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Analysis of calprotectin, PMN elastase, MMP-8 and IL-1β in microliter volumes of gingival crevicular fluid - suitability as prognostic factors for the recession after soft tissue management

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Abbreviations

ANOVA	Analysis of variance
BOP	Bleeding On Probing
EbM	evidence based medicine
ELISA	Enzyme-linked Immunosorbent Assay
GCF	gingival crevicular fluid
IgA	Immunoglobulin A
IL-1β	interleukin-1β
MAP	Multianalyte profiling
MeSH	Medical Subject Headings
MMP-8	matrix metalloproteinase-8
MMP-9	matrix metalloproteinase-9
MRP 8/14	myeloid-related protein 8/14
PGE2	Prostaglandine E2
PU	Periotron Unit
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF-α	Tumor Necrosis Factor

1 Introduction

A crown or other form of long-term prosthetic restoration of a tooth can be produced only indirectly. The elastomeric impression materials were developed to transfer the patient situation to the dental laboratory. The correct acquisition of the preparation margin or the finishing line is an issue of primarily greatest importance. A very accurate soft tissue management is essential in order to achieve a clear reproduction of the finishing line in dental impressions and in particular its subgingival areas. Different gingival retraction methods are therefore available. These methods should not cause any permanent damage, show no systemic side effects and allow a healing of the locally affected tissue within 14 days. Thereby the gingival sulcus must be expanded efficiently and reliably while controlling bleeding and moisture the same time [11].

Currently available effective approaches of soft tissue management are mechanical displacing or chemical methods, and combination of both techniques. In addition, other methods, such as Laser and electro-surgical and surgical procedures, could also be used [48]. The combinations of gingival tissue displacement (e.g. inserting a thin cord in the gingival sulcus) and chemical substances (hypomolar solutions) are the most widely used type of retraction. In addition those methods of gingival retraction may be used in different combinations.

In vitro studies suggested that regardless of the impression material the required minimum of sulcus widening must be at least 0.15 mm [4]. Prior clinical studies concluded that the critical range of the widened sulcus is approximately 0.2 mm. This should provide the sufficient tensile strength of the impression material and maximum accuracy can be achieved [31].

All retraction approaches cause a slight acute injury of the adjacent soft tissues and a temporary local inflammation. The inflammatory responses may differ depending on the applied retraction method. The extent of the damage and trauma and the inflammatory response moreover depend on the strength and technique of the cord insertion, the type and concentration of the chemical agent, the duration of stay of the retraction cord, the applied pressure during the cord placement, and the health of the gingival tissue [3, 32]. The extent of that kind of gingival trauma may present itself in form of clinically variable degrees of gingival recession.

Comparative studies in the prosthetic and restorative dentistry have examined the amount of the gingival recession after the use of different displacement methods. They showed that for example retraction cords harm the periodontal tissues by not only causing a degeneration of the underlying tissue, but also by decelerating the healing process [3]. *Liu et al.* [32] showed in an in vitro study that the retraction cord had a significant potential for gingival injury. Non-impregnated retraction cords had the slightest and DL-epinephrine-impregnated cord the highest potential for damage. *Azzi et al.* [3] examined in a comparative study the gingival recessions after curettage by rotating instruments, electro surgery, and use of retraction cords. They proved that all the methods caused serious side effects, such as injury to sulcular epithelium (up to a certain degree), edema, and disruption and loss of fibers in the surrounding connective tissues. The results showed that the recessions and their clinical relevance were most frequent and largest by the appliance of rotating curettage (after 6 hours: crevicular fluid flow rate 11-30 Periotron Units (PU), -0,5 to -0,8 mm recession, after 14 days: crevicular fluid flow rate PU 0-2, recession -0,1 to -1 mm), they were less distinct after electro surgery (after 6 hours: crevicular fluid flow rate 6-47 PU, recession -0,1 to -0,5 mm, after 14 days: crevicular fluid flow rate 0 PU, recession 0 to – 0,5 mm) and were practically negligible by the use of retraction cords (after 6 hours: crevicular fluid flow rate 5-30 PU , 0 mm recession, after 14 days: crevicular fluid flow rate 0 PU, 0 mm recession). Microscopically all three methods caused soft tissue injuries but generally any permanent damage, so were all gingival tissues were completely recovered after 14 days.

A similar study compared the recession formation by the appliance of electro surgery, copper band technique and retraction cord method. Again, the retraction cord technique showed advantages in regeneration capability of the soft tissues compared to the electro surgery. The authors could also demonstrate that the wound healing has been characterized by infiltration of polymorph nuclear leukocytes [47].

Locally applied epinephrine exhibited local and systemic side effects. The systemic effect of locally applied epinephrine has been the subject of extensive studies. Most scientists conclude that epinephrine should not be applied routinely for soft tissue management [12, 16, 23, 54]. Even though the systemic reaction of

epinephrine in healthy subjects is of little significance, the subjective and objective assessment of the epinephrine-effect demonstrated large differences.

Long lasting recessions or gingival retraction are important aspects of potential adverse effects of soft tissue management. These side effects are undesirable when visible in the anterior region. In some aspects the changes during the breakdown of the gingival fiber system and especially in the epithelial attachment and connective tissue fiber system induced by cord retraction may parallel the changes observed with periodontitis [42].

It is not well established which enzymes, proteases or cytokines from the gingival crevicular fluid (GCF) are involved in the development of dental recessions during soft tissue displacement. Gingival recession induced by mechanical trauma such as soft tissue management measures represents gingival inflammation with clinical attachment loss but with no radiographic evidence of bone loss. Collagen is a major structural protein in the periodontal complex, and its degradation by proteinases is crucial to periodontal disease progression. An extended PUBMED literature search (URL: http://www.ncbi.nlm.nih.gov/pubmed/) showed that the gingival tissue damage after the use of retraction cords is caused by factors released from activated granulocytes. These factors are suspected to be involved in inflammation-related alteration and repair of the periodontal tissues. The neutrophil elastase concentration and enzyme activity as well as the concentration of matrix metalloproteinase-8 (MMP-8) and myeloid-related protein 8/14 (MRP8/14, calprotectin) over time in the gingival crevicular fluid should be investigated in terms of their suitability as a prognostic factor of progression of attachment loss after gingival retraction. The markers were selected as initial targets because of their key role in the turnover of collagen in periodontal tissues.

The aim of the present prospective study with 40 participants and a split mouth design was to develop a model of immunological analysis. This model should help recording the variation trend of the concentrations and the enzyme activity over time. It also should help forecast the gingival recession after soft tissue management if the initial concentration of the enzymes MMP-8, PMN-Elastase and MRP 8/14 in the GCF with healthy gingival conditions and with gingivitis were known. Sensitivity and specificity as well as positive and negative predictive values of those enzymes during soft tissue displacement and the occurring gingival recession are to be determined. This study should demonstrate and compare the

levels of degradation-related molecules before and after the soft tissue management, which would contribute to a better understanding of the occurrence of gingival recessions. Therefore, the purpose of the present study is to evaluate the gingival crevicular fluid levels of IL-1β, MMP-8, MRP 8/14 and neutrophil elastase

- in gingival healthy patients before soft tissue management and
- after the appliance of gingival retraction cords and
- to compare the parameters within the group and
- to determine the relationship between clinical parameters and gingival crevicular fluid levels of IL-1β, MMP-8, MRP 8/14 and neutrophil elastase.

1.1 Inflammatory mechanisms in gingival tissues and biochemical analysis of enzymes, markers and their inhibitors

Studies in Periodontology have investigated the reasons of soft tissue degeneration only during periodontal diseases such as gingivitis and periodontitis. In this context it should be emphasized as a special feature that gingivitis or periodontitis represent a particular infection, which differs in many aspects from other infections [26, 41] .

The inflammation defines a chemical and cellular response of the body tissue to a physical, chemical or biological caused injury or irritation. This process is complemented by disturbance of the electrolyte balance, electrolyte transport and the migration of neutrophils and monocytes through the blood vessel walls with the purpose of phagocytosis of the noxious agents and damaged or necrotic cells. Furthermore there is an invasion of lymphocyte effector cells and as well of eosinophils, which triggers the immune response. Due to the activation of the complement system a release of the fragments C3a and C5a is carried out, these fragments and mediators together with interleukins IL-8 have a chemotactic effect on neutrophil granulocytes and monocytes [26].

Healthy gingiva has several special features. It contains practically always neutrophils and lymphocytes. Unlike other tissues, the endothelial cells of the gingival complex express vascular adhesion molecules such as E-selectin. Thereby there is a constant flow of neutrophils leaving the plexus and migrating through the connective tissue and junctional epithelium towards the gingival

sulcus. Even in periodontal healthy humans, approximately 500 000 leukocytes are reaching the oral cavity each minute. That connotes a release of a wide variety of hydrolytic enzymes from stromal cells, epithelia cells and cells of the hematopoietic lineage in the periodontal tissues[26, 41, 42].

Gingival crevicular fluid is a transudate that originates from the gingival plexus of blood vessels in the gingival connective tissue, close to the epithelium lining of the dentogingival space. It also includes resident host cells and microorganisms in the microbial biofilm and also their cellular products [5]. The gingival crevicular fluid provides therefore the opportunity to measure the inflammatory mediators by collecting them on an easily non-invasive way [6]. The gingival crevicular fluid components are drawn from 4 main sources:

1) breakdown of the host epithelial and connective tissues;
2) products of host cells in the periodontium;
3) plasma derived proteins;
4) Products derived from the subgingival microbial plaque.

Each of these groups has multiple factors which could be considered as possible candidates for predictors of disease activity [9].

A large number of those enzymes and their inhibitors could be detected in the gingival crevicular fluid and were identified as implicated in the course of periodontal disease and enable the comprehension of disease pathogenesis and may be used to predict disease progression [34, 44, 58]

Gingival crevicular fluid and its proteases, enzymes and cytokines were measured in several previous studies; those resulted to be suitable to measure the local inflammatory response [6, 27, 28, 30, 34, 39, 40, 44, 58]. Attempts have been made to correlate the amounts and presence of these substances/ molecules as indicators of gingival inflammation [6-8, 10, 14, 17, 18, 26, 27, 30, 34, 39-41, 58]. In order to follow the progress of healthy gingival tissues to gingivitis and its progression to periodontitis the concentration or activity of the following parameters seem to be eligible:

- neutrophil elastase [10, 13, 14, 18, 19, 21, 34, 37, 55]
- alkaline phosphatase [7, 10, 37, 39]
- lactate dehydrogenase [27, 28]
- β-glucuronidase [10, 28, 37, 39]
- arylsulphatase [28]

- Interleukin-1β [17, 22, 36, 40, 44, 45, 51, 53]
- Metal-matrix-proteases (MMPs) [10, 19, 26, 34, 37, 40, 41, 43, 50, 51].
- Calprotectin MRP 8/14 [2, 15, 25, 56, 57]

These studies have shown that gingivitis and periodontitis are associated with increases in several mediators of inflammation including Interleukin-1β, metal-matrix-proteases and neutrophil elastase.

These inflammatory markers are either present in a latent pro-form or in an active form or bound to inhibitors in the GCF. If the latent form is converted into the active form or if the relation between the inhibitor and the active form would alter in favor of the active form, then it comes to tissue degradation, in particular by collagenases (MMPs) and elastase, the connective tissue components collagen, fibronectin, laminin and proteoglycans break down and thus results in pathological catabolism of the extracellular matrix of the periodontium [28, 30, 41].

IL-1 β is a multifunctional pro-inflammatory mediator with important regulatory functions in inflammatory and immunologic reactions [39, 40, 45, 51]. It is certified that IL-1β has systemic and local effects a large number of cells, for example fibroblasts, chondrocytes, bone cells, neutrophils and lymphocytes. It has many diverse biologic activities through its effects on the regulation of a variety of markers that are active during inflammation. IL-1β is the most potent of all osteoclast activating factors and is associated with periodontal destruction and repair in gingival tissues and seems to be examined on a very profound level. The profiles of that cytokine are of considerable value when developing a methodology of immunoassay verification. It has a dual function in collagen digestion by inhibiting the intracellular phagocytic pathway and promoting at the same the extracellular digestion by inducing the release of collagenases (MMPs).

Neutrophil elastase is one of the major proteases released by human neutrophils, it is always released into the gingival tissue during the beginning stages of inflammation and this might be the reason of its increased levels in gingival crevicular fluid. Its major effect is the contribution of tissue destruction and attachment loss. GCF always contains active elastase and this is reflected either in the activity of the migrating neutrophil granulocytes or the relative lack of inhibitors in the crevice. *Eley at al.* showed that elastase activity gingival crevicular fluid mainly derives from PMNs by centrifuging the samples which removed the contained cells and reduced the elastase activity in the samples [14].

Similarities and correlations between both MMP-8, andMMP-9 and also elastase have been noticed by the majority of the studies. This reflects probably the fact that both enzymes are mostly associated with neutrophils. MMP-8 is also expressed by gingival fibroblasts in response to pro-inflammatory mediators such as IL-1β and TNF-α [8, 22, 34, 36, 43]. The neutrophil collagenase is also released in latent form from secretory granules, and it both forms –latent and active- are detectable in gingival crevicular fluid [14, 28, 29]. All these enzymes are able to degrade interstitial collagens and several extracellular matrix proteins.

Calprotectin, MRP 8/14 or myeloid-related protein is expressed in the cytosol of neutrophils, monocytes, activated macrophages and keratinocytes and released during activation or death of these cells. It has several functions in inflammatory reactions. It is considered as pro-inflammatory factor because it acts as a chemotactic factor and regulates the adhesion and migration of neutrophils and monocytes. Elevated levels of myeloid-related protein (MRP) 8/14, a calcium-binding protein secreted predominantly by neutrophils and monocytes, has been found in many sites of inflammation and in the extracellular fluid of patients with many types of inflammatory conditions [2, 38, 52]. Andersen et al. reported that MRP8/14 was significantly higher at the sites in periodontal disease than in healthy subjects [2]. Clinical improvement was associated with a significant decrease of MRP8/14 from baseline [2].

The abovementioned markers are promising enzymes for diagnostic tests because of:

- its crucial role in the periodontal attachment loss and disease progression
- evidence of positive correlations between enzyme levels and attachment loss
- availability of sensitive and specific assay to quantify these enzymes.

Within the scope of biochemical and molecular biological analysis of gingival crevicular fluid different methods for representing the enzyme activity in gingival crevicular fluid were compared. *Lamster* revealed that the analysis of the total enzyme activity and total enzyme activity multiplied by the amount of gingival fluid (gingival crevicular fluid × µl) per mm of probing depth is that best method for the differentiation between active and inactive stages of periodontal disease, that was the best method of data presentation in order to distinguish between patients displaying clinical attachment loss from patients that didn’t lose attachment [29].

The total enzyme activity increased independently of the GCF volume and the volume of fluid in the crevice recovers more rapidly than constituents derived primarily from host cells [14, 24]. There are controversial references in respect of the data presentation of gingival crevicular fluid assays such as concentration, total amounts or total amount per timed sample [28]. *Yamalik et al.* reported that when data presented as enzyme activity the healthy group exhibited lower enzyme activity, but when reported as concentration the highest elastase levels were seen in the healthy group [55]. *Nakashima et al.* expressed biochemical parameters as ratios to the number of PMNs and say that an analysis using total amounts of the markers has more significant diagnostic values [37]. In addition to this, concerning the acquisition of the extracted proteins, different filter paper strips for gingival crevicular fluid sampling were examined. The results showed that Periopaper® was the most suitable according to perform best analysis of gingival crevicular fluid [20].

Lamster and Ahlo examined the prostaglandin E2 (PGE2), neutrophil elastase and β-glucuronidase depending on the oral and systemic diseases and concluded that the GCF analysis can be used for the measurement of the interactions between systemic and periodontal diseases [30]. Even in the monitoring of the periodontal status of smokers and nonsmokers with chronic periodontitis has been found that regular controls of MMP-8 concentrations in GCF may be a reliable extension of traditional diagnosis. This applies in particular for patients with poor response to conventional periodontal therapy [33]. *Sorsa et al.* compared four different tests for GCF sample MMP-8 detection and couldn't make a conclusion about the predictive value of MMP-8 but at the same time they suggested that MMP-8 should be a valuable diagnostic aid supplementing other diagnostic methods [50].

Söder et al. analyzed the behavior / the conduct of neutrophil elastase, MMP-8 and prostaglandin E2 in the gingival crevicular fluid of smokers and nonsmokers and were able to show that despite of the existing higher elastase levels in smoker's gingival crevicular fluid, compared with nonsmokers there were no significant differences in the other inflammatory markers [33]. On the contrary *Rawlinson et al.* showed that there was a significant statistical difference in IL-1β concentrations in gingival crevicular fluid in deep bleeding sites [46]. Also there was significant difference in MMP-8 concentration in GCF of smokers and non-smokers [43], but however the MMP-8 concentrations correlated with the disease

severity in both groups. *Zhong et al*. examined the IL-1β-concentration and PGE2 concentration in relationship to the host risk factors for periodontal and inflammatory diseases. The concentration increase of the aforementioned markers was significantly associated with the appearance of clinical symptoms, e.g. increased probing depths and bleeding on probing. The increased concentrations were not bound to the habits, behavior, or existing risk factors (smoking, obesity, frequency of dental visits) [58].

Although associations have been established between levels of inflammation markers and presence of periodontal disease in general, large inter- and intraindividual variations suggest that that these parameters are influenced by multitude of other factors which so far have not been examined and specified enough.

Collectively all those studies measured the collagenolytic activity by several methods including an enzyme-linked immunosorbent assays, checkerboard immunoblotting technique, gelatin enzymography, release of radioactivity from radiolabeled collagen fibrils, and fluorogenic assays based on synthetic peptides that copy the cleavage stand of collagenase. All have proved that sites with history of periodontal destruction have greater proteolytic activity than those sites in health. However, comparison between studies is often difficult because GCF has not always been collected in a standardized protocol and there is also no commonly accepted ‘’gold standard’’ with which to compare the test results [20, 29, 34]. *Curtis et al.* also tried to examine the total protein concentration in GCF in order to standardize the volume of GCF used for the analysis e.g. sample loading on gel electrophoresis (SDS-PAGE) [9]. *Hanioka et al.* evaluated the potential of gingival crevicular fluid assay as screening methodology and also found that the relationships between the total quantity of the inflammation markers and periodontal disease status were unclear. However they also showed that a combination assay of IgA and neutrophil elastase in gingival crevicular fluid examining the total quantities of those substances may be crucial for prediction of periodontal disease status [21]. They however did not measure the gingival crevicular fluid volume which makes the concentration and activity analysis impossible. On the other side there are recommendations to use combinations of crevicular parameters for more precise identification of disease activity [37].

1.2 Gingival crevicular fluid sampling

The analysis of gingival crevicular fluid offered the best results when searching for reliable methods which can at best predict the prognosis of the periodontal disease and the progress of attachment loss. The biochemical analysis of GCF provides an widely recognized atraumatic approach to this issue and it's sampling provides several useful features (e.g. ease of access, rapid equilibration with whole pool, can vary time and site, repeated sampling possible) [34].

The gingival crevicular fluid also has unique features such as the very limited volume and the extreme volumetric variations among individuals and sites [28, 49]. Furthermore, many factors affect fluid volume [24, 28, 29, 49] and problems are associated with the process of precise volume determination [24, 29] which also may affect its diagnostic potential. The spectrum of fluid volume is disease related in general. However, the wide range of volumetric distribution, the site-specific nature, and the clear impact of the distinct sampling site on volume are important volumetric features on this biologic fluid.

Volumetric features of gingival crevicular fluid are under the influence of many factors, including sampling variables. Standardizing such factors may enable a more precise methodology. Volumetric measures are also highly sensitive to sampling techniques [28]. For the collection of gingival crevicular fluid were used three sampling methods [10]:

(1) gingival washings;
(2) by means of micropipettes;
(3) by absorbent paper strips

The gingival washing methods are most suitable when cell types and numbers are to be explored, if large volumes of gingival crevicular fluid are needed the most suitable technique is the micropipettes collection but the collected fluid may contain high plasma component because this procedure may be disruptive to the crevicular epithelium. It has been well shown that mechanical injury during sampling can increase vascular and sulcular epithelium permeability and the flow rate of the fluid, thus diluting the actual gingival crevicular fluid [28]. To avoid this absorbent filter paper strips are widely employed. It is clear that the diagnostic potential of gingival crevicular fluid depends on the appropriateness of the gingival crevicular fluid samples and the sampling strategy [28].

Various gingival crevicular fluid sampling protocols are available but however the accurate and reproducible sampling of fluid is not trivial [34]. Some specific recommendations such as the preference of the shortest sampling time and the procedure to cause the least interference with the site and dilution effect and to allow comparison between samples are frequently made [6, 24, 28]. Currently there is no definitely superior proven method to control the basic and intrinsic problems as collection and analysis of GCF [34]. The need for standardization of factors with the potential for altering the actual resting volume/ flow rate is frequently underlined [6, 9, 10, 28, 29].

It can also be seen that the preference of different sampling sites such as maxillary, mandibular, posterior, anterior or various index teeth and approximal, labial-buccal, or palatinal-lingual approaches are all available [24, 49].

Appropriate location of the sampling area is crucial because the sampling site has a consistent effect on both resting volume and GCF flow. The clear site-specific resting volume variations are attributed to the differences in the surface area of the crevices, contact area of the strips with the sulcus/ pocket epithelium, and unique dimensional features of distinct teeth groups. Furthermore gravity, accessibility of the site and extent of risk of contamination with plaque and saliva are also of specific concern [43].

The limitation of the methods for measurement of host inflammation markers in gingival crevicular fluid lies in the small number of samples, sites, subjects and markers that can be analyzed simultaneously [6]. Added to this a single periodontal site produces a negligible volume of gingival crevicular fluid approximately less than 0,5 µl which has to be diluted or pooled after sampling in order to provide enough volume for the analysis of several analytes/ agents. A verified and standardized method for neutrophil elastase determination in gingival crevicular fluid using a commercially available PMN-Elastase Enzyme-linked immunosorbent assay (ELISA) was also established.

For this reason in a previous study, we followed up the enzyme activity changes and concentration during and after gingival tissue retraction. The purpose was to establish the initial steps of developing a methodology for reproducible analysis of neutrophil elastase and MRP8/14 calprotectin in microliter volumes of GCF, by using well-examined factors such as MMP-8, MMP-9 and IL-1β as standard

controls for both sampling GCF and the successive marker analysis. We have developed a verified and standardized method for determination of neutrophil elastase in GCF using a commercially available PMN-Elastase linked immunosorbent assay (ELISA).

2. Material and Methods

2.1 Literature Survey - Preliminary notes on the methodology of literature research

The literature research was modeled on the methodological approach of evidence based medicine (EbM). That approach enabled the reproducibility of the conducted systematic literature review by:

- the application of strict entry and exclusion criteria and
- The quality control of the researched literature.

The literature review was carried out during the period of 04/01/2010 until 14/05/2015 and was based on database searches in PUBMED (National library of Medicine, NLM; URL: http://ncbi.nlm.nih.gov/Pubmed).

The search terms and Medical Subject Headings (MeSH) listed in Table 1 and Table 2 were based on the principal question which molecular markers of soft tissue breakdown were investigated so far (Table 1) and how to provide quality control and reproducibility of the gingival crevicular fluid sampling and enzyme marker analysis (Table 2). An electronic search was conducted for human studies presenting clinical data for the sampling and analyzing the reproducibility and specificity of the molecular markers in gingival crevicular fluid. Only *in vivo* human studies from the period of 1980 up to 2015 which fulfilled the inclusion criteria were selected. All levels of evidence were included. Studies based on medical influences of pharmacy products and common and infection diseases were excluded.

The PUBMED database search, regarding the question which molecular marker in GCF and which sampling and analysis methods are most reliable, resulted in 463 articles of possible relevance. The first extraction based on the titles led to the elimination of 357 articles. Subsequently 54 articles were excluded using the abstracts. At the end after full text reading and sorting out of 21 papers a total number of 31 relevant articles resulted.

A second electronic literature search for the pilot study was performed. Thus was based on the cognitions of the first literature survey. The objective of this research was to hold the scientific knowledge on the reproducibility and controllability of the crevicular fluid assays. From all hits found in the electronic PUBMED database

arose a number of 327 related articles. The extraction of the papers was realized using the same concept: sorting by title, by abstracts and at last by full text. In total, there were a number of 14 relevant full text articles.

The last PUBMED search for the terms “soft tissue management” ,”retraction cord”, ”GCF”, “biomarkers”, ”analysis” and all their combinations didn’t show any results and this led to the conclusion that the above mentioned problem was not investigated so far.

The chapters 1.1 and 5 are based on a literature survey which consists of publications which fulfilled the literature search criteria and from publications which the authors cited.

The systematic approach and reproducibility of the literature search and evaluation is ensured by the application of the methods of EbM. A detailed presentation of the exclusion reasons and exclusion criteria of individual publications such as systematical reviews and publications considering too many modulation factors was omitted because of the extensive questioning.

Table 1: Search terms and results of the literature review in PUBMED regarding the molecular marker, sampling methods and biochemical analysis of molecular marker

No.	Search terms	Quantity	Sorted by title	Sorted by abstract	Sorted by full text	Final selection
1	crevicular fluid assays [MeSH Terms]	129	70	28	13	18
2	crevicular, fluid, assay [MeSH Terms]AND sampling	139	105	22	6	6
3	crevicular, fluid, assays [MeSH Terms]AND sampling AND methods	64	63	0	0	1
4	(crevicular fluid assay) AND sample AND method	116	106	4	1	5
5	(crevicular fluid assay) AND method AND analyze	15	13	0	1	1
Total		463	357	54	21	31

Table 2: search terms and results of the literature review in PUBMED regarding the reproducibility and controllability of the crevicular fluid assays

No.	Search terms	Quantity	Excluded by title	Excluded by abstract	Excluded by full text	Final selection
1	crevicular, fluid, assay [MeSH Terms]AND sensitivity	156	115	18	13	10
2	crevicular, fluid, assays [MeSH Terms]AND sensitivity	22	13	4	4	1
3	crevicular, fluid, assays [MeSH Terms]AND sensitivity AND reproducible	4	2	1	1	0
4	crevicular, fluid, assays [MeSH Terms]AND diagnostic accuracy	4	1	0	3	0
5	crevicular, fluid, assays [MeSH Terms]AND diagnostic accuracy AND sensitivity	4	3	1	0	0
6	crevicular, fluid, assay [MeSH Terms]AND reproducible	18	8	6	1	3
7	crevicular, fluid, assays [MeSH Terms]AND sensitivity AND elastase	3	0	0	3	0
8	crevicular, fluid, assays [MeSH Terms]AND sensitivity AND elastase AND interleukin 1beta	0	0	0	0	0
9	crevicular, fluid, assays [MeSH Terms]AND sensitivity AND elastase AND IL-1b	0	0	0	0	0
10	crevicular fluid assay AND sensitivity AND specificity	116	76	17	23	0
Total		327	218	47	48	14

2.2 Subject population and clinical examination

The patients participating in the study were recruited from the students and employees of the University of Ulm between May 2011 and January 2012. A total of 40 systemically healthy subjects were included in the study. The purpose and the procedures were explained to all subjects prior to participation and all participants gave written informed consent in accordance with the Helsinki declaration. The study protocol was approved by the Ethical Committee of the University of Ulm. Complete medical and dental history was taken from every patient.

Patient selection was done according to the following including criteria:

- The subjects were demanded to have naturally healthy teeth and for this reason the target age was set between 18 and 50 years
- Do not have a history of any systemic or infectious disorder
- Female subjects should not be pregnant.
- No tobacco use, alcohol or drug abuse may be present.
- the eligible subjects don’t have allergy to the materials used in this study
- the PSI (Periodontal Screening Index) is equal or below 2
- The examined teeth are naturally healthy and free of defects, if they are restored than the restauration should have at least 1 mm supragingival situated restoration margins, the teeth should not have crowns.

The following additional exclusion criteria were as follows:

- subjects for which no short-term restoration is possible or a complete cure could not be assured prior to the start of the intervention phase of the study
- subjects with restorations of the examined teeth and their neighboring teeth, the margins of which are not at least 1mm supragingival
- subjects with crowns on the teeth mentioned above
- Left-handed subjects
- Presence of systemic disease that could affect the periodontal tissues (diabetes mellitus, cancer, cardiovascular and respiratory diseases)

2.3 Study course

The study had a total duration of 14 days and was divided in 6 visits starting with the participant selection and ending with the final follow up appointment on the 14th day.

After the initial patient selection and the obtaining of the signed informed consent form the subjects, each patient underwent one intervention during appointment 3 and GCF collection during the visits 2, 3, 4, 5, and 6. GCF sampling during appointment 3 was performed immediately after intervention. The intervention consisted of packing of aluminum impregnated retraction cord in double cord technique into the sulcus of the examined teeth for 10 minutes.

A workflow chart of the clinical study is presented in Figure 1.

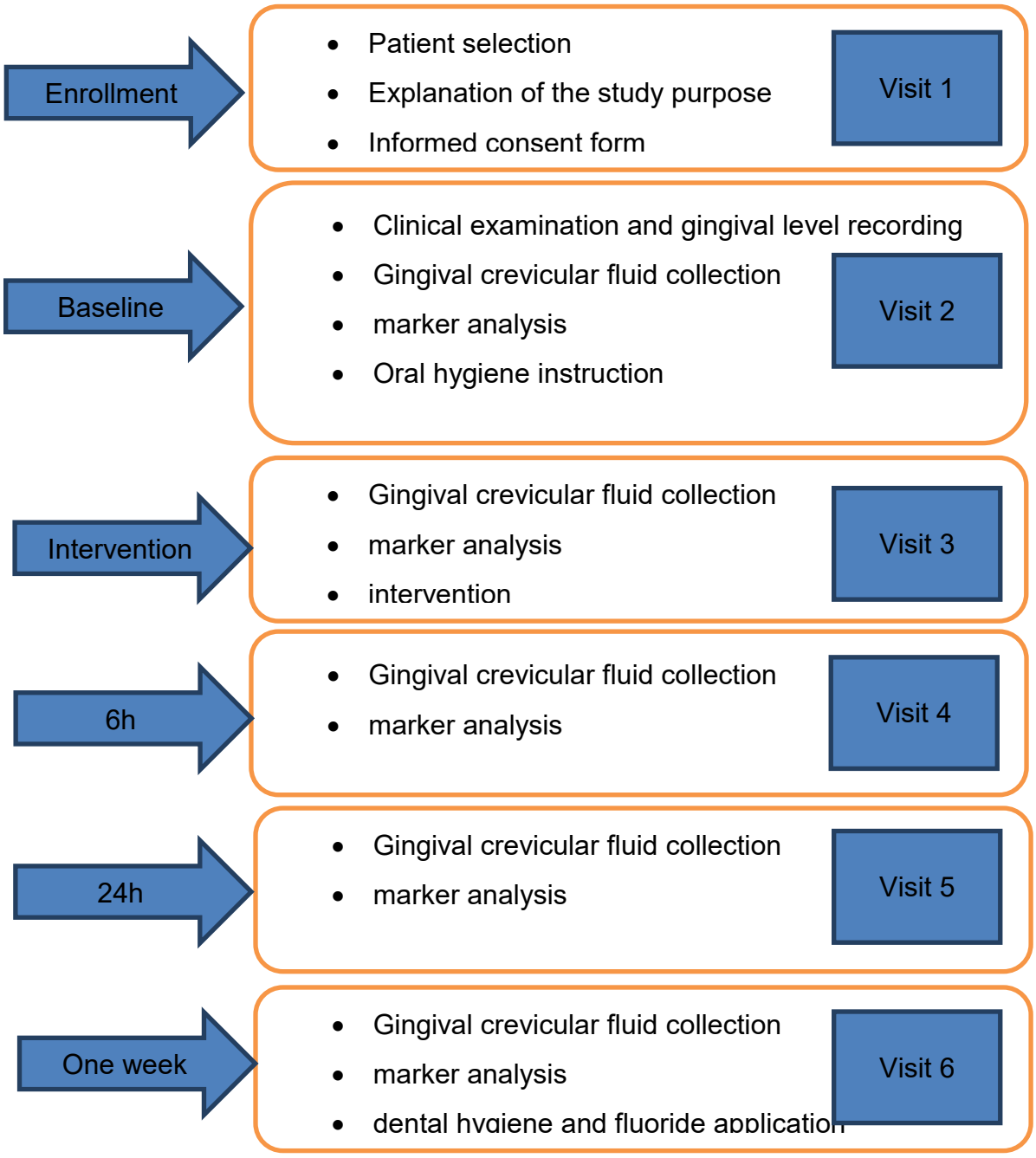

Fig.1 Workflow chart presenting the procedures during every course appointment

2.4 Clinical examination and gingival crevicular fluid sampling

The subjects were clinically evaluated using the following parameters: probing pocket depth and clinical attachment level. Full mouth clinical data were obtained from every patient. All parameters were recorded at six sites per tooth from the test sites (distobuccally, median-buccal, mesiobuccally, mesiopalatinal, median-palatal and distopalatal) by the same clinician with periodontal Goldmann probe calibrated in millimeters.

To enable the accessibility and isolation, GCF samples were obtained only from the buccal and palatal and in particular from the buccal-median and median-palatinal sites of the maxillary central incisors and first molars, and for the same reason, maxillary second and third molars were excluded. GCF sampling was performed at sites with clinical periodontal health preferably no restorations or teeth which restoration margins were laid at least 1mm supragingival.

To minimize the risk of contamination with saliva, the selected sites were rinsed with water and isolated by cotton rolls, to eliminate the risk of plaque contamination the supragingival plaque was carefully removed with a scaler. The teeth were gently air-dried and GCF was collected using standardized paper strips (Periopaper®, Oraflow Inc., New York, USA,) that were inserted into the sulcus/ pocket until mild resistance was encountered and left there for 30 seconds. Samples with evidence of gingival bleeding were discarded.

2.5 Analysis of neutrophil elastase, MMP-8, IL-1β, and MRP 8/14 (Calprotectin)

In terms to determine the volume of the absorbed GCF liquid the Periopaper® strips were processed as follows: 1. 5 ml micro centrifuge tubes were weighed, the Periopaper® strips were placed in the micro centrifuge tube and both were weighed together. After GCF sampling the strips were immediately put back in the plastic tube and immediately weighed together again. The difference between the two weights gave the total mass of the collected fluid.

All the samples were measured in 10 batches: 80 samples in batches 1 – 9 each, 66 samples in the batch 10 (80*9 + 66 = 786 samples). From each sample IL-1ß, MMP-8, neutrophil elastase, and MRP8/14 (calprotectin) were measured. Because of the different abundance of the different proteins, the samples were differentially

pre-diluted with assay buffer (for IL-1ß without dilution, for MMP-8 1:25, for elastase 1:50, and for MRP8/14 1:500). The values in the lists were already calculated with the corresponding dilution factors.

The weight of the fluid of each strip, expressed in µg, was converted to GCF volume in µl by assuming that the density of GCF is 1, 0 mg/ml. After sampling and weighing the samples were immediately stored at -80°C until assayed. The absorbed GCF fluid was extracted from the strips with 160 µl assay buffer (Bio-Plex, Cat. 171-304000, Bio-Rad, Hercules CA, USA) containing 0, 5% bovine serum albumin in terms of sample stabilization. The extract was collected after centrifugation at 13000 rpm for 10 min at 4°C. From this, the enzymes and cytokines were analyzed.

Neutrophil elastase was measured with the Human Elastase ELISA Kit according to manufacturer's instructions (HyCult Biotechnology, Uden, Netherlands). The optical density was measured at 450 nm.

The matrix metalloproteinase-8 and were simultaneously quantified with the Fluorokin MAP Multiplex Human MMP Panel (R & D Systems GmbH, Wiesbaden, Germany) on the Luminex 200 system (Bio-Rad Laboratories, Inc. CA, USA). IL-1β and were measured with Bio-Plex Cytokine Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) on the Luminex 200 system.

The assay sensitivity for neutrophil elastase, MMP-8, and IL-1ß, was 0.4 ng/ml, 8.9 pg/ml, and 0.2 pg/ml, respectively.

MRP 8/14 was measured with the MRP 8/14 ELISA kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland). The optical density of that kit was measured at 450nm too.

The samples were confirmed free of blood contamination with the Combur-Test® Strip.

2.6 Statistical Analysis

In order to analyze the differences among the changes of the concentration of the IL-1β, MMP-8, neutrophil elastase, and MRP8/14 calprotectin in gingival crevicular fluid over time as well as their associated variation, an ANOVA (analysis of variance) data analysis was performed using a statistical package (SPSS Version 21, IBM Corp, New York, USA). A retrospective power analysis was performed for every marker. The Levene-Test was not significant and for this reason only the

ANOVA results for MRP8/14 Calprotectin and Il-1β were used. The Levene-Test was significant for the MMP-8 and neutrophil elastase and for this reason the ANOVA results for this two enzymes could not be used. The interpretation of the data was carried out via descriptive analysis. The Student-Newman-Keuls procedure was used for evaluation among the groups as post-hoc analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

During the study course 4 samples per visit from each subject were collected during the visits 2, 3, 4, 5, and 6. From every subject 20 samples were obtained , which results in an overall of 796 samples for the complete course. Four samples are missing because one subject did not complete the study course. All samples that were contaminated with blood or in which enzymes were beyond the lower limit of the measuring range of the biochemical test were discarded. The values for weight per ml were calculated with the corresponding dilution factors in order to achieve comparable results. Table 3 shows the mean values of every examined marker and their standard deviation per appointment. Table 8 presents and compares the power calculation and the sample size calculation for predetermined power.

3.1 MRP8/14 Calprotectin

From 796 GCF samples, 695 in total were eligible and not discarded because of blood presence; also the presence of calprotectin was not below the measures limit. With this the amount of the valid samples was 86,9% or 139 samples per group resulting of an analysis power of 0.986.The distribution of the total concentration of GCF calprotectin is shown in Fig. 2 and Table 4. Significant differences were found between the groups ($p<0,001$). The six hours group had higher GCF MRP 8/14 calprotectin concentration compared to the other four groups. The GCF MRP8/14 calprotectin concentration of Baseline, Intervention and “one day” were similar to each other. The “one week” group had also significantly higher GCF calprotectin concentrations than the groups baseline, intervention and “one day”. MRP8/14 calprotectin also showed a good correlation to the theoretically assumed level of inflammation: GCF Calprotectin concentration is enhanced with the time of mechanical trauma (e.g. dental cord insertion) and significantly elevated in the 6 hours group, compared to other study groups.

3.2 MMP-8

The MMP-8 results support the MRP8/14 findings. From the overall of 796 GCF Samples MMP-8 was present and measurable in 675 samples. The power calculation for these 84.4% valid samples was 0.869. This enzyme also showed a good correlation to the theoretically assumed level of inflammation (Fig. 4).

Although the Levene-Test was significant (p=0.041), an increase of the enzyme concentration 6 hours after the intervention was recorded compared to the group of measurements for Baseline, Intervention, 24 hours and one week.

IL-1β did not show the expected changes over time-between baseline, intervention, 6 hours measurements, 24 hours measurements and one week measurements (p=0.351) and seemed to remain stable throughout the whole investigation. The results are shown on Fig. 3 and Table 5.

3.3 Interleukin-1β

IL-1β was present in 500 samples or in 62.5% of the overall amount, the carried out power calculation was 0.271 and this marker did not show the expected changes over time. The results showed homogeneity between baseline, intervention, 6 hours measurements, 24 hours measurements and one week measurements (p=0.351) and this marker seemed to remain stable throughout the whole investigation. The results are shown on Fig. 4 and Table 6.

3.4 Neutrophil Elastase

Neutrophil Elastase was present in 690 of the taken samples but showed the most inhomogeneous results (Fig. 5 and Table 7). In this case the power calculation for the 86.3% valid samples was 0.044. While Intervention, 24 hours measurements and one week measurements did not show significant difference (p=0.080) as well as Baseline, 6 hours measurements and one week measurements (p=0.069). These two groups did show significant difference (p<0.0001).

Table 3: Presentation of the concentrations data as mean values and their standard deviation. Because of the different abundance of the markers a pre-dilution of the assay buffers varied accordingly for myeloid-related protein 8/14 (MRP 8/14) 1:500, for matrix metalloproteinase-8 (MMP-8) 1:25, for elastase 1:50, and for interleukin-1ß (IL-1ß) without dilution

	MRP 8/14 (ng/µl)	**Neutrophil Elastase (ng/µl)**	**MMP-8 (ng/µl)**	**IL-1β (ng/µl)**
Baseline	484,40 ± 373,36	365,33± 271,14	27218,73 ± 23987,31	123,62 ± 138,36
Intervention	423,19 ± 431,10	296,22 ± 271,88	21094,86 ± 28456,55	118,58 ± 174,90
Six hours	670,83 ± 429,95‡	373,69 ±213,44*	36205,52 ± 29485,89‡	150,04 ± 162,44*
One day (24h)	442,70 ± 348,25	254,06±215,87	20545,13 ± 21456,36	120,98 ± 166,39
One week	520,89 ± 444,64	313,05 ± 221,71	27646,34 ± 30173,88	116,15 ± 140,60
Overall	507,59 ± 415,58	320,84 ± 244,43	26558,16 ± 27437,93	126,13 ± 156,00

‡ significant difference from six hours and baseline groups

*** Slight increase of the concentration in the 6 hours group**

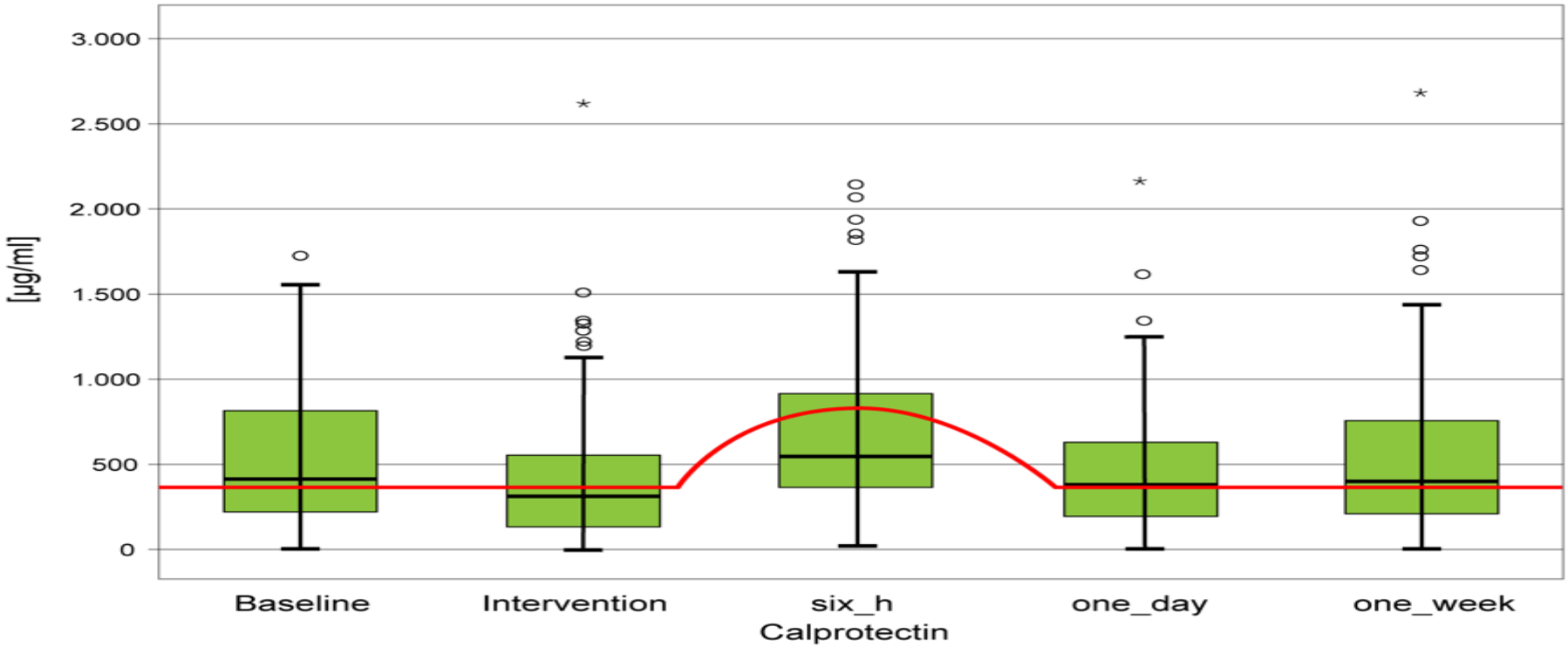

Fig. 2: Presentation of the myeloid-related protein 8/14 (MRP 8/14) concentration ng/µl after 1: 500 dilution. The enzyme was abundant at all measurements, showing correlation with the expected concentration values (red graph) and presenting a significant increase in the 6 hour measurements compared to all other groups (six_h = six hours)

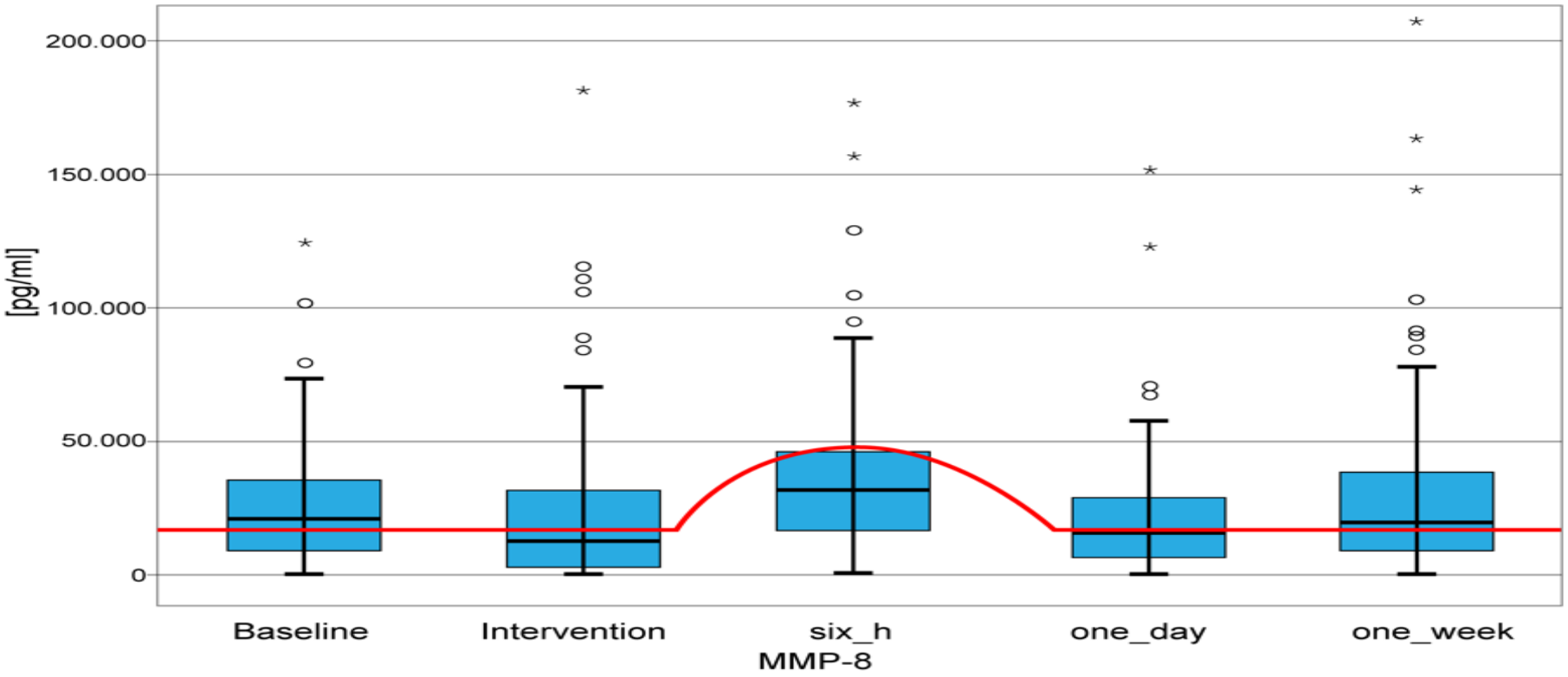

Fig. 3: Presentation of the matrix metalloproteinase-8 (MMP-8) concentrations in ng/µl after 1:25 dilution. The increase of the enzyme concentration 6 hours after the intervention was significant compared to the group of measurements for Baseline, Intervention, 24 hours and one week (six_h = six hours, red graph = expected concentration values).

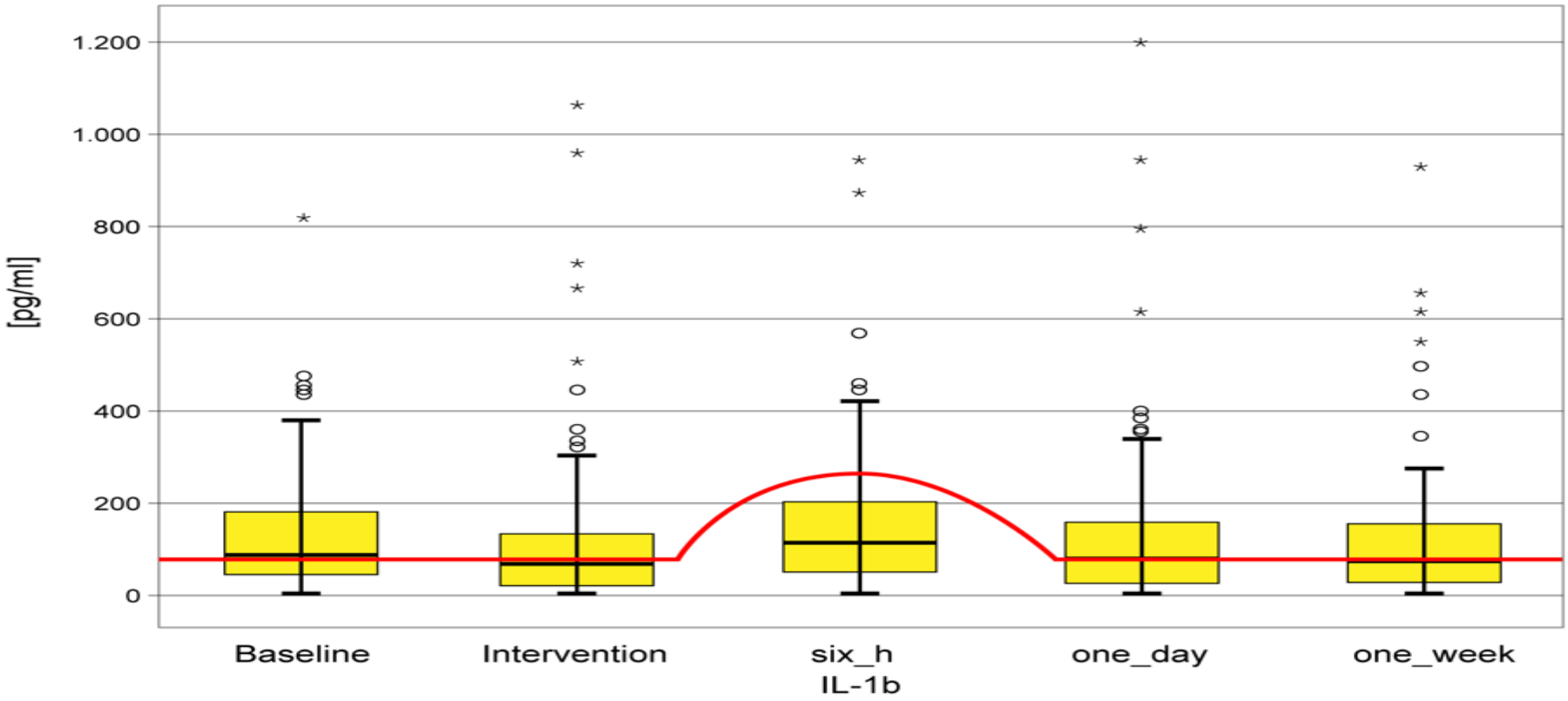

Fig. 4: Presentation of the concentration behavior of the interleukin-1β (IL-1ß) over time. The cytokine concentration didn't change significantly (p=0.351) throughout the whole examination and measurements, except for a slight increase six hours after intervention (value: 150,0438 pg/µl, red graph = expected concentration values).

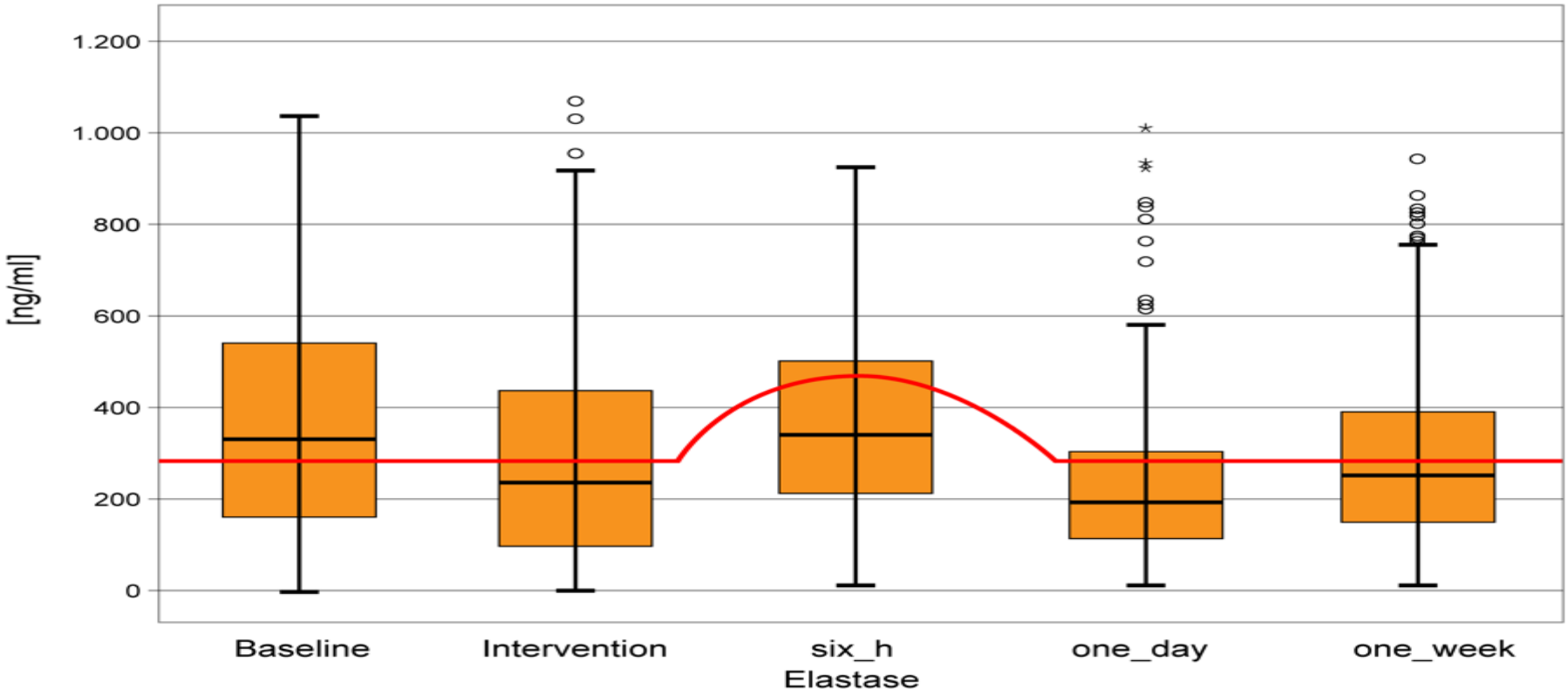

Fig. 5: Presentation of the behavior of neutrophil elastase expressed as concentration in pg/µl after 1:50 dilution. There is no significant difference (p=0.080) between Intervention, 24 hour measurements and one week measurements (group 1), as well as Baseline, 6 hour measurements and one week measurements (p=0.069, group 2). Significant difference (p<0.0001) can be observed between the 2 groups (red graph = expected concentration values).

Table 4: Presentation of the explorative analysis of the myeloid-related protein 8/14 calprotectin (MRP8/14) concentration changes over time presented per time/ sample group and pg/µl. In this case each group has n=139. The confidence intervals are also included.

pg/µl / Time	Mean Value	Standard Deviation	Minimum	Maximum	95% Confidence Interval
Baseline	509,77	385,02	0,00	1.731,28	445,20- 574,34
Intervention	429,59	414,26	0,00	2.623,03	360,11- 499-06
Six hours	678,06	444,15	24,41	2.150,20	603,57- 752,54
One day	450,51	345,653	5,00	2.159,84	392,54- 508,48
One week	526,83	444,18	4,06	2.690,28	452,32- 601,32

Table 5: Presentation of the explorative analysis of the matrix metalloproteinase-8 (MMP-8)concentration changes over time presented per time/ sample group and pg/μl. In this case each group has 135 sample cases (n=135). The confidence intervals are also included.

pg/μl / Time	Mean Value	Standard Deviation	Minimum	Maximum	95% Confidence Interval
Baseline	25.686,15	22.315,61	0,00	124.235,02	21.887,5- 29.484,81
Intervention	21.702,70	26.995,44	21,39	181.651,15	17.107,43- 26.297,98
Six hours	36.816,14	29.512,96	844,28	177.250,61	31.792,33- 41.839,96
One day	20.906,24	21.370,43	0,45	151.968,90	17.268,47- 24.543,00
One week	28.794,92	30.636,85	213,00	20.7993,00	23.534,79- 33.965,05

Table 6: Presentation of the explorative analysis of the interleukin-1β (IL-1ß) concentration changes over time presented per time/ sample group and pg/µl. In this case each group has 100 samples (n=100). The confidence intervals are also included.

pg/µl / Time	Mean Value	Standard Deviation	Minimum	Maximum	95% Confidence Interval
Baseline	140,42	144,49	3,12	820,56	111,75-169,08
Intervention	126,67	182,25	3,00	1.064,27	90,49- 162,85
Six hours	159,18	164,23	3,22	945,58	126,59- 191,76
One day	138,41	187,70	3,30	1.199,40	101,16 -175,64
One week	123,17	151,90	4,50	928,61	93,02- 153,30

Table 7: Presentation of the explorative analysis of the neutrophil elastase concentration changes over time presented per time/ sample group and pg/µl. In this case each group has 138 sample cases (n=138). The confidence intervals are also included.

Time	Mean Value `	Standard Deviation	Minimum	Maximum	95% Confidence Interval
Baseline	377,42	276,15	0,00	1.038,51	330,93- 423,90
Intervention	306,45	271,91	0,00	1.066,20	269,68- 352,22
Intervention/ Cord placement	372,98	207,94	13,05	922,23	337,97- 407,98
One day	257,40	211,18	8,89	1.011,98	221,85- 292,94
One Week	310,17	219,54	8,18	943,02	273,21- 347,12

Table 8: Presentation of the actual calculated power and actual provided valid sample size of this clinical trial versus calculation of the needed sample size for predetermined power of 0.80

	Clinical trial results		Needed data for predetermined power 0.80	
Enzymes/Markers	**Power**	**Sample size per Group**	**Power**	**Needed sample size**
MRP 8/14 calprotectin	0.986	139	0.80	64
MMP-8	0.869	135	0.80	113
Interleukin-1β	0.271	100	0.80	431
Neutrophil elastase	0.044	138	0.80	16.533

4. Discussion

The aim of this study was to determine whether with the aid of the presented model it is possible to record the variation trend of the enzyme concentration. Also it should be determined, if a forecast of the gingival recession after soft tissue management were possible by knowing the initial concentration of MMP-8, PMN-Elastase, IL-1 β, and MRP 8/14 in the GCF with healthy gingival conditions compared with their variation over time after applying retraction cords. The results showed that IL-1β did not show the expected changes over time but seemed to remain stable throughout the whole investigation. Neutrophil elastase showed inhomogeneous results and for this reason both of these enzymes cannot be considered as predictable markers for the forecast of gingival recession after dental cord appliance. MMP-8 showed a good correlation to the theoretically assumed level of inflammation. This also applied to the MRP8/14 Calprotectin concentration, which was first raised after the mechanical trauma and then lowered again to the starting level.

Generally, biomarkers of disease have gained considerable attention during the past years and decades. These markers generally fall into three categories [1]

1. Current disease activity indicators
2. Future disease progression predictors
3. Predictors of future disease initiation at currently healthy sites

On the other hand the potential biomarkers in the GCF have been grouped into three generalized categories [1]

- Host derived enzymes (MMP-8 and neutrophil elastase)
- Inflammatory mediators and products (cytokines as IL-1β)
- Tissue breakdown products (Calprotectin MRP8/14)

In this study we have examined the concentrations of IL-1β, MMP-8, neutrophil elastase and MRP 8/14 calprotectin in infinitesimal volumes of gingival crevicular fluid in order to follow up transient changes in gingival crevicular fluid during soft tissue management. All these enzymes are suspected to be involved in the early collagen matrix break down and this pathway also involved in the formation of gingival recession after gingival tissue management and they also fall into all the categories mentioned above.

Regarding the literature survey the analysis for this methodology revealed that:

(1) As GCF is an oral cavity specific fluid, it has been studied in order to determine which constituents could be used as biomarkers for the prognostic of periodontal diseases
(2) There are no studies which tried to examine the enzymes and markers of gingival recession after soft tissue management
(3) There exists no single marker or combination of markers that can disclose periodontal tissue destruction adequately [5].

From the preliminary tests could be concluded that the gingival crevicular fluid sampling using Periopaper® is a reliable method since all aforementioned enzymes could be recovered [35]. Although some authors concluded that the gingival crevicular fluid collection by means of Periopaper® strips for a relatively short time is a reproducible and reliable collection method [20, 27] not all samples in the presented study were valid. This seems to be in agreement with previous studies reporting that the collection of gingival crevicular fluid for only 30 s is too short time for collecting some cytokines in detectable amounts [45]. This could be the reason of the missing detection of the markers in the some of the samples. Analysis of gingival crevicular fluid samples obtained with paper strips is a widely used method and provides reliable data. However, most studies and investigations are conducted using different sampling methodology, different assay analyzing and data presentation methodology, which leads to a limitation for the direct comparison of result. To a certain level this also leads to a conflict in the interpretation of the obtained data. Regarding standardization of the sampling methods, the laboratory assays and presentation of the laboratory data seem to be of great importance.

The decision to perform an analysis of matrix metalloproteinase contained in gingival crevicular fluid was was based on the fact that these collagenases represent the most important family of proteinases that participate in the normal turnover of periodontal tissues and as responsible enzymes for the degradation of the most matrix proteins. These proteins should be present at any time in the gingival crevicular fluid. The results of this study were in general agreement with the findings of previous studies e.g. the increased amount of MMP-8 molecules corresponded to the disease severity [19, 26, 34, 40, 41, 43].

Although IL-1β did not show the expected concentration changes over time and remained stable, its concentration always ensured additionally the reproducibility of the obtained values and the quality control of the samples. Comparing the IL-1β concentration over time during the course of the study contributed to the verification also of the diagnostic accuracy of the immune-linked enzyme assay. The findings for this enzyme corresponded with these of *Rawlinson et al.* whose group also showed stability in this enzyme's concentration and demonstrated no statistical differences between IL-1β concentration between healthy sites in diseased patients and diseased sites in healthy controls [45].

It is difficult to compare the results with most of the previously performed studies, due to variations in experiment´s set-up and mathematical treatment of the results[10]. Literature values for the amount of IL-1β, MMP-8, neutrophil elastase and MRP 8/14 (calprotectin) are very variable. There are probably two reasons for this:

(1) Evaporative losses prior to volume determination

(2) Time of gingival crevicular fluid collection

In clinical human medicine, data of fluid analysis is presented as concentration. But there are difficulties to apply this to gingival crevicular fluid analysis because of its special features like very small volumes, difficult volume standardization, evaporation at transport and contamination at sampling [9, 29, 55]. *Lamster et al.* suggested in regard to this a standardization of collection time instead of volume of gingival crevicular fluid. This leads to simplification of the data presented as activity [29].

In the present study, all enzyme data were presented as concentration and the results of *Lamster et al.* could lead to the conclusion that the neutrophil elastase, despite of its inhomogeneous outcome, might have had showed other behavior if instead of its concentration its total enzyme activity had been calculated and presented.

Some authors recommend measurements of the total enzyme activity [8, 27-29] but never the less there were no efforts to discriminate between active and latent enzymes, so it is conceivable that diagnostic information may be lost. In order to distinguish between active and inactive sites the enzyme activity should be measured, which is represented by the µl/ mm probing depth / 30 sec. For this also the probing depth of each site should be recorded.

The results in this study also exhibit very low gingival crevicular fluid levels and strikingly high concentrations of the examined markers in the healthy subjects when reported as concentration. They also show strikingly high mean deviations within in enzyme groups. Although the sampled sites were all clinically similar with no attachment loss or clinical signs of gingivitis they were completely different concerning the expression of the enzymes which also reflected in the shown mean deviations. *Lamster et al., Curtis et al.* and *Yamalik et al.* have mentioned the same problem when data were contributed as concentration [9, 28, 29, 55]. On the other hand *Yamalik et al.* also reported that there was no constant and consistent correlation between elastase activity, elastase concentration and disease severity. While neutrophil elastase was not found to be a promising marker for the aim of this trial, while presented as concentration, it should be considered to examine the elastase in its total enzyme activity. Total enzyme activity based on standardized collection time seems to be more precise in reflecting the actual enzyme content of gingival crevicular fluid [9, 28, 29].

The analytical method used in this test enabled the simultaneous measurement of multiple inflammation markers within gingival crevicular fluid samples in order to determine mediator concentration at each site. The results of the standard markers MMP-8 and MRP 8/14 Calprotectin corresponded to the expected values according to the literature research. However, the findings from the preliminary tests have shown that the transportation / analysis time is very critical because the volume obtained using Periopaper® can be a maximum of 1.7 µl and the enzymes are very sensitive to delays in analysis and the amount of enzyme content is partly below the detection limit of the assays. This may be caused by gingival crevicular fluid evaporation from the samples.

As found in the aforementioned studies the levels of gingival crevicular fluid IL-1β, MMP-8, neutrophil elastase and calprotectin were elevated in periodontitis patients and decreased after periodontal therapy and after professional tooth cleaning. In our study, IL-1β did not express the expected changes overtime as well as the neutrophil elastase delivered inhomogeneous results. The reason for this might be that our patients were of good general health but had either chronic periodontitis or gingivitis.

Because the study at hand used only one group of subjects which presented gingival health, no correlation between clinical parameters and gingival crevicular fluid levels of IL-1β, neutrophil elastase, MMP-8 and MRP 8/14 could be presented

As a conclusion of the performed trial the following can be stated:

(1) MRP 8/14 (calprotectin) was always found in abundance and was very stable compared to the other markers under examination.
(2) The MMP-8 results support the MRP8/14 calprotectin findings
(3) While neutrophil elastase was not found to be a promising marker for the aim of this trial, calprotectin may be an auspicious biomarker for the prediction and follow-up of inflammatory reaction.

The sensitivity and specificity of neutrophil elastase and IL-1β as verification standards of disease progress has not been reliably demonstrated because of the huge mean deviations within the enzyme concentration among the groups and because of the small sample size in this experiment. The sensitivity and specificity of the MRP8/14 Calprotectin and MMP-8 as markers predicting the disease progression were reliably demonstrated showing high concentration peaks 6 hours after cord insertion (mechanical trauma, e.g. intervention).

An efficient periodontal biomarker should be able to predict future attachment loss and gingival recession in a susceptible subject. Therefore the analysis of sites of patients in treatment (which would undergo a restorative treatment with soft tissue management) might help in the identification of which factors are already present and what might indicate future breakdown. An identification of the marker's standard baseline concentration is required in order to provide reliable evaluations. For this, further controlled clinical studies on a larger scale could help to understand the complex associations among the inflammatory markers and the destructive process of the gingival recessions.

5 Summary

Gingival tissue displacement by retraction cords may lead to gingival tissue system break down. It is not exactly known which enzymes and cytokines are involved in the development of gingival recessions but since IL-1β, MMP-8, neutrophil elastase and MRP 8/14 calprotectin are involved in the collagen turnover in periodontal tissues; it is suspected that this pathway may also be involved in the gingival recession formation.

The aim of the study was to develop a methodology for the reproducible analysis of neutrophil elastase; MMP-8, MRP8/14 (calprotectin) and IL-1β in µL gingival crevicular fluid (GCF) volumes by using well examined factors such as IL-1β as control values for the interpretation of GCF sampling and its analysis. The intentions was to determine, if these enzymes may be used as markers to predict the occurrence of gingival recessions after soft tissue management. This is provided that the initial concentrations and their concentrations trends during the treatment process are examined and known.

The study consisted of 6 appointments in order to asses and follow up on the development of the concentration and change trends over time and to decide, if these enzymes may be used as prognosis markers. The study was carried out on 40 systemically healthy patients. Gingival crevicular fluid samples were obtained from healthy sites of the first incisors and first molars using Periopaper® by a standardized sampling protocol. Neutrophil elastase, IL-1β, MMP-8 and MRP8/14 calprotectin were analyzed using commercial ELISA kit and Bio-Plex Cytokine Assay Kit. MMPs were measured using the Fluorokin MAP Multiplex Human MMP Panel on the Luminex 200 system. All markers were measured in nearly all samples. The obtained results were reproducible for every patient and in accordance with the expected values. The aforementioned markers can be measured simultaneously in infinitesimal volumes of GCF. Hereby the IL-1β did not show the expected changes over time and the neutrophil elastase measurements showed inconsistent results. MMP-8 and MRP8/14 calprotectin both showed a good correlation to the theoretically assumed level of inflammation. The increase after 6 hours was significant for both markers.

MRP8/14 (calprotectin) was always found in abundance and was very stable compared to the other markers under examination. The MMP-8 results support the MRP8/14 findings. While neutrophil elastase was not found to be a promising

marker for the aim of this trial, calprotectin may be an auspicious biomarker for the prediction and follow-up of inflammatory reaction.

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35. Mock D. Entwicklung einer Methodik der Analyse von PMN-Elastase, MMP-8 und IL-1β in µL-Volumina in Gingivasulkusflüssigkeit. . Masterarbeit, Masterstudiengang Zahnmedizinische Prothetik: Universität Greifswald (2011).
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ANNEX

Zustimmungserklärung der Ethikkommission bzw. Seite aus urheberrechtlichen Gründen entfernt

Publications

1. Mock, Deniza. Entwicklung einer Methodik der Analyse von PMN-Elastase, MMP-8 und IL-1β in µL-Volumina in Gingivasulkusflüssigkeit. Masterarbeit, Masterstudiengang Zahnmedizinische Prothetik, Universität Greifswald (2011)

2. Luthardt RG, Rudolph H, Mock D, Bachem M, Zhou S, Groß H-J
   Calprotectin as predictor for inflammation in Gingival Crevicular Fluid
   8th Conference of the European Federation of Periodontology, EuroPerio8, June 3-6, 2015, London, United Kingdom, Poster Session D14, #087

3. Luthardt RG, Rudolph H, Mock D, Bachem M, Zhou S, Groß H-J
   Calprotectin as predictor for inflammation in Gingival Crevicular Fluid
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