.13) were not significant, but time (p<0.001) was. Interactions between graft x time (p=0.70) and graft x gender x time (p=0.46) were not significant either, but the graft x gender interaction was significant (p<0.001). This means that, overall, the WF production of Geistlich Mucograft®-treated sites (13.8 [+2.6, -2.2] PS) exceeded that of CTG-treated sites (10.7 [+2.1, -1.8] PS) in females (Figure 30a). On the other hand, in males, the opposite was found: Geistlich Mucograft®-treated sites had less WF (6.9 [+1.5, -1.2] PS) than CTG-treated sites (10 [+2.4, -1.9] PS) (Figure 30b).

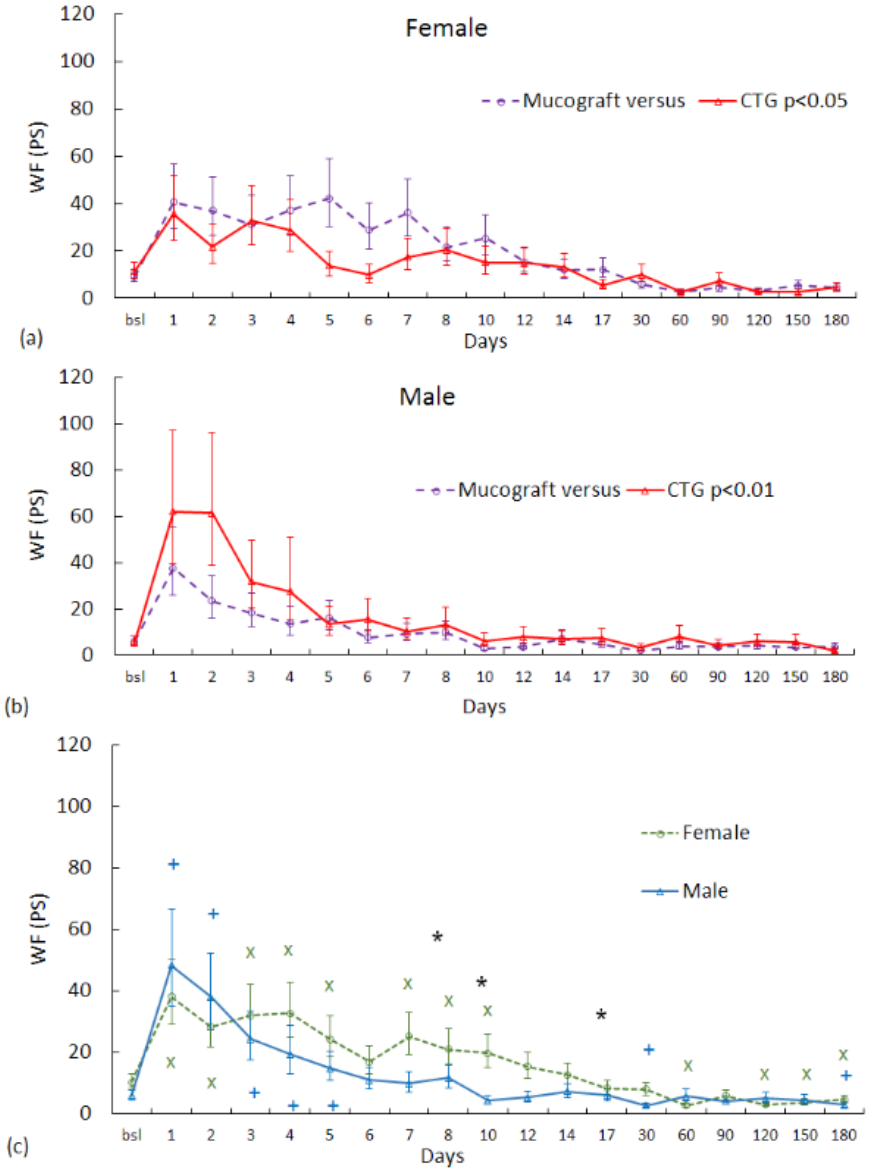

Figure 30: The effect of time, graft and gender on wound fluid (WF) production. The two upper graphs (a and b) show the interaction between graft and gender in wound fluid (WF) production during the whole period, expressed in Periotron Scores (PS). The lower plot (c) shows the changes of WF production over time when the graft data were grouped. Time points include preoperative data (bsl.) and postoperative days (1 to 180). Data are presented as means ± SE. Statistically significant differences of the postoperative values versus bsl. are indicated by × in females and by + in males. The differences between the genders are indicated by ∗ ($p < 0.05$, adjusted by the Benjamini and Hochberg method).

As time interacted with gender ($p<0.001$), pairwise comparison was made at each time point (Figure 30c). PSs increased dramatically in both genders on the first day after surgery. They remained significantly higher than the baseline until day 10 in females and until day 5 in males. On the first two days, WF looked similar in both genders but from day 3 the values in males dropped steeper than in females. One month after the surgery, WF tended to be lower than the respective baseline values for both genders.

6.8.5 The correlation between WF and blood flow

During the early healing period, blood flow in Zone A showed a moderate inverse correlation with WF production on day 4 ($r=-0.55$, $p<0.05$), day 5 ($r=-0.49$, $p<0.05$), day 6 ($r=-0.51$, $p<0.05$) and day 7 ($r=-0.61$, $p<0.01$).

6.8.6 Clinical parameters

Baseline GRD0 and GRW0 were very similar in the Geistlich Mucograft®- and the CTG-treated groups (Table 9), however, the initial KT0 was significantly less in the CTG-treated group. Gains in the depth (REC) and width (RW) of the recessions were similar in the two groups. The increase in KT at Geistlich Mucograft®-treated sites was significantly less than at CTG-treated sites (Table 9).

Table 9: Gingival recession characteristics obtained at baseline and six months after the surgery.

GRD0: gingival recession depth at baseline; GRW0: gingival recession width at baseline; KT0: width of the keratinized tissue at baseline; REC: recession depth reduction; RW: recession width reduction; KT: increase in the width of the keratinized tissue. # represents statistically significant differences between the graft types; $p < 0.05$.

	GEISTLICH MUCOGRAFT® (n = 14 SITES) MEAN ± SE IN MM	**CTG (n = 13 SITES) MEAN ± SE IN MM**
GRD0	2.4 ± 0.23	2.8 ± 0.27
GRW0	3.2 ± 0.28	3.2 ± 0.26
KT0	3 ± 0.41#	1.4 ± 0.35
REC	1.9 ± 0.29	2.6 ± 0.27
RW	2.1 ± 0.47	2.7 ± 0.40
KT	−0.7 ± 0.29#	0.7 ± 0.41

No statistically significant differences were observed between females and males either in the baseline values or in REC, RW and KT (data not shown). REC and RW were positively correlated with the baseline values (r=0.92, p<0.001 and r=0.64, p<0.001). In contrast, KT was negatively correlated with KT0 (r=-0.79, p<0.001).

7 DISCUSSION

7.1 The relationship between periodontal inflammation, smoking and blood flow, measured by LDF (exp. I, II, III)

We found that a short application of heat on the human gingiva resulted in a temporary increase in GBF. To the best of our knowledge, there are only two references in the literature on the application of a heat test on the human gingiva. Similarly to our results, after a hot water flush, a hyperemic response was observed by Baab et al. (116) measured by Laser Doppler Flowmetry and the application of hot water produced a significant increase in pulse amplitude in the healthy gingiva measured by reflection photoplethysmography (79). However, neither the characteristics, nor the mechanism of the vascular changes have been described yet.

The mechanism of vasodilation after a heat challenge in the skin is extensively researched (166-168). Thermal hyperemia in the skin is characterized by a biphasic increase in skin blood flow. A rapid initial peak is observed within 1 to 2 minutes after the onset of heat application, which mostly depends on a local sensory nerve axon reflex since it can be significantly attenuated by local anesthesia (169). The initial peak is followed by a prolonged plateau which mainly depends on the release of nitric oxide (170, 171). It should be noted that in our experiment only one peak was observed which may be explained by the short application of heat (30 s or 80 s). Contrary to skin experiments where it is continuous throughout the measurement, with our technique, recording is not possible during the application of heat. Another difference compared to the skin is the magnitude of the response. In the human skin, it is possible to bring about even a 10-fold increase by the application of 42 °C heat without a pain stimulus. However, based on our results, the increase was less than 2-fold in the human gingiva. Subsequent application of light (for a total of 300 s) was able to induce a further dose-dependent increase in GBF (data not shown), but the maximum response was only 3-fold and already accompanied by slight pain in some of the patients. The pain itself could evoke nerve-mediated vasoregulatory alterations which may interfere with heat-induced vascular control mechanisms. Therefore, the pain-induced component of GBF increase has to be avoided during the heat test.

Interestingly, the increase in GBF after heat application was solely due to the increase in the average speed of blood cells without a change in CMBC. By contrast, for the skin, most studies found little or no increase in velocity but did so in CMBC (172, 173). Fredriksson et al. used velocity-resolved quantitative Laser Doppler Flowmetry in the skin and found that blood flow, in parallel with CMBC, greatly increased during local heat provocation in the high-velocity region but not in the low-velocity region (174). Based on these findings they concluded that increased flow due to heat provocation is shunted from the artery to the vein side. In healthy gingival tissue, the microvessels supplying the marginal gingiva run perpendicular to the outer surface, forming long hairpin capillary loops (175-177). They receive their arterial blood supply mainly from the vessels within the periosteum of the alveolar process running parallel to the epithelium. In our experiments the laser Doppler probe was positioned perpendicular to the surface of the gingival margin to measure the capillary flow of blood cells moving mostly parallel with the laser beam. We assume that the increase in flow observed in the terminal capillaries was due to proximal arteriolar dilation without opening new capillaries, which resulted in an increase in the speed without a change in CMBC. Theoretically, the relatively larger dilation of the arterioles than that of the venules could also contribute to the selectively increased Speed observed. The main function of the increased flow after heat stress is to eliminate excessive local heat, but the metabolic demand of the tissue does not require the opening of inactive capillaries.

The differences in thermal response (magnitude and speed) between the skin and the gingiva may be explained by the higher thermal conductance and the lower basal temperature of the skin. Furthermore, basal cutaneous blood flow is much lower than that of the oral mucosa (178), which may account for the higher increase in the skin. One of the fundamental roles of skin blood flow control is the thermoregulation of the body. However, the gingiva does not have such a function. It should rather have its own defense mechanisms against heat stress which may be activated during the consumption of hot food or during smoking. Overall, the gingiva seems to have a distinct thermoregulatory mechanism, but further human and animal studies are required to understand the vascular regulatory mechanism in the gingiva subsequent to a thermal challenge.

Heat challenge to the skin is usually applied using a thermostatic heating probe device made of solid metal (179-181). But this method is not practical in the case of the gingiva

because its surface is curved. In addition, the heating device could mechanically compress the thin gingival tissues, including vessels. We used two relatively simple clinically applicable techniques, namely warm saline and light-induced heat provocation. We found that both methods can be applied on the human gingiva with distinct benefits. Warmed saline is routinely applied in the oral cavity as a daily rinse after tooth extraction to prevent alveolar osteitis (182) and it may improve edema as well by stimulating lymphatic circulation (183). Based on our experiments, local warming can have additional benefits after oral flap surgery as it may increase blood flow in the low-perfused flap, especially after augmentation procedures. However, dropping saline on the gingiva may cause mechanical stimulation of the mucosa which may also interfere with the examination method, while the spreading fluid can cause a certain degree of discomfort to the patient and may also interfere with LDF measurements. Light-induced heat has advantages in experimentation as it involves no mechanical stimulation, causes no discomfort and allows for a better timing of heat application (start and end). Despite similar peak responses, light resulted in a prolonged increase in GBF, which is possible due to the deeper penetration of heating light beams and to the longer stimulus.

In this pilot study, we also investigated the relationship between blood flow in the marginal gingiva and periodontal inflammation. No correlation was found between periodontal inflammation and GBF at rest. Studies investigating the effect of periodontal inflammation on basal gingival blood flow found conflicting results. Some animal studies (69, 70) demonstrated increased blood flow in the inflamed gingiva involving bone loss (a combination of gingivitis and chronic periodontitis). However, in the same species, Baab and Öberg (71) found no significant correlation between the gingival index, GCF and blood flow, and the elimination of the inflammation did not result in a decrease in blood flow either. In humans, experimentally induced gingivitis resulted in decreased blood flow to the gingiva (72, 73) whereas naturally occurring gingivitis resulted in increased blood flow (72). GBF volume at rest was found to be lower in periodontitis (74) and the treatment of gingivitis (75) or periodontitis (76) reduced blood flow. A possible explanation for conflicting results are variations in gingival blood flow as a function of time and the location of the laser Doppler probe. Temporal variation related to biological variation may be influenced by many physiological factors in addition to the inflammation, such as circadian rhythm (77), blood pressure (78), temperature (79) or

tooth brushing (72, 80, 81). Furthermore, although no data are available about the effects of disinfectant mouth rinses, eating and drinking on GBF they may influence the recordings. That is why we kept all these factors standardized before and during the measurements. To overcome this problem and to better control the temporal and spatial variation of blood flow, a heat provocation test, which is a relative functional measurement, was implemented on the gingiva instead of an absolute measurement. As a result of the application of heat of standard temperature, a relationship was discovered between inflammation and gingival circulation. In contrast to GBF at rest, the MAX value, the GFPA-bsl and the GFPA-heat values were found to be positively correlated to GCF. GBF also returned to the level measured at rest more quickly in the case of more severe inflammation. These findings suggest that the heat provocation test could be a useful tool to detect vascular changes in periodontal inflammation.

Similarly to our results, faster recovery of the blood flow after cooling was found in periodontitis (184), but according to another study (185) the temperature of the gingiva seems to recover slower in periodontitis. These data suggest that the observed shorter vascular response after a thermal challenge does not necessarily mean better thermoregulation; instead, regulation can be impaired as well. As we could not measure the surface temperature of the gingiva during the provocation test we cannot draw a conclusion on whether thermoregulation was changed by the inflammation. However, we found that increased GCF is accompanied by increased absolute MAX GBF which suggests that increased flow may eliminate the excess heat more quickly. It is also possible that the preconditioning effect of the continuous inflammatory insult may also promote gingival vascular responsiveness. Furthermore, in periodontitis, there is an increase in vascular density with more dilated and collateral vessels than in the healthy gingiva, which is a possible explanation for the higher magnitude of heat-induced hyperemia and faster recovery in the case of periodontal inflammation (65, 12, 13).

An increasing number of studies have suggested that cutaneous circulation can serve as a model for generalized microvascular dysfunction (186, 187, 167) which can be an early sign of disease before the chief symptoms appear (188, 189, 187, 190). Whether the vasodilatory function of gingival microvessels can be used as a substitute measurement for the small vessel function in the same manner as in the skin model (186) needs further investigation, but our implemented heat provocation test could be a way to test this in

human subjects. Our results are in concord with the findings of an animal experiment (20) where both the periodontal inflammation and diabetes influenced gingival vascular reactivity; therefore, we need to carefully distinguish between the effects of local inflammation and systemic conditions.

Our results showed that smokers have similar GBF values at rest as non-smokers. Similarly, other studies found no difference in the GBF at rest of non-smoking and smoking periodontitis patients (74). However, the cessation of smoking improved GBF and restored GCF to the normal non-smoker level in chronic smokers (147), suggesting that smokers may have reduced gingival blood flow. Unfortunately, no comparison was performed with a non-smoking group. We found a similar extent of vasodilation after the heat challenge in the gingiva of smokers and non-smokers. This is another difference to the skin where vasodilation after heat provocation is attenuated in smokers, possibly due to the decreased availability of nitric oxide in smokers (191). However, in our study, blood flow recovered slower after heat stress in smokers from similar MAX values as in non-smokers, and contrary to non-smokers, heat decreased relative pulse amplitude. Furthermore, the correlation of blood flow parameters with GCF were weaker in smokers. However, the lower range of GCF values in our smoking subjects may as well be the reason for our findings as GCF can be reduced by smoking both in healthy and inflamed periodontal tissue compared with a corresponding non-smoker group (192, 193). This suggests that smoking may suppress the symptoms of inflammation (194). Previous studies (195, 196) showed that the GCF volume serves as an index for the extent of periodontal destruction and the severity of clinical inflammation, but that may not be applicable to smokers. The limitation of the present study is that only GCF measurement was used for assessing the current degree of inflammation, regardless of the classification of the periodontal disease (gingivitis, chronic periodontitis or aggressive periodontitis) and previous treatment, if there was any. Consequently, we cannot conclude based on this pilot study that there is a vascular impairment in the gingiva of smokers even though we found some differences in vascular parameters. The clinical classifications of periodontitis tend not to be correlated with tissue and molecular reactions (197), which calls for the setting up of a new biologically based model for the classification of periodontal disease. Using one of these novel molecular methods to correlate vascular

reactivity with the severity of the disease may answer the question of vascular impairment in smokers.

Local functional heat challenge tests may be a useful method to examine the vascular reactivity of the gingival tissue. The light-induced thermal test seems to be more advantageous under clinical conditions than the warm saline induced test. Compared to the skin, the increase in GBF after heat provocation is much smaller and, interestingly, only the increase in the speed of flowing blood cells is responsible for it while the increase in their concentration is not. Furthermore, periodontal inflammation promotes increased peak flow and faster restoration after heating in non-smokers, but not in smokers. Our data suggest that moderate periodontal inflammation may facilitate gingival vascular responsiveness which, however, is suppressed by smoking. At the same time, as smoking itself is able to suppress the clinical symptoms of periodontal inflammation (including GCF), further investigations are needed to compare the vascular reactions of non-smokers and smokers, with a more detailed biological classification of periodontal disease status based on molecular markers.

7.2 Utilizing LSCI methods to measure blood flow in the human oral mucosa (exp. IV, V, VI, VIII)

We found no systemic effect of lip retraction or using a mirror in consecutive measurements of GBF with low measurement error. Unexpectedly, the incidence angle has some effect on the mean of the measurements. Contrary to our study on the gingiva, the incidence angle does not influence LSCI measurements in the skin even when a much wider range of angles (up to 45 degrees) is used (129, 155). In our experiment, the patient's head was turned by only 10 degrees approximately, a limit imposed by the field of view. However, since the surface of the gingiva is curved unevenly in three dimensions due to the alveolar arch and root prominences, the actual incidence angle of the laser light may be higher. Standardizing this angle under clinical circumstances would be demanding, especially when multiple sites within multiple patients are monitored. Therefore, it is advisable to control the possible effect of incidence angle statistically. One solution is to take repeated snapshots from different views and to average out the difference.

The coefficient of variation was the smallest in the experiment involving turning of the head in which the lips were retracted constantly by a lip retractor. As no direct intervention on the gingiva was applied between two measurements, this CV could be considered an inherent component of clinical LSCI measurements. These values (5.4%–6.0%) were very similar to the residual error of LSCI measurements in animal experiments (198, 199) where most clinical factors (retraction, reflection, most of the movements) can be eliminated. This suggests that it is difficult to further reduce this component, which may include not only technical factors such as the selection of regions by visual inspection, motion artefacts and the internal error of the LSCI instrument (155), but also short-term biological variation due to breathing, pulse and vasomotion.

In the retraction, mirror and surgical experiment, repeatability was considerably lower. Furthermore, a higher error was recorded for the zones closer to the vestibulum (Zone A<Zone B<Zone C). The attached gingiva is mainly supplied from the alveolar mucosa. Vessels of moveable alveolar mucosa may be sensitive to retraction as the retraction tool partially compresses arteries. This could induce either a drop in GBF, or inversely, reactive hyperemia, which may cause random variation in GBF in the neighboring attached gingiva, especially in Zone C.

In a great number of situations, particularly in surgery, there is no direct access to the area investigated and using a photo mirror to obtain indirect vision is inevitable. However, when a mirror is used, the distance between the camera and the patient's face shrinks due to the fixed 10 cm focal distance, which complicates manipulation. Fogging has to be controlled, more movement artefacts are involved due to the handhold mirror and the adjustment of the angle is even more difficult. This could be the reason for the lowest repeatability value in this experiment.

Inter-day variation was found to be higher than intra-day variation in spite of the standardized conditions. The blood flow of the gingiva may have a day-to-day variability due to unknown factors that cannot be controlled. The effect of some intra-session components could be minimized by repeated intra-session measurements. For example, a sample size of 10 patients in a longitudinal study could be decreased to 7 by four repeats, but more repeats practically do not improve reliability and are therefore unnecessary. The repeats not only decrease the total number of patients needed but may also warn of

accidental mechanical stimuli. This is very important in a surgical study where a failed reading cannot be repeated or corrected later.

In a clinical trial, the reference sites display higher inter-day coefficient of variation than in a standardized test-retest experiment. The reference sites were chosen in every patient so as they were as far from the surgery site as possible, and preferably the contralateral side or other jaw were chosen. However, after surgery, the whole mouth is affected due to several reasons (antibiotics, mouthwash, change in eating habits or in cleaning etc.) and these general effects on the oral cavity may influence the blood flow of the gingiva at the reference sites. Accordingly, responses to the factors affecting the whole mouth may have high individual variability. We presumed that the effect of such systemic factors could be controlled by measuring GBF at the reference sites. However, we found in our clinical trial (125) that normalization of blood flow values at surgical sites to the reference sites did not improve the measurement error. Also, there is evidence that local painful stimuli may evoke a contralateral change in the blood flow of the oral mucosa (200), which may further complicate the control measurements.

Interestingly, we found lower between-subject variability (9.5–23.7%) of gingival blood flow than other studies carried out by LSCI in the skin (23–31%) (201), in the human liver (28%) (127) or in the human stomach (34%) (130). Between-subject variability in our experiment is close to the variability of GBF in humans (12–20%) measured by an absolute technique such as the hydrogen clearance method (105, 202). This suggests that between-subject variability in LSCI measurements mainly depends on biological variation rather than the method used. Most available data on human GBF are obtained by the single-point LDF technique. These data show high between-subject variability of GBF, ranging from 26% to 72% (136, 72, 135, 203, 77). The superior reproducibility of LSCI compared with LDF (122, 123) may probably be explained by its smaller sensitivity to positioning owing to its imaging properties.

We have concluded that the LSCI technique is a non-invasive and non-contact method of excellent reliability for monitoring changes in the microcirculation of the oral mucosa, not only in short-term experiments but also during long-term studies following up disease progression or wound healing. Reproducibility of the long-term measurements can be improved by repetitions of inter-session readings due to the fact that soft tissue retraction, angulation or using a photo mirror do have a minor influence on the measurements.

7.3 Blood flow and wound fluid measurements on a healing periodontal flap after root coverage surgery (exp. VII)

Our primary aim was to introduce LSCI as a method for monitoring the microcirculation of the oral mucosa after periodontal plastic surgery interventions. This imaging technique allows us to observe many various areas on the flap at the same time in contrast to single-point techniques such as Laser Doppler Flowmetry. Another unique property of LSCI is rapid imaging which reduces movement artefacts and decreases the time of each measurement session, especially when multiple images have to be captured within the mouth. These features facilitate patient compliance over many visits. This new method has not been tested before on postoperative mucosal flaps, therefore, we had no data available about intra-day and inter-day variability. In order to get the best estimation of the time-course, we performed multiple repeats on each day and we made measurements on numerous days during the wound healing period. It is important to note that the great number of measurements resulted in a tremendous amount of ROIs (~8000 total, ~1000 per patient, ~150 per tooth-site), which were selected manually through laborious data processing work. In this exploratory study, we managed to define the most characteristic days (1, 3, 7 and 10) from the point of view of flap circulation, which may decrease the number of necessary measurement sessions in further high-scale studies. The split mouth design is also a good way to decrease the number of the patients involved as it reduces the error rate due to the low relative standard deviation (<7%) of the gingival sites within a patient. This low deviation also shows that the effect of the surgical intervention on variability between parallel tooth-sites was managed to be kept fairly standardized, which is probably due to the single experienced operator performing the interventions. Involving some reference sites to normalize the values at the test sites only slightly decreased the error rate, therefore, this step was omitted. The single-point laser Doppler technique is compromised by the spatial variability of tissue blood flow, day-to-day variability due to the repositioning of the probe and the factors of the distance and angulation of the probe, whereas the LSCI method is not (129, 204, 201, 123). In our study, spatial variability was decreased by using thousands of pixels for each ROI spanning 10 to 20 mm^2 and the instrument was set to a fixed focal distance. Overall, these careful settings in our design resulted in low variability among the patients (<11%), which promoted better power for the evaluation of between-group effects, such as gender. The LSCI method was able to

capture not only the massive effect of the surgical intervention but also small differences in the surgical technique used. We assume that this method may help to understand physiological and pathophysiological changes during mucosal healing. Understanding the mechanism would help us to readjust postoperative care and select the best available surgical technique, including incision and flap design, suture, graft size and type etc.

According to our results, the most apical area (Zone C) was the least influenced by the surgery, as this was repositioned directly to vital tissues without an intermediate graft. Moreover, it is a more distensible mucosal area with the best collateral circulation. On the other hand, the most severe ischemia was observed in the marginal area (Zone A) due to the intrasulcular incisions which cut off the main collateral circulation (with the periodontal plexus) of this area. Furthermore, the suspended sutures and the underlying grafts caused probably the highest tension in Zone A. Blood flow in this area returned to the baseline level within 14 days in both sexes in all cases and in some subjects much earlier. In addition, in males, a hyperemic response was also observed after hypoperfusion, between day 4 and day 8 postoperatively.

Furthermore, xenogenic matrices do not have a vasculature contrary to autologous grafts, where revascularization can occur earlier by inosculation (205, 110). The vascularization of the xenogenic graft area only begins after the graft is almost disrupted, which takes months (206, 111). According to an animal study (207), vascularization begins very slowly and sparsely after the application of a Geistlich Mucograft® collagen matrix. The quick restoration of the blood flow long before the expected revascularization confirmed that MCAT only minimally compromised the mucosal vascular architecture and full graft vascularization is not necessary for mucosal regeneration. The early recovery of flap circulation helps to cover and protect the grafted area as well as to promote tissue integration. Similarly, it was observed previously (92) that a careful and less invasive surgical approach – e.g. employing micro- rather than macro-surgical techniques – may better maintain circulation and speeds up revascularization. This also resulted in better clinical performance (208, 209). Favorable flap circulation and the relatively intact periosteal plexus can provide a good double-layered recipient bed for the grafts. They also ensure abundant nutritive supply to both auto- and xenogenic grafts by imbibition until new vessels develop within them.

In spite of the fact that flap circulation slightly favored the xenogenic matrix, the CTG resulted in similar root coverage compared to Geistlich Mucograft®, with comparable mean baseline recession depth in both groups. This is in accordance with the findings of randomized clinical trials (RCT) (155) where the percentage of root coverage by Geistlich Mucograft® remained only slightly below that of the CAF+CTG. Although the gain in keratinized tissue width was slightly less in the Geistlich Mucograft® than in the CTG group, which is also confirmed by an RCT (210), another RCT found no difference (211, 212). Mean KT at baseline was slightly different across the different groups which may have some effect on the clinical outcome.

To be able to compare our results to other studies, we are constrained here to group our data and plot them without gender separation (Figure 31), since unfortunately none of those studies made a comparison regarding the gender effect despite the fact that their subject population was a mixture of both genders.

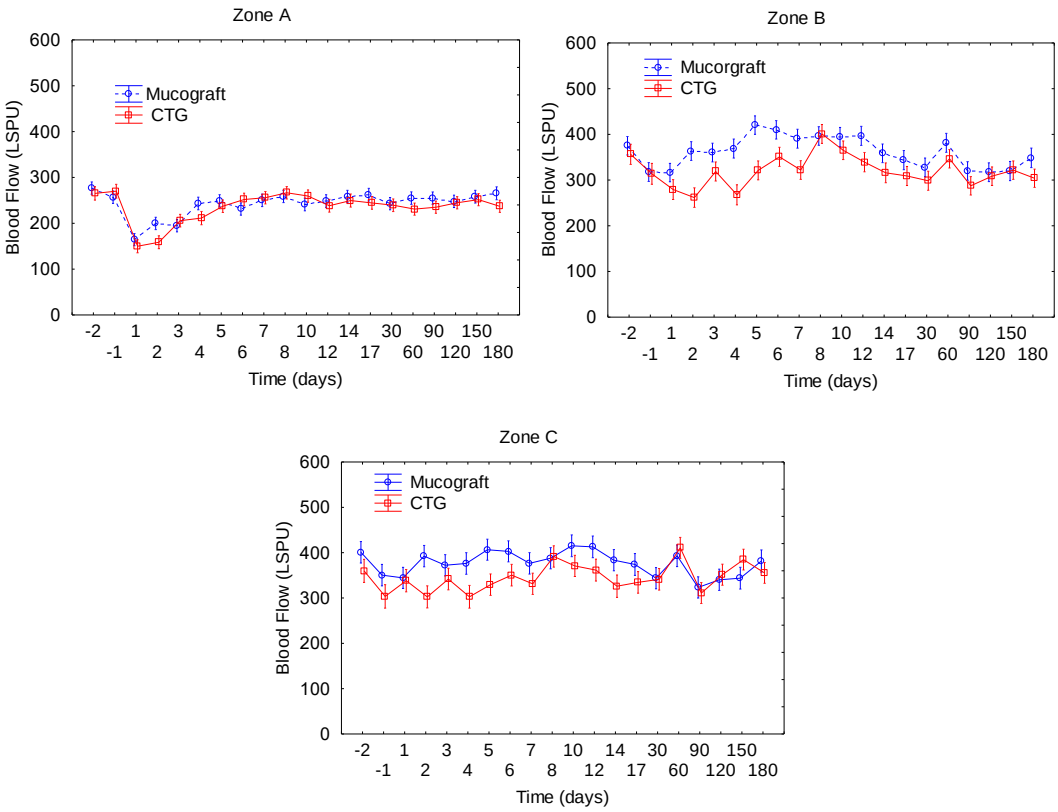

Figure 31: Blood flow values with no gender separation in the Mucograft® and CTG group.

Donos et al. (94) investigated the treatment of single Miller Class I and II recessions with a 3D xenogenic collagen matrix or connective tissue graft (in 3 males and 5 females). The xenogenic collagen matrix exhibited a greater hyperemia than the CTG group at the mucogingival junction and at the coronal area, similarly to what we found in male subjects or in a grouped population. In another study (93), after periodontal surgery or surgical crown lengthening (in 16 males and 14 females), the microcirculation returned within 3 days to unimpaired levels and no hyperemia was observed at the buccal papilla, which is again similar to our findings in a male or in a grouped population.

In a recent study (91), LDF was used to follow up early wound healing after a single tooth extraction with socket preservation or guided bone regeneration (in 8 males and 7 females). Hyperemia recorded at the marginal gingiva was observed from day 3 to one month, with a higher increase observed in the guided bone regeneration group and in case of wound exposure. Ischemia was not observed, however, no measurements were taken on day 1 and 2.

Interestingly, unlike the moderate effects of graft types, gender made a considerable impact on flap circulation. This was an unexpected result compelling us to split our data into two subgroups. Ignoring the gender factor would have resulted in a failure to correctly assess the recovery time of blood flow as the two curves would have cancelled each other out. Furthermore, the effect of the different grafts on microcirculation in both Zone A and B would not have been assessed either. Males had an ischemic phase lasting a few days, followed by a hyperemic response in the marginal gingival zone whereas females were characterized by a slower recovery of blood flow. All baseline clinical parameters of the gingiva, including crevicular fluid and tissue morphology (e.g. recession depth, recession width, width of the keratinized mucosa, thickness of the keratinized mucosa) were similar in the two gender groups. Accordingly, neither the initial subclinical inflammation nor the morphology of the gingival recession can explain the gender-specific postoperative alteration in blood flow. Differences in blood flow between the genders were less pronounced in Zone B and disappeared in Zone C, implying that the gender effect is more important in the marginal zone where the circulation is most severed. To the best of our knowledge, there are no data available on the effect of gender on flap microcirculation in the oral mucosa. Clinical observations (52-55) and findings in an experimental excisional palatal wound model (56) suggest that

mucosal wound healing is faster in males than in females, however, blood flow was not measured in these studies. It can only be supposed that better blood flow recovery may have facilitated wound healing in males.

The MCAT recovered earlier than the expected complete revascularization period, suggesting that higher blood flow in males occurred due to the vasodilation of the remaining vessels. We can further suppose that there may be differences in terms of vascular reactivity between the genders. After surgery, blood flow was reduced as the vascular supply of the flap was compromised, which may result in low-flow mediated vasoconstriction. In the brachial artery, an in vivo prolonged low-flow condition augmented vasoconstriction during occlusion and attenuated hyperemic response (213). Furthermore, low-flow mediated vasoconstriction seems to be more intense in women (214).

Arteriogenesis or 'collateralization' is another important mechanism to maintain perfusion in the case of reduced vascularity before neovascularization can be completed. This involves a proliferative increase in the diameter and length of the arterioles to compensate for the reduced perfusion of the flap (215). In an ischemic limb mouse model, blood flow recovered faster in males and more alpha smooth muscle cell positive vessels – arterioles – were found (216), suggesting greater arteriogenesis. Furthermore, higher maximal vasodilation was recorded in male ischemic limbs in response to acetylcholine and nitroglycerin. Similarly, in patients with stable angina and chronic total occlusion of at least one major epicardial coronary artery (217) and in a rat myocardial infraction model the remodeling of arteriolar vessels (arteriogenesis) was found to be reduced in females (218). There is evidence that the gingiva reacts by collateralization to pathophysiological stimuli such as periodontitis (65, 219, 12). As a conclusion, we hypothesized that in the gingival tissues of males there may be more native collaterals and/or increased collateralization after surgery and/or higher reactivity to vasodilation agents which might be attributable to gender differences.

Until the recovery of graft vascularization, a vascular leak maintains the nutritive supply of the graft via imbibition (110, 220). However, not only the graft but also the distal part of the mucosal flap is supplied by nutrition via extravasation in the early ischemic period of healing (23). In the present study, wound fluid production was measured in order to indirectly and non-invasively assess vascular permeability. Interestingly, blood flow

recovery showed a correlation with recovery to normal tissue transudation. In males, this happened earlier – within 2–3 days for blood flow and within 6 days for wound fluid – whereas in females, it was 8–14 days and 12 days, respectively. As in the case of blood flow in the flap (in Zone A), differences in vascular leakage between the grafts showed some gender-specificity. In females, Geistlich Mucograft®-treated sites had higher fluid production which may be a compensatory mechanism of the lower blood flow while in males the tissue may require less diffusive nutrition due to the superior blood flow compared to CTG-treated sites. Similarly, it was observed in male mice (221) that skin graft angiogenesis peaked at 10 days after grafting and this was coincident with maximum vascular leakage.

Recently, it has been found (154) that GCF measured at day 10 and month 1 was elevated after root coverage surgery and the CTG group had a higher increase at day 10 than the than the group treated with platelet rich fibrin. In another study (93), GCF measurement was performed more frequently and earlier, and it was found – similarly to our results – that GCF was elevated already at day 1 and day 3. The application of Emdogain and biphasic calcium phosphate for the regeneration of bony defects resulted in a higher and prolonged elevation of GCF compared with simple surgical crown lengthening. These results suggest that more complex surgical procedures and the inclusion of more graft material may result in higher and more prolonged vascular leakage.

Similarly, GCF was found to be elevated at day 3, 6 and 9 after surgical tooth removal, regardless of whether socket preservation or guided bone regeneration had been performed (91).

It can be concluded that the application of both grafts resulted in excellent recirculation patterns in the flap. Gender was the most substantial influencing factor. Males showed a more rapid re-establishment of mucosal blood flow. Further study is necessary to investigate the mechanism of gender-specific blood flow regulation in the healing mucosal flap. We found that earlier blood flow recovery is strongly associated with the earlier normalization of vascular permeability. It is conceivable that the opposite changes observed in blood flow versus vascular permeability are equally able to ensure the supply required for the tissues to heal. This, however, did not influence the apparent clinical

outcome: favorable recession coverage could be achieved with both CTG and Geistlich Mucograft® in males and females.

8 CONCLUSIONS

I.: Local functional heat challenge tests could be useful methods to examine the vascular reactivity of the gingival tissue. Based on our experiments, local warming can have additional benefits after oral flap surgery as it may increase blood flow in the low-perfused flap, however, dropping saline on the gingiva may cause mechanical stimulation of the mucosa which may also interfere with the examination method, while the spreading fluid can cause a certain degree of discomfort to the patient and may also interfere with LDF measurements.

II.: The light induced thermal test seems to be more advantageous under clinical conditions than the warm saline induced one. Despite similar peak responses, light resulted in a prolonged increase in GBF, which is possible due to the deeper penetration of heating light beams and to the longer stimulus.

III.: Periodontal inflammation promotes increased peak flow and faster restoration after heating in non-smokers, but not in smokers. Our data suggest that moderate periodontal inflammation may facilitate gingival vascular responsiveness which, however, is suppressed by smoking. At the same time, as smoking itself is able to suppress the clinical symptoms of periodontal inflammation (including GCF), further investigations are needed to compare the vascular reactions of non-smokers and smokers.

IV.: Unexpectedly, in the intraday repeatability of LSCI in oral mucosa measurement incidence angle has some effect on the mean of the measurements, therefore standardizing this angle under clinical circumstances would be demanding, especially when multiple sites within multiple patients are monitored.

V.: Laser Speckle Contrast Imaging has good short- and long-term reliability. Inter-day variation was found to be higher than intra-day variation in spite of the standardized conditions. The effect of some intra-session components could be minimized by repeated intra-session measurements. The repeatability coefficient showed if the repeated value was 1.36 times more or less than the previous measurement, indicating that an artificial

stimulus might have occurred. In this case, it is recommended to repeat the measurement immediately.

VI.: Fogging has to be controlled, more movement artefacts are involved due to the handhold mirror and the adjustment of the angle is even more difficult. This could be the reason for the lowest repeatability value in this series of experiments. It is preferable to use the lip retractors for LSCI measurements.

VII.: In the clinical trial, the reference sites display higher inter-day coefficient of variation than in a standardized test-retest experiment. Responses to the factors that affecting the whole mouth after surgery may have high individual variability. We presumed that the effect of such systemic factors could be controlled by measuring GBF at the reference sites. This technique seems to be appropriate for the long-term clinical non-invasive follow-up of gingival microcirculation.

VIII.: The LSCI method seems to be feasible way to characterize the postoperative flap circulation in oral mucosa. It can be concluded that the application of both grafts resulted in excellent recirculation patterns in the flap. Gender was the most substantial influencing factor. Males showed a more rapid reestablishment of mucosal blood flow. We found that earlier blood flow recovery is strongly associated with earlier normalization of vascular permeability. It is conceivable that the opposite changes observed in blood flow versus vascular permeability are equally able to ensure the supply required for the tissues to heal. This, however, did not influence the apparent clinical outcome; favorable recession coverage could be achieved with both CTG and Geistlich Mucograft® in males and females.

9 SUMMARY

LDF readings are suitable for marginal gingival measurements, e. g. for measuring blood flow changes in gingivitis. It is easier to set the Doppler probe when it is fixed with a manipulator. By connecting a manipulator, it is possible to perform measurements relating to interventions in the distal part of the oral cavity with a small probe. The flowmeter itself is not so expensive, and different probes can be mounted onto the new models, or they can be expanded with different racks, e. g. by a pulsoxymeter.

LSCI is a promising tool to evaluate the microcirculation of the gingiva in two dimensions, real-time and in a non-invasive way, with good repeatability and reproducibility. The main advantage of LSCI readings is that a whole mucogingival flap can be made visible in a speckle image. Since LSCI provides greater image areas, it is more suitable for exploring local regulatory differences than LDF. Differences arising from the spatial variation of blood flow are averaged during evaluation. Having pictures as a result of a measurement enables graphical data collection by defining ROIs. Treated and non-treated sites can also be measured in parallel in one image, if these sites are close to each other. Factors inherent in oral cavity measurements, such as the retraction of the lips and cheeks, using reflected images or a change in incidence angle only slightly influence the readings. The disadvantages of LSCI are its robustness and that it is very sensitive to movements, for example to fluid flow in the oral cavity. It is also costlier than LDF. Additionally, it is important that when the mucosa is heated with a lamp during a local heat test, the light of the lamp interferes with the laser signal of the LSCI and LDF measuring devices, but after the illumination is complete, the measurement can be continued.

Overall, both laser light induced non-invasive blood flow measurement methods are reliable, but one should take the area of application into consideration and choose the method which is best suited to it.

As to future perspectives, LSCI may open up a new perspective for investigating the long-term changes of human gingival microcirculation in clinical situations as well.

10 Összefoglalás

Az LDF alkalmazása megfelelő eszköz gingiva marginális véráramlás mérésekhez pl.: gingivitisben véráramlás változás érzékelésére. A Doppler szondát könnyebb beállítani a megfelelő pozícióba, ha manipulátorral van rögzítve. Manipulátor csatlakoztatásával lehetőség van a szájüreg disztális részén lévő beavatkozásokkal kapcsolatos méréseket is elvégezni kis méretű szondával. Az áramlásmérő mérsékelten költséges, az új modellekhez különböző szondákat is lehet alkalmazni, illetve akár különféle modulokkal bővíthető, pl. pulzoximéterrel.

Az LSCI ígéretes eszköz a gingiva mikrocirkuláció valós idejű vizsgálatára (két dimenzióban, non-invazív módon, jó ismételhetőséggel, reprodukálhatósággal). Az LSCI-mérések fő előnye, hogy egy egész mukogingivális lebeny válik láthatóvá a speckle képen. Mivel nagyobb képterületeket biztosít, mint az LDF, jobban alkalmas a helyi mikrocirkuláció szabályozási különbségeinek feltárására. A véráram térbeli ingadozásából adódó különbségek az értékelés során átlagolódnak. A mérések eredményeként kapott adatok grafikusan kiértékelhetők, ROI-k meghatározásával. A kezelt és kezeletlen területek egyidőben is mérhetők, ha ezek az oldalak térben egymáshoz közel helyezkednek el. A szájüregi mérésekben rejlő tényezők, mint például az ajkak és az orca eltartása, a visszatükröződött képek használata, vagy az incidencia szögének változása, csak kis mértékben befolyásolják a méréseket. Az LSCI hátránya a robusztussága, valamint érzékenysége különböző mozgásokra, például a folyadék áramlására a szájüregben. Továbbá költségesebb is, mint az LDF. Emellett fontos, hogy amikor a nyálkahártyát lámpával melegítik helyi hővizsgálat során, a lámpa fénye megzavarja a LSCI és LDF mérőeszközök lézerjelét, ugyanakkor a megvilágítás befejezése után a mérés folytatható.

Összességében: az alkalmazási terület helyes megválasztása esetén mindkét, lézerfény által kiváltott non-invazív véráramlás mérési módszer megbízható.

A jövőbe tekintően az LSCI új perspektívát nyithat meg a humán gingivális mikrocirkuláció hosszú távú változásainak vizsgálatára klinikai körülmények között is.

11 BIBLIOGRAPHY

1. Jepsen S, Blanco J, Buchalla W, Carvalho JC, Dietrich T, Dorfer C, Eaton KA, Figuero E, Frencken JE, Graziani F, Higham SM, Kocher T, Maltz M, Ortiz-Vigon A, Schmoeckel J, Sculean A, Tenuta LM, van der Veen MH, Machiulskiene V. (2017) Prevention and control of dental caries and periodontal diseases at individual and population level: consensus report of group 3 of joint EFP/ORCA workshop on the boundaries between caries and periodontal diseases. J Clin Periodontol, 44 Suppl 18: S85-S93.
2. Meyle J, Chapple I. (2015) Molecular aspects of the pathogenesis of periodontitis. Periodontol 2000, 69: 7-17.
3. Marcenes W, Kassebaum NJ, Bernabe E, Flaxman A, Naghavi M, Lopez A, Murray CJ. (2013) Global burden of oral conditions in 1990-2010: a systematic analysis. J Dent Res, 92: 592-597.
4. Buset SL, Walter C, Friedmann A, Weiger R, Borgnakke WS, Zitzmann NU. (2016) Are periodontal diseases really silent? A systematic review of their effect on quality of life. J Clin Periodontol, 43: 333-344.
5. Czukor J. (1994) [WHO epidemiologic studies in Hungary in 1985 and 1991]. Fogorv Sz, 87: 223-235.
6. Hermann P, Gera I, Borbely J, Fejerdy P, Madlena M. (2009) Periodontal health of an adult population in Hungary: findings of a national survey. J Clin Periodontol, 36: 449-457.
7. Humphrey LL, Fu R, Buckley DI, Freeman M, Helfand M. (2008) Periodontal disease and coronary heart disease incidence: a systematic review and meta-analysis. J Gen Intern Med, 23: 2079-2086.
8. Kuo LC, Polson AM, Kang T. (2008) Associations between periodontal diseases and systemic diseases: a review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. Public Health, 122: 417-433.
9. Scannapieco FA. (2004) Periodontal inflammation: from gingivitis to systemic disease? Compend Contin Educ Dent, 25: 16-25.

10. Ohlrich EJ, Cullinan MP, Seymour GJ. (2009) The immunopathogenesis of periodontal disease. Aust Dent J, 54 Suppl 1: S2-10.
11. Seymour RA, Ellis JS, Thomason JM. (1993) Drug-induced gingival overgrowth and its management. J R Coll Surg Edinb, 38: 328-332.
12. Zoellner H, Chapple CC, Hunter N. (2002) Microvasculature in gingivitis and chronic periodontitis: disruption of vascular networks with protracted inflammation. Microsc Res Tech, 56: 15-31.
13. Zoellner H, Hunter N. (1991) Vascular expansion in chronic periodontitis. J Oral Pathol Med, 20: 433-437.
14. Bascones-Martinez A, Arias-Herrera S, Criado-Camara E, Bascones-Ilundain J, Bascones-Ilundain C. (2012) Periodontal disease and diabetes. Adv Exp Med Biol, 771: 76-87.
15. Brooks BA, Franjic B, Ban CR, Swaraj K, Yue DK, Celermajer DS, Twigg SM. (2008) Diastolic dysfunction and abnormalities of the microcirculation in type 2 diabetes. Diabetes Obes Metab, 10: 739-746.
16. Leite RS, Marlow NM, Fernandes JK. (2013) Oral health and type 2 diabetes. Am J Med Sci, 345: 271-273.
17. Sanz M, Ceriello A, Buysschaert M, Chapple I, Demmer RT, Graziani F, Herrera D, Jepsen S, Lione L, Madianos P, Mathur M, Montanya E, Shapira L, Tonetti M, Vegh D. (2017) Scientific evidence on the links between periodontal diseases and diabetes: Consensus report and guidelines of the joint workshop on periodontal diseases and diabetes by the International Diabetes Federation and the European Federation of Periodontology. J Clin Periodontol.
18. Scardina GA, Messina P. (2007) Microvascular periodontal alterations: A possible relationship between periodontitis and rheumatoid arthritis. Clin Hemorheol Microcirc, 37: 229-235.
19. Lockhart CJ, Agnew CE, McCann A, Hamilton PK, Quinn CE, McCall DO, Plumb RD, McClenaghan VC, McGivern RC, Harbinson MT, McVeigh GE. (2011) Impaired flow-mediated dilatation response in uncomplicated Type 1 diabetes mellitus: influence of shear stress and microvascular reactivity. Clin Sci, 121: 129-139.

20. Sugiyama S, Takahashi SS, Tokutomi FA, Yoshida A, Kobayashi K, Yoshino F, Wada-Takahashi S, Toyama T, Watanabe K, Hamada N, Todoki K, Lee MC. (2012) Gingival vascular functions are altered in type 2 diabetes mellitus model and/or periodontitis model. J Clin Biochem Nutr, 51: 108-113.
21. Grace PA. (1994) Ischaemia-reperfusion injury. Br J Surg, 81: 637-647.
22. Carroll WR, Esclamado RM. (2000) Ischemia/reperfusion injury in microvascular surgery. Head Neck, 22: 700-713.
23. McLean TN, Smith BA, Morrison EC, Nasjleti CE, Caffesse RG. (1995) Vascular changes following mucoperiosteal flap surgery: a fluorescein angiography study in dogs. J Periodontol, 66: 205-210.
24. Folkman J, Shing Y. (1992) Angiogenesis. J Biol Chem, 267: 10931-10934.
25. Lazarus GS, Cooper DM, Knighton DR, Percoraro RE, Rodeheaver G, Robson MC. (1994) Definitions and guidelines for assessment of wounds and evaluation of healing. Wound Repair Regen, 2: 165-170.
26. Singer AJ, Clark RA. (1999) Cutaneous wound healing. N Engl J Med, 341: 738-746.
27. Wikesjo UM, Selvig KA. (1999) Periodontal wound healing and regeneration. Periodontol 2000, 19: 21-39.
28. Clark R. Wound repair. Overview and general considerations. In: R Clark (szerk.), The Molecular and Cellular Biology of Wound Repair. Plenum Press, New York U.S.A., 1996: 3–50.
29. Woo YC, Park SS, Subieta AR, Brennan TJ. (2004) Changes in tissue pH and temperature after incision indicate acidosis may contribute to postoperative pain. Anesthesiology, 101: 468-475.
30. Robson MC, Steed DL, Franz MG. (2001) Wound healing: biologic features and approaches to maximize healing trajectories. Curr Probl Surg, 38: 72-140.
31. Reinke JM, Sorg H. (2012) Wound repair and regeneration. Eur Surg Res, 49: 35-43.
32. Eming SA, Krieg T, Davidson JM. (2007) Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol, 127: 514-525.

33. Profyris C, Tziotzios C, Do Vale I. (2012) Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics Part I. The molecular basis of scar formation. J Am Acad Dermatol, 66: 1-10; quiz 11-12.
34. Arnold F, West DC. (1991) Angiogenesis in wound healing. Pharmacol Ther, 52: 407-422.
35. Bauer SM, Bauer RJ, Velazquez OC. (2005) Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. Vasc Endovascular Surg, 39: 293-306.
36. Madden JW, Peacock EE, Jr. (1971) Studies on the biology of collagen during wound healing. 3. Dynamic metabolism of scar collagen and remodeling of dermal wounds. Ann Surg, 174: 511-520.
37. Eckes B, Nischt R, Krieg T. (2010) Cell-matrix interactions in dermal repair and scarring. Fibrogenesis Tissue Repair, 3: 4.
38. Nauta A, Gurtner G, Longaker MT. (2011) Wound healing and regenerative strategies. Oral Dis, 17: 541-549.
39. Gurtner GC, Evans GR. (2000) Advances in head and neck reconstruction. Plast Reconstr Surg, 106: 672-682; quiz 683.
40. Tziotzios C, Profyris C, Sterling J. (2012) Cutaneous scarring: Pathophysiology, molecular mechanisms, and scar reduction therapeutics Part II. Strategies to reduce scar formation after dermatologic procedures. J Am Acad Dermatol, 66: 13-24; quiz 25-16.
41. Wong JW, Gallant-Behm C, Wiebe C, Mak K, Hart DA, Larjava H, Hakkinen L. (2009) Wound healing in oral mucosa results in reduced scar formation as compared with skin: evidence from the red Duroc pig model and humans. Wound Repair Regen, 17: 717-729.
42. Whitby DJ, Ferguson MW. (1991) The extracellular matrix of lip wounds in fetal, neonatal and adult mice. Development, 112: 651-668.
43. Graves DT, Nooh N, Gillen T, Davey M, Patel S, Cottrell D, Amar S. (2001) IL-1 plays a critical role in oral, but not dermal, wound healing. J Immunol, 167: 5316-5320.
44. Zuhr O, Rebele SF, Cheung SL, Hürzeler MB. Surgery without papilla incision: tunneling flap procedures in plastic periodontal and implant surgery. Periodontol 2000, 0.

45. Choi YJ, Lee D-W, Kim K-H, Chung CJ. (2015) Scar formation and revision after the removal of orthodontic miniscrews. Korean Journal of Orthodontics, 45: 146-150.
46. Gaberthuel TW, Mormann W. (1978) The angiographic tension test in mucogingival surgery. J Periodontol, 49: 395-399.
47. Chen L, Arbieva ZH, Guo S, Marucha PT, Mustoe TA, DiPietro LA. (2010) Positional differences in the wound transcriptome of skin and oral mucosa. BMC Genomics, 11: 471.
48. Schrementi ME, Ferreira AM, Zender C, DiPietro LA. (2008) Site-specific production of TGF-beta in oral mucosal and cutaneous wounds. Wound Repair Regen, 16: 80-86.
49. Szpaderska AM, Walsh CG, Steinberg MJ, DiPietro LA. (2005) Distinct patterns of angiogenesis in oral and skin wounds. J Dent Res, 84: 309-314.
50. Mak K, Manji A, Gallant-Behm C, Wiebe C, Hart DA, Larjava H, Hakkinen L. (2009) Scarless healing of oral mucosa is characterized by faster resolution of inflammation and control of myofibroblast action compared to skin wounds in the red Duroc pig model. J Dermatol Sci, 56: 168-180.
51. Politis C, Schoenaers J, Jacobs R, Agbaje JO. (2016) Wound Healing Problems in the Mouth. Front Physiol, 7: 507.
52. Adeyemo WL, Ladeinde AL, Ogunlewe MO. (2006) Clinical evaluation of post-extraction site wound healing. J Contemp Dent Pract, 7: 40-49.
53. Benediktsdottir IS, Wenzel A, Petersen JK, Hintze H. (2004) Mandibular third molar removal: risk indicators for extended operation time, postoperative pain, and complications. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 97: 438-446.
54. Conrad SM, Blakey GH, Shugars DA, Marciani RD, Phillips C, White RP, Jr. (1999) Patients' perception of recovery after third molar surgery. J Oral Maxillofac Surg, 57: 1288-1294; discussion 1295-1286.
55. Phillips C, White RP, Jr., Shugars DA, Zhou X. (2003) Risk factors associated with prolonged recovery and delayed healing after third molar surgery. J Oral Maxillofac Surg, 61: 1436-1448.

56. Engeland CG, Bosch JA, Cacioppo JT, Marucha PT. (2006) Mucosal wound healing: the roles of age and sex. Arch Surg, 141: 1193-1197; discussion 1198.
57. Ashcroft GS, Dodsworth J, van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, Ferguson MW. (1997) Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. Nat Med, 3: 1209-1215.
58. Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM, Ferguson MW. (1999) Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. Am J Pathol, 155: 1137-1146.
59. Ashcroft GS, Mills SJ. (2002) Androgen receptor-mediated inhibition of cutaneous wound healing. J Clin Invest, 110: 615-624.
60. Gilliver SC, Ruckshanthi JP, Atkinson SJ, Ashcroft GS. (2007) Androgens influence expression of matrix proteins and proteolytic factors during cutaneous wound healing. Lab Invest, 87: 871-881.
61. Gilliver SC, Wu F, Ashcroft GS. (2003) Regulatory roles of androgens in cutaneous wound healing. Thromb Haemost, 90: 978-985.
62. Engeland CG, Sabzehei B, Marucha PT. (2009) Sex hormones and mucosal wound healing. Brain Behav Immun, 23: 629-635.
63. Szabo A, Vollmar B, Boros M, Menger MD. (2006) Gender differences in ischemia-reperfusion-induced microcirculatory and epithelial dysfunctions in the small intestine. Life Sci, 78: 3058-3065.
64. Nuki K, Hock J. (1974) The organisation of the gingival vasculature. J Periodontal Res, 9: 305-313.
65. Kennedy JE. (1974) Effect of inflammation on collateral circulation of the gingiva. J Periodontal Res, 9: 147-152.
66. Tsilibary EC. (2003) Microvascular basement membranes in diabetes mellitus. J Pathol, 200: 537-546.
67. Vracko R, Benditt EP. (1970) Capillary basal lamina thickening. Its relationship to endothelial cell death and replacement. J Cell Biol, 47: 281-285.
68. Sadzeviciene R, Paipaliene P, Zekonis G, Zilinskas J. (2005) The influence of microvascular complications caused by diabetes mellitus on the inflammatory pathology of periodontal tissues. Stomatologija, 7: 121-124.

69. Hock JM, Kim S. (1987) Blood flow in healed and inflamed periodontal tissues of dogs. J Periodontal Res, 22: 1-5.
70. Kaplan ML, Jeffcoat MK, Goldhaber P. (1982) Blood flow in gingiva and alveolar bone in beagles with periodontal disease. J Periodontal Res, 17: 384-389.
71. Baab DA, Oberg PA. (1987) Laser Doppler measurement of gingival blood flow in dogs with increasing and decreasing inflammation. Arch Oral Biol, 32: 551-555.
72. Gleissner C, Kempski O, Peylo S, Glatzel JH, Willershausen B. (2006) Local gingival blood flow at healthy and inflamed sites measured by laser Doppler flowmetry. J Periodontol, 77: 1762-1771.
73. Matheny JL, Abrams H, Johnson DT, Roth GI. (1993) Microcirculatory dynamics in experimental human gingivitis. J Clin Periodontol, 20: 578-583.
74. Mavropoulos A, Brodin P, Rosing CK, Aass AM, Aars H. (2007) Gingival blood flow in periodontitis patients before and after periodontal surgery assessed in smokers and non-smokers. J Periodontol, 78: 1774-1782.
75. Vag J, Fazekas A. (2002) Influence of restorative manipulations on the blood perfusion of human marginal gingiva as measured by laser Doppler flowmetry. J Oral Rehabil, 29: 52-57.
76. Hinrichs JE, Jarzembinski C, Hardie N, Aeppli D. (1995) Intrasulcular laser Doppler readings before and after root planing. J Clin Periodontol, 22: 817-823.
77. Svalestad J, Hellem S, Vaagbo G, Irgens A, Thorsen E. (2010) Reproducibility of transcutaneous oximetry and laser Doppler flowmetry in facial skin and gingival tissue. Microvasc Res, 79: 29-33.
78. Sasano T, Kuriwada S, Sanjo D. (1989) Arterial blood pressure regulation of pulpal blood flow as determined by laser Doppler. J Dent Res, 68: 791-795.
79. Ikawa M, Ikawa K, Horiuchi H. (1998) The effects of thermal and mechanical stimulation on blood flow in healthy and inflamed gingiva in man. Arch Oral Biol, 43: 127-132.
80. Perry DA, McDowell J, Goodis HE. (1997) Gingival microcirculation response to tooth brushing measured by laser Doppler flowmetry. J Periodontol, 68: 990-995.

81. Tanaka M, Hanioka T, Kishimoto M, Shizukuishi S. (1998) Effect of mechanical toothbrush stimulation on gingival microcirculatory functions in inflamed gingiva of dogs. J Clin Periodontol, 25: 561-565.
82. Cafffesse RG, Castelli WA, Nasjleti CE. (1981) Vascular response to modified Widman flap surgery in monkeys. J Periodontol, 52: 1-7.
83. Cutright DE. (1969) The proliferation of blood vessels in gingival wounds. J Periodontol, 40: 137-141.
84. Mormann W, Ciancio SG. (1977) Blood supply of human gingiva following periodontal surgery. A fluorescein angiographic study. J Periodontol, 48: 681-692.
85. Lindeboom JA, Mathura KR, Aartman IH, Kroon FH, Milstein DM, Ince C. (2007) Influence of the application of platelet-enriched plasma in oral mucosal wound healing. Clin Oral Implants Res, 18: 133-139.
86. Milstein DM, Lindeboom JA, Ince C. (2010) Intravital sidestream dark-field (SDF) imaging is used in a rabbit model for continuous noninvasive monitoring and quantification of mucosal capillary regeneration during wound healing in the oral cavity: a pilot study. Arch Oral Biol, 55: 343-349.
87. Milstein DM, Mathura KR, Lindeboom JA, Ramsoekh D, Lindeboom R, Ince C. (2009) The temporal course of mucoperiosteal flap revascularization at guided bone regeneration-treated implant sites: a pilot study. J Clin Periodontol, 36: 892-897.
88. Wuchenich GT, Torabinejad M, Gilbert RD, McMillan P. (1996) Measurement of blood flow with radiolabeled microspheres in reflected mucogingival flaps in cats. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 82: 330-334.
89. Retzepi M, Tonetti M, Donos N. (2007) Comparison of gingival blood flow during healing of simplified papilla preservation and modified Widman flap surgery: a clinical trial using laser Doppler flowmetry. J Clin Periodontol, 34: 903-911.
90. Retzepi M, Tonetti M, Donos N. (2007) Gingival blood flow changes following periodontal access flap surgery using laser Doppler flowmetry. J Clin Periodontol, 34: 437-443.

91. Alssum L, Eubank TD, Roy S, Erdal BS, Yildiz VO, Tatakis DN, Leblebicioglu B. (2017) Gingival Perfusion and Tissue Biomarkers During Early Healing of Postextraction Regenerative Procedures: A Prospective Case Series. J Periodontol, 88: 1163-1172.
92. Burkhardt R, Lang NP. (2005) Coverage of localized gingival recessions: comparison of micro- and macrosurgical techniques. J Clin Periodontol, 32: 287-293.
93. Kaner D, Soudan M, Zhao H, Gassmann G, Schonhauser A, Friedmann A. (2017) Early Healing Events after Periodontal Surgery: Observations on Soft Tissue Healing, Microcirculation, and Wound Fluid Cytokine Levels. Int J Mol Sci, 18.
94. Tatarakis N, Gkranias N, Darbar U, Donos N. (2017) Blood flow changes using a 3D xenogeneic collagen matrix or a subepithelial connective tissue graft for root coverage procedures: a pilot study. Clin Oral Investig.
95. Ohba S, Yokoyama J, Fujimaki M, Ito S, Kojima M, Shimoji K, Ikeda K. (2012) Significant improvement in superselective intra-arterial chemotherapy for oral cancer by using indocyanine green fluorescence. Oral Oncol, 48: 1101-1105.
96. Mormann W, Lutz F. (1974) Fluoreszenzangiographie der Gingiva. Acta Parodontologica, in Schweizerische Monatsschrift für Zahnheitkunde, 84: 798-816.
97. Mormann W, Bernimoulin JP, Schmid MO. (1975) Fluorescein angiography of free gingival autografts. J Clin Periodontol, 2: 177-189.
98. Mormann W, Meier C, Firestone A. (1979) Gingival blood circulation after experimental wounds in man. J Clin Periodontol, 6: 417-424.
99. Soderholm G, Atistrom R. (1977) Vascular permeability during initial gingivitis in dogs. J Periodontal Res, 12: 395-401.
100. Fazekas A, Vindisch K, Posch E, Gyorfi A. (1990) Experimentally-induced neurogenic inflammation in the rat oral mucosa. J Periodontal Res, 25: 276-282.
101. Aukland K, Bower BF, Berliner RW. (1964) MEASUREMENT OF LOCAL BLOOD FLOW WITH HYDROGEN GAS. Circ Res, 14: 164-187.
102. Fazekas A, Posch E, Zelles T. (1982) Pressure-flow relations in the vascular bed of the rabbit submandibular gland. J Dent Res, 61: 66-68.

103. Sasano T, Shoji N, Kuriwada S, Sanjo D. (1995) Calibration of laser Doppler flowmetry for measurement of gingival blood flow. J Periodontal Res, 30: 298-301.
104. Basa S, Ercan MT, Aras T, Araz K. (1987) Blood flow to palatal mucosal grafts in mandibular labial vestibuloplasty measured by 133Xe clearance technique. Int J Oral Maxillofac Surg, 16: 548-553.
105. Demirkol A, Demirkol MO, Demirel K, Meric H, Cantez S. (2001) Blood flow of free gingival grafts measured by xenon-133 clearance. Periodontal Clin Investig, 23: 15-19.
106. Blumenthal NM. (1988) The use of collagen membranes to guide regeneration of new connective tissue attachment in dogs. J Periodontol, 59: 830-836.
107. Janson WA, Ruben MP, Kramer GM, Bloom AA, Turner H. (1969) Development of the blood supply to split-thickness free ginival autografts. J Periodontol, 40: 707-716.
108. Kim JI, Choi BH, Li J, Xuan F, Jeong SM. (2009) Blood vessels of the peri-implant mucosa: a comparison between flap and flapless procedures. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 107: 508-512.
109. Nobuto T, Imai H, Suwa F, Kono T, Suga H, Jyoshi K, Obayashi K. (2003) Microvascular response in the periodontal ligament following mucoperiosteal flap surgery. J Periodontol, 74: 521-528.
110. Oliver RC, Loe H, Karring T. (1968) Microscopic evaluation of the healing and revascularization of free gingival grafts. J Periodontal Res, 3: 84-95.
111. Vergara JA, Quinones CR, Nasjleti CE, Caffesse RG. (1997) Vascular response to guided tissue regeneration procedures using nonresorbable and bioabsorbable membranes in dogs. J Periodontol, 68: 217-224.
112. Kouadio AA, Jordana F, Koffi NJ, Le Bars P, Soueidan A. (2018) The use of laser Doppler flowmetry to evaluate oral soft tissue blood flow in humans: A review. Arch Oral Biol, 86: 58-71.
113. Doppler CJ. (1842) Uber das farbige Licht der Doppelsterne und einiger anderer Gestirne des Hiramels. Abhandlunsen der Königlich BShmischen Gesselschaft der Wissenschaften, vol. 1: 467-482.

114. Vongsavan N, Matthews B. (1993) Some aspects of the use of laser Doppler flow meters for recording tissue blood flow. Exp Physiol, 78: 1-14.
115. Svensson H, Pettersson H, Svedman P. (1985) Laser Doppler flowmetry and laser photometry for monitoring free flaps. Scand J Plast Reconstr Surg, 19: 245-249.
116. Baab DA, Oberg PA, Holloway GA. (1986) Gingival blood flow measured with a laser Doppler flowmeter. J Periodontal Res, 21: 73-85.
117. Meekin TN, Wilson RF, Scott DA, Ide M, Palmer RM. (2000) Laser Doppler flowmeter measurement of relative gingival and forehead skin blood flow in light and heavy smokers during and after smoking. J Clin Periodontol, 27: 236-242.
118. Ambrosini P, Cherene S, Miller N, Weissenbach M, Penaud J. (2002) A laser Doppler study of gingival blood flow variations following periosteal stimulation. J Clin Periodontol, 29: 103-107.
119. Justus T, Chang BL, Bloomquist D, Ramsay DS. (2001) Human gingival and pulpal blood flow during healing after Le Fort I osteotomy. J Oral Maxillofac Surg, 59: 2-7; discussion 7-8.
120. Donos N, D'Aiuto F, Retzepi M, Tonetti M. (2005) Evaluation of gingival blood flow by the use of laser Doppler flowmetry following periodontal surgery. A pilot study. J Periodontal Res, 40: 129-137.
121. Bay B, Hilliges M, Weidner C, Sandborgh-Englund G. (2009) Response of human oral mucosa and skin to histamine provocation: laser Doppler perfusion imaging discloses differences in the nociceptive nervous system. Acta Odontol Scand, 67: 99-105.
122. Roustit M, Millet C, Blaise S, Dufournet B, Cracowski JL. (2010) Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. Microvasc Res, 80: 505-511.
123. Tew GA, Klonizakis M, Crank H, Briers JD, Hodges GJ. (2011) Comparison of laser speckle contrast imaging with laser Doppler for assessing microvascular function. Microvasc Res, 82: 326-332.
124. Fazekas R, Molnar E, Lohinai Z, Dinya E, Toth Z, Windisch P, Vag J. (2018) Functional characterization of collaterals in the human gingiva by laser speckle contrast imaging. Microcirculation, 25: e12446.

125. Molnar E, Molnar B, Lohinai Z, Toth Z, Benyo Z, Hricisak L, Windisch P, Vag J. (2017) Evaluation of Laser Speckle Contrast Imaging for the Assessment of Oral Mucosal Blood Flow following Periodontal Plastic Surgery: An Exploratory Study. Biomed Res Int, 2017: 4042902.
126. Eriksson S, Nilsson J, Sturesson C. (2014) Non-invasive imaging of microcirculation: a technology review. Med Devices (Auckl), 7: 445-452.
127. Eriksson S, Nilsson J, Lindell G, Sturesson C. (2014) Laser speckle contrast imaging for intraoperative assessment of liver microcirculation: a clinical pilot study. Med Devices (Auckl), 7: 257-261.
128. Hecht N, Woitzik J, Dreier JP, Vajkoczy P. (2009) Intraoperative monitoring of cerebral blood flow by laser speckle contrast analysis. Neurosurg Focus, 27: E11.
129. Lindahl F, Tesselaar E, Sjoberg F. (2013) Assessing paediatric scald injuries using Laser Speckle Contrast Imaging. Burns, 39: 662-666.
130. Milstein DM, Ince C, Gisbertz SS, Boateng KB, Geerts BF, Hollmann MW, van Berge Henegouwen MI, Veelo DP. (2016) Laser speckle contrast imaging identifies ischemic areas on gastric tube reconstructions following esophagectomy. Medicine (Baltimore), 95: e3875.
131. Groner W, Winkelman JW, Harris AG, Ince C, Bouma GJ, Messmer K, Nadeau RG. (1999) Orthogonal polarization spectral imaging: a new method for study of the microcirculation. Nat Med, 5: 1209-1212.
132. Lindert J, Werner J, Redlin M, Kuppe H, Habazettl H, Pries AR. (2002) OPS imaging of human microcirculation: a short technical report. J Vasc Res, 39: 368-372.
133. Dennis MJ. (2000) Plethysmography: the new wave in haemodynamic monitoring--a review of clinical applications. Aust Crit Care, 13: 14-20.
134. Rajendra Acharya U, Paul Joseph K, Kannathal N, Lim CM, Suri JS. (2006) Heart rate variability: a review. Med Biol Eng Comput, 44: 1031-1051.
135. Molnar E, Lohinai Z, Demeter A, Mikecs B, Toth Z, Vag J. (2015) Assessment of heat provocation tests on the human gingiva: the effect of periodontal disease and smoking. Acta Physiol Hung, 102: 176-188.

136. Csillag M, Nyiri G, Vag J, Fazekas A. (2007) Dose-related effects of epinephrine on human gingival blood flow and crevicular fluid production used as a soaking solution for chemo-mechanical tissue retraction. J Prosthet Dent, 97: 6-11.
137. Fazekas A, Csempesz F, Csabai Z, Vág J. (2002) Effects of pre-soaked retraction cords on the microcirculation of the human gingival margin. Oper Dent, 27: 343-348.
138. Yamaguchi K, Nanda RS, Kawata T. (1991) Effect of orthodontic forces on blood flow in human gingiva. Angle Orthod, 61: 193-203; discussion 203-194.
139. Hashimoto S, Yamamura T, Shimono M. (1986) Morphometric analysis of the intercellular space and desmosomes of rat junctional epithelium. J Periodontal Res, 21: 510-520.
140. Schroeder HE. (1969) Ultrastructure of the junctional epithelium of the human gingiva. Helv Odontol Acta, 13: 65-83.
141. Schroeder HE, Munzel-Pedrazzoli S. (1970) Morphometric analysis comparing junctional and oral epithelium of normal human gingiva. Helv Odontol Acta, 14: 53-66.
142. Yamasaki A, Nikai H, Niitani K, Ijuhin N. (1979) Ultrastructure of the junctional epithelium of germfree rat gingiva. J Periodontol, 50: 641-648.
143. Griffiths GS. (2003) Formation, collection and significance of gingival crevice fluid. Periodontology 2000, 31: 32-42.
144. Schroeder HE, Listgarten MA. (1997) The gingival tissues: the architecture of periodontal protection. Periodontol 2000, 13: 91-120.
145. Bosshardt DD, Lang NP. (2005) The junctional epithelium: from health to disease. J Dent Res, 84: 9-20.
146. Lange D, Schroeder HE. (1971) Cytochemistry and ultrastructure of gingival sulcus cells. Helv Odontol Acta, 15: Suppl 6:65-86.
147. Morozumi T, Kubota T, Sato T, Okuda K, Yoshie H. (2004) Smoking cessation increases gingival blood flow and gingival crevicular fluid. J Clin Periodontol, 31: 267-272.
148. Klinger G. (1982) [Sulcus fluid flow rate in relation to hormonal influence]. Stomatol DDR, 32: 53-55.

149. Drummond S, Canavarro C, Perinetti G, Teles R, Capelli J, Jr. (2012) The monitoring of gingival crevicular fluid volume during orthodontic treatment: a longitudinal randomized split-mouth study. Eur J Orthod, 34: 109-113.
150. Alfano MC. (1974) The origin of gingival fluid. Journal of Theoretical Biology, 47: 127-136.
151. Pashley DH. (1976) A mechanistic analysis of gingival fluid production. Journal of Periodontal Research, 11: 121-134.
152. Bevilacqua L, Biasi MD, Lorenzon MG, Frattini C, Angerame D. (2016) Volumetric Analysis of Gingival Crevicular Fluid and Peri-Implant Sulcus Fluid in Healthy and Diseased Sites: A Cross-Sectional Split-Mouth Pilot Study. Open Dent J, 10: 131-138.
153. Armitage GC. (2004) Analysis of gingival crevice fluid and risk of progression of periodontitis. Periodontol 2000, 34: 109-119.
154. Eren G, Tervahartiala T, Sorsa T, Atilla G. (2016) Cytokine (interleukin-1beta) and MMP levels in gingival crevicular fluid after use of platelet-rich fibrin or connective tissue graft in the treatment of localized gingival recessions. J Periodontal Res, 51: 481-488.
155. Zotterman J, Mirdell R, Horsten S, Farnebo S, Tesselaar E. (2017) Methodological concerns with laser speckle contrast imaging in clinical evaluation of microcirculation. PLoS ONE, 12: e0174703.
156. Loe H, Holm-Pedersen P. (1965) ABSENCE AND PRESENCE OF FLUID FROM NORMAL AND INFLAMED GINGIVAE. Periodontics, 3: 171-177.
157. Morelli T, Neiva R, Nevins ML, McGuire MK, Scheyer ET, Oh TJ, Braun TM, Nor JE, Bates D, Giannobile WV. (2011) Angiogenic biomarkers and healing of living cellular constructs. J Dent Res, 90: 456-462.
158. Vangeneugden T, Laenen A, Geys H, Renard D, Molenberghs G. (2004) Applying linear mixed models to estimate reliability in clinical trial data with repeated measurements. Control Clin Trials, 25: 13-30.
159. Molnar B, Aroca S, Keglevich T, Gera I, Windisch P, Stavropoulos A, Sculean A. (2013) Treatment of multiple adjacent Miller Class I and II gingival recessions with collagen matrix and the modified coronally advanced tunnel technique. Quintessence Int, 44: 17-24.

160. Bland JM, Altman DG. (1996) Measurement error proportional to the mean. BMJ, 313: 106.
161. Atkinson G, Nevill AM. (1998) Statistical methods for assessing measurement error (reliability) in variables relevant to sports medicine. Sports Med, 26: 217-238.
162. Euser AM, Dekker FW, le Cessie S. (2008) A practical approach to Bland-Altman plots and variation coefficients for log transformed variables. J Clin Epidemiol, 61: 978-982.
163. Gaverth J, Sandgren M, Lindberg PG, Forssberg H, Eliasson AC. (2013) Test-retest and inter-rater reliability of a method to measure wrist and finger spasticity. J Rehabil Med, 45: 630-636.
164. Bland JM, Altman DG. (2007) Agreement between methods of measurement with multiple observations per individual. J Biopharm Stat, 17: 571-582.
165. Brown H, Prescott R. Other applications of mixed models. In: (szerk.), Applied Mixed Models in Medicine. John Wiley & Sons, Ltd, 2014: 329-451.
166. Kellogg DL, Jr. (2006) In vivo mechanisms of cutaneous vasodilation and vasoconstriction in humans during thermoregulatory challenges. J Appl Physiol (1985), 100: 1709-1718.
167. Minson CT. (2010) Thermal provocation to evaluate microvascular reactivity in human skin. J Appl Physiol (1985), 109: 1239-1246.
168. Roustit M, Cracowski JL. (2013) Assessment of endothelial and neurovascular function in human skin microcirculation. Trends Pharmacol Sci, 34: 373-384.
169. Arildsson M, Asker CL, Salerud EG, Stromberg T. (2000) Skin capillary appearance and skin microvascular perfusion due to topical application of analgesia cream. Microvasc Res, 59: 14-23.
170. Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR. (2006) Methodological issues in the assessment of skin microvascular endothelial function in humans. Trends Pharmacol Sci, 27: 503-508.
171. Minson CT, Berry LT, Joyner MJ. (2001) Nitric oxide and neurally mediated regulation of skin blood flow during local heating. J Appl Physiol (1985), 91: 1619-1626.

172. Freccero C, Holmlund F, Bornmyr S, Castenfors J, Johansson AM, Sundkvist G, Svensson H, Wollmer P. (2003) Laser Doppler perfusion monitoring of skin blood flow at different depths in finger and arm upon local heating. Microvasc Res, 66: 183-189.
173. Wipke-Tevis DD, Williams DA. (2007) Effect of oral hydration on skin microcirculation in healthy young and midlife and older adults. Wound Repair Regen, 15: 174-185.
174. Fredriksson I, Larsson M, Nystrom FH, Lanne T, Ostgren CJ, Stromberg T. (2010) Reduced arteriovenous shunting capacity after local heating and redistribution of baseline skin blood flow in type 2 diabetes assessed with velocity-resolved quantitative laser Doppler flowmetry. Diabetes, 59: 1578-1584.
175. Folke LE, Stallard RE. (1967) Periodontal microcirculation as revealed by plastic microspheres. J Periodontal Res, 2: 53-63.
176. Naumova EA, Dierkes T, Sprang J, Arnold WH. (2013) The oral mucosal surface and blood vessels. Head Face Med, 9: 8.
177.
178. Johnson GK, Squier CA, Johnson WT, Todd GL. (1987) Blood flow and epithelial thickness in different regions of feline oral mucosa and skin. J Oral Pathol, 16: 317-321.
179. Heimhalt-El Hamriti M, Schreiver C, Noerenberg A, Scheffler J, Jacoby U, Haffner D, Fischer DC. (2013) Impaired skin microcirculation in paediatric patients with type 1 diabetes mellitus. Cardiovasc Diabetol, 12: 115.
180. Nicotra A, Asahina M, Young TM, Mathias CJ. (2006) Heat-provoked skin vasodilatation in innervated and denervated trunk dermatomes in human spinal cord injury. Spinal Cord, 44: 222-226.
181. Petrofsky J, Alshammari F, Khowailed IA, Rodrigues S, Potnis P, Akerkar S, Shah J, Chung G, Save R. (2013) The effect of acute administration of vitamin D on micro vascular endothelial function in Caucasians and South Asian Indians. Med Sci Monit, 19: 641-647.
182. Osunde OD, Adebola RA, Adeoye JB, Bassey GO. (2014) Comparative study of the effect of warm saline mouth rinse on complications after dental extractions. Int J Oral Maxillofac Surg, 43: 649-653.

183. Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Fujimura A, Nozaka Y. (2000) Improvement of macromolecular clearance via lymph flow in hamster gingiva by topical warming and massage. J Periodontal Res, 35: 310-317.
184. Baab DA, Oberg A, Lundstrom A. (1990) Gingival blood flow and temperature changes in young humans with a history of periodontitis. Arch Oral Biol, 35: 95-101.
185. Mormann WH, Bosiger P, Grau P, Scaroni F. (1985) The thermodynamic behaviour of labial gingiva in patients with destructive periodontal disease. J Clin Periodontol, 12: 477-493.
186. Holowatz LA, Thompson-Torgerson CS, Kenney WL. (2008) The human cutaneous circulation as a model of generalized microvascular function. J Appl Physiol (1985), 105: 370-372.
187. Khan F, Elhadd TA, Greene SA, Belch JJ. (2000) Impaired skin microvascular function in children, adolescents, and young adults with type 1 diabetes. Diabetes Care, 23: 215-220.
188. IJzerman R, de Jongh RT, Beijk MA, van Weissenbruch MM, Delemarre-van de Waal HA, Serne EH, Stehouwer CD. (2003) Individuals at increased coronary heart disease risk are characterized by an impaired microvascular function in skin. Eur J Clin Invest, 33: 536-542.
189. Khan F, Belch JJ, MacLeod M, Mires G. (2005) Changes in endothelial function precede the clinical disease in women in whom preeclampsia develops. Hypertension, 46: 1123-1128.
190. Kruger A, Stewart J, Sahityani R, O'Riordan E, Thompson C, Adler S, Garrick R, Vallance P, Goligorsky MS. (2006) Laser Doppler flowmetry detection of endothelial dysfunction in end-stage renal disease patients: correlation with cardiovascular risk. Kidney Int, 70: 157-164.
191. Edvinsson ML, Andersson SE, Xu CB, Edvinsson L. (2008) Cigarette smoking leads to reduced relaxant responses of the cutaneous microcirculation. Vasc Health Risk Manag, 4: 699-704.
192. Luthra K, Grover HS, Aggarwal N, Luthra S. (2012) Smoking swings of gingival crevicular fluid secretion. J Indian Soc Periodontol, 16: 101-103.

193. Persson L, Bergstrom J, Gustafsson A, Asman B. (1999) Tobacco smoking and gingival neutrophil activity in young adults. J Clin Periodontol, 26: 9-13.
194. Hedin CA, Ronquist G, Forsberg O. (1981) Cyclic nucleotide content in gingival tissue of smokers and non-smokers. J Periodontal Res, 16: 337-343.
195. Oliver RC, Holm-Pederen P, Loe H. (1969) The correlation between clinical scoring, exudate measurements and microscopic evaluation of inflammation in the gingiva. J Periodontol, 40: 201-209.
196. Perozini C, Chibebe PC, Leao MV, Queiroz Cda S, Pallos D. (2010) Gingival crevicular fluid biochemical markers in periodontal disease: a cross-sectional study. Quintessence Int, 41: 877-883.
197. Kebschull M, Demmer RT, Grun B, Guarnieri P, Pavlidis P, Papapanou PN. (2014) Gingival tissue transcriptomes identify distinct periodontitis phenotypes. J Dent Res, 93: 459-468.
198. Ambrus R, Strandby RB, Svendsen LB, Achiam MP, Steffensen JF, Sondergaard Svendsen MB. (2016) Laser Speckle Contrast Imaging for Monitoring Changes in Microvascular Blood Flow. Eur Surg Res, 56: 87-96.
199. Wada Y, Higashide T, Nagata A, Sugiyama K. (2016) Longitudinal Changes in Optic Nerve Head Blood Flow in Normal Rats Evaluated by Laser Speckle Flowgraphy. Invest Ophthalmol Vis Sci, 57: 5568-5575.
200. Kemppainen P, Avellan NL, Handwerker HO, Forster C. (2003) Differences between tooth stimulation and capsaicin-induced neurogenic vasodilatation in human gingiva. J Dent Res, 82: 303-307.
201. Rousseau P, Mahe G, Haj-Yassin F, Durand S, Humeau A, Leftheriotis G, Abraham P. (2011) Increasing the "region of interest" and "time of interest", both reduce the variability of blood flow measurements using laser speckle contrast imaging. Microvasc Res, 82: 88-91.
202. Ozcan G, Taner IL, Aras T, Ercan MT, Balos K. (1992) Blood flow to human gingiva measured by the 133Xe clearance technique. J Nihon Univ Sch Dent, 34: 208-213.
203. Patino-Marin N, Martinez F, Loyola-Rodriguez JP, Tenorio-Govea E, Brito-Orta MD, Rodriguez-Martinez M. (2005) A novel procedure for evaluating gingival perfusion status using laser-Doppler flowmetry. J Clin Periodontol, 32: 231-237.

204. Mahe G, Haj-Yassin F, Rousseau P, Humeau A, Durand S, Leftheriotis G, Abraham P. (2011) Distance between laser head and skin does not influence skin blood flow values recorded by laser speckle imaging. Microvasc Res, 82: 439-442.
205. Laschke MW, Menger MD. (2012) Vascularization in tissue engineering: angiogenesis versus inosculation. Eur Surg Res, 48: 85-92.
206. Schwarz F, Rothamel D, Herten M, Sager M, Becker J. (2006) Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat. Clin Oral Implants Res, 17: 403-409.
207. Ghanaati S, Schlee M, Webber MJ, Willershausen I, Barbeck M, Balic E, Gorlach C, Stupp SI, Sader RA, Kirkpatrick CJ. (2011) Evaluation of the tissue reaction to a new bilayered collagen matrix in vivo and its translation to the clinic. Biomed Mater, 6: 015010.
208. Andrade PF, Grisi MF, Marcaccini AM, Fernandes PG, Reino DM, Souza SL, Taba M, Palioto DB, Novaes AB. (2010) Comparison between micro- and macrosurgical techniques for the treatment of localized gingival recessions using coronally positioned flaps and enamel matrix derivative. J Periodontol, 81: 1572-1579.
209. Nizam N, Bengisu O, Sönmez Ş. (2015) Micro- and Macrosurgical Techniques in the Coverage of Gingival Recession Using Connective Tissue Graft: 2 Years Follow-Up. Journal of Esthetic and Restorative Dentistry, 27: 71-83.
210. Aroca S, Molnar B, Windisch P, Gera I, Salvi GE, Nikolidakis D, Sculean A. (2013) Treatment of multiple adjacent Miller class I and II gingival recessions with a Modified Coronally Advanced Tunnel (MCAT) technique and a collagen matrix or palatal connective tissue graft: a randomized, controlled clinical trial. J Clin Periodontol, 40: 713-720.
211. Cardaropoli D, Tamagnone L, Roffredo A, Gaveglio L. (2012) Treatment of gingival recession defects using coronally advanced flap with a porcine collagen matrix compared to coronally advanced flap with connective tissue graft: a randomized controlled clinical trial. J Periodontol, 83: 321-328.

212. McGuire MK, Scheyer ET. (2010) Xenogeneic collagen matrix with coronally advanced flap compared to connective tissue with coronally advanced flap for the treatment of dehiscence-type recession defects. J Periodontol, 81: 1108-1117.
213. Rakobowchuk M, Parsloe ER, Gibbins SE, Harris E, Birch KM. (2013) Prolonged Low Flow Reduces Reactive Hyperemia and Augments Low Flow Mediated Constriction in the Brachial Artery Independent of the Menstrual Cycle. PLoS ONE, 8: e55385.
214. Levenson J, Pessana F, Gariepy J, Armentano R, Simon A. (2001) Gender differences in wall shear–mediated brachial artery vasoconstriction and vasodilation. J Am Coll Cardiol, 38: 1668-1674.
215. Merz K, Schweizer R, Schlosser S, Giovanoli P, Erni D, Plock JA. (2012) Distinct microhemodynamic efficacy of arteriogenesis and angiogenesis in critically ischemic skin flaps. Microvasc Res, 83: 249-256.
216. Peng X, Wang J, Lassance-Soares RM, Najafi AH, Sood S, Aghili N, Alderman LO, Panza JA, Faber JE, Wang S, Epstein SE, Burnett MS. (2011) Gender differences affect blood flow recovery in a mouse model of hindlimb ischemia. Am J Physiol Heart Circ Physiol, 300: H2027-2034.
217. Shen Y, Ding FH, Zhang RY, Zhang Q, Lu L, Shen WF. (2015) Serum Cystatin C Reflects Angiographic Coronary Collateralization in Stable Coronary Artery Disease Patients with Chronic Total Occlusion. PLoS ONE, 10: e0137253.
218. Dedkov EI, Oak K, Christensen LP, Tomanek RJ. (2014) Coronary vessels and cardiac myocytes of middle-aged rats demonstrate regional sex-specific adaptation in response to postmyocardial infarction remodeling. Biol Sex Differ, 5: 1.
219. Soderholm G, Egelberg J. (1973) Morphological changes in gingival blood vessels during developing gingivitis in dogs. J Periodontal Res, 8: 16-20.
220. Wang X, Zhang Y, Han C. (2014) Topical negative pressure improves autograft take by altering nutrient diffusion: A hypothesis. Med Sci Monit, 20: 61-63.
221. Shaterian A, Borboa A, Sawada R, Costantini T, Potenza B, Coimbra R, Baird A, Eliceiri BP. (2009) Real-time analysis of the kinetics of angiogenesis and vascular permeability in an animal model of wound healing. Burns, 35: 811-817.

12 BIBLIOGRAPHY OF THE CANDIATE'S PUBLICATIONS

12.1 Related publications

I. Molnár, E., Lohinai, Z., Demeter, A., Mikecs, B., Tóth, Z., Vág, J. Assessment of heat provocation tests on the human gingiva: the effect of periodontal disease and smoking. Acta Physiol Hung. 2015 Jun;102(2):176-88. doi: 10.1556/036.102.2015.2.8.
IF: 0,814

II. Molnár, E., Molnár, B., Lohinai, Z., Tóth, Z., Benyó, Z., Hricisák, L., Windisch, P., Vág, J. Evaluation of Laser Speckle Contrast Imaging for the Assessment of Oral Mucosal Blood Flow following Periodontal Plastic Surgery: An Exploratory Study BioMed Research International, vol. 2017, Article ID 4042902, 11 pages, 2017. doi:10.1155/2017/4042902
IF: 2,583

III. Molnár, E., Fazekas, R., Lohinai, Z., Tóth, Z., Vág, J. Assessment of the test-retest reliability of human gingival blood flow measurements by Laser Speckle Contrast Imaging in a healthy cohort. Microcirculation. 2018 Feb;25(2). doi: 10.1111/micc.12420.
IF: 2,797

12.2 Not related publications

I. Fazekas, R., Molnár, E., Lohinai, Z., Dinya, E., Tóth, Z., Windish, P., Vág, J. Functional characterization of collaterals in the human gingiva by laser speckle contrast imaging. Microcirculation. 2018 Apr;25(3):e12446. doi: 10.1111/micc.12446.
IF: 2,797

II. Fazekas, R., Molnár, E., Nagy, P., Mikecs B., Windisch, P., Vág, J. A proposed method for assessing the appropriate timing of early implant placements: a case report. J Oral Implantol. 2018 Jun 5. doi: 10.1563/aaid-joi-D-17-00295.

IF: 1,212

III. Fazekas, R., Molnár, E., Mikecs, B., Lohinai Z., Vág. J. A Novel Approach to Monitoring Graft Neovascularization in the Human Gingiva. J Vis Exp. 2019 Jan 12;(143). doi: 10.3791/58535.
IF: 1,184

IV. Molnár, B., Molnár, E., Fazekas, R., Gánti, B., Mikecs, B., Vág J. Assessment of palatal mucosal wound healing following connective tissue harvesting by Laser Speckle Contrast Imaging: observational, case series study. The International Journal of Periodontics & Restorative Dentistry, 2019 Mar/Apr;39(2):e64-e70. doi: 10.11607/prd.3878.
IF: 1,249

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14 Appendix

14.1 List of Tables

14.2 List of Figures