Spring 4-30-2015

Milk Fat Globule-Epidermal Growth Factor-Factor 8 (MFG-E8) as a Novel Biomarker for Periodontal Disease

Fatimah A. Meshikhes  
*University of Pennsylvania, fmesh@dental.upenn.edu*

Follow this and additional works at: [http://repository.upenn.edu/dental_theses](http://repository.upenn.edu/dental_theses)

Part of the [Investigative Techniques Commons](http://repository.upenn.edu/investigativedtechniques), [Oral Biology and Oral Pathology Commons](http://repository.upenn.edu/oralbiologyandoralpathology), and the [Periodontics and Periodontology Commons](http://repository.upenn.edu/periodonticsperiodontology)

**Recommended Citation**  

This paper is posted at ScholarlyCommons. [http://repository.upenn.edu/dental_theses/2](http://repository.upenn.edu/dental_theses/2)  
For more information, please contact repository@pobox.upenn.edu.
Milk Fat Globule-Epidermal Growth Factor-Factor 8 (MFG-E8) as a Novel Biomarker for Periodontal Disease

Abstract

AIM:

To detect the presence of milk fat globule-epidermal growth factor-factor 8 (MFG-E8) in human gingival crevicular fluid (GCF) and to determine its potential role as a biomarker for periodontal disease activity.

MATERIALS & METHODS:

GCF was collected from a total of 230 sites from with seven subjects with gingivitis, twelve subjects with chronic moderate periodontitis, fourteen subjects with chronic severe periodontitis, six subjects with localized severe periodontitis as well as seven clinically healthy subjects. Subjects from the severe periodontitis group received nonsurgical therapy and were re-evaluated after 4 weeks. GCF was re-collected from thirty sites in five subjects at that point. Pocket reduction surgery was performed on the same subgroup and GCF was collected again at the 4-month postoperative appointment. GCF collection was performed using paper strips and analyzed for the presence of MFG-E8 and cytokines using multiplexing magnetic bead immunoassays (Luminex xMAP with MagPlex beads). Each sample was tested using an MFG-E8 kit, a human cytokine/chemokine kit and a human bone panel kit.

RESULTS:

MFG-E8 was detected at higher levels in sites with gingivitis and gingival health as compared to all periodontitis groups, suggesting that MFG-E8 production is down regulated in periodontitis. Consistent with this notion, MFG-E8 was found to significantly increase following non-surgical therapy of subjects with severe periodontitis. Furthermore, the levels of MFG-E8 significantly increased after surgical treatment correlating with decreased probing pocket depths. IL-1α, IL-1β, RANKL, OPG, IL-6 and IL-17A were detected at levels consistent with those found in earlier studies. In the periodontitis treatment subgroup, the levels of RANKL, IL-6 and IL-17A decreased with decreasing probing pocket depths.

CONCLUSIONS:

MFG-E8 was detected in human GCF collected from healthy, gingivitis and periodontitis subjects using a magnetic bead-based immunoassay. The levels of MFG-E8 were negatively related to the level of gingival inflammation and increased after both non-surgical and surgical treatment of periodontal disease. These data suggest the potential of MFG-E8 as a novel biomarker of periodontal disease.

Degree Type

Thesis

Degree Name

MSOB (Master of Science in Oral Biology)

Primary Advisor

George Hajishengallis

This thesis is available at ScholarlyCommons: http://repository.upenn.edu/dental_theses/2
University of Pennsylvania Dental Medicine

Milk Fat Globule-Epidermal Growth Factor-Factor 8 (MFG-E8) as a Novel Biomarker for Periodontal Disease

THESIS

Fatimah Meshikhes, BDS
4/30/2015

Presented to the Faculty of Penn Dental Medicine in Fulfillment of the Requirements for the Degree of Master of Science in Oral Biology

Dr. George Hajishengallis
Research Advisor

Dr. Keisuke Wada
Committee Member

Dr. Jonathan Korostoff
Committee Member

Dr. Joseph Fiorellini
Committee Member
Milk Fat Globule-Epidermal Growth Factor 8 (MFG-E8) as a Novel Biomarker for Periodontal Disease

THESIS

Fatimah Meshikhes, BDS
4/30/2015

Presented to the Faculty of Penn Dental Medicine in Fulfillment of the Requirements for the Degree of Master of Science in Oral Biology
AKNOWLEDGEMENTS

I would like to thank Dr. George Hajishengallis for welcoming me in his lab and giving me an opportunity to work on this project. I would also like to extend my gratitude to everyone in Dr. Hajishengallis’s lab for allowing me to share their space and equipment. My research would not have been possible without the help and guidance of Dr. Tomoki Maekawa who took from his time to teach me the basics and offered continuous support and assistance throughout the data collection, analysis and the manuscript writing process.

I would like to thank Dr. Jonathan Korostoff for his help during the IRB process and for his invaluable role in my periodontal clinical training.

Many thanks to Dr. Joseph Fiorellini and Dr. Keisuke Wada for making time in their busy schedules to be part of my thesis committee and for their continuing support during the past years.

I am forever grateful to my parents for all that they have done for me and for being my role models, both personally and professionally. I am also grateful to my husband for his unwavering support and encouragement and my son for being my biggest motivation. I wouldn’t be where I am today if it wasn’t for them.

I would finally like to thank to all my friends and co-residents for their continuing support and humor that helped me through this journey.
ABSTRACT

AIM:
To detect the presence of milk fat globule-epidermal growth factor-factor 8 (MFG-E8) in human gingival crevicular fluid (GCF) and to determine its potential role as a biomarker for periodontal disease activity.

MATERIALS & METHODS:
GCF was collected from a total of 230 sites from with seven subjects with gingivitis, twelve subjects with chronic moderate periodontitis, fourteen subjects with chronic severe periodontitis, six subjects with localized severe periodontitis as well as seven clinically healthy subjects. Subjects from the severe periodontitis group received nonsurgical therapy and were re-evaluated after 4 weeks. GCF was re-collected from thirty sites in five subjects at that point. Pocket reduction surgery was performed on the same subgroup and GCF was collected again at the 4-month postoperative appointment. GCF collection was performed using paper strips and analyzed for the presence of MFG-E8 and cytokines using multiplexing magnetic bead immunoassays (Luminex xMAP with MagPlex beads). Each sample was tested using an MFG-E8 kit, a human cytokine/chemokine kit and a human bone panel kit.

RESULTS:
MFG-E8 was detected at higher levels in sites with gingivitis and gingival health as compared to all periodontitis groups, suggesting that MFG-E8 production is down regulated in periodontitis. Consistent with this notion, MFG-E8 was found to significantly increase following non-surgical therapy of subjects with severe periodontitis. Furthermore, the levels of MFG-E8 significantly increased after surgical treatment correlating with decreased probing pocket depths. IL-1α, IL-1β, RANKL, OPG, IL-6 and IL-17A were detected at levels consistent with those found in earlier studies. In the periodontitis treatment subgroup, the levels of RANKL, IL-6 and IL-17A decreased with decreasing probing pocket depths.

CONCLUSIONS:
MFG-E8 was detected in human GCF collected from healthy, gingivitis and periodontitis subjects using a magnetic bead-based immunoassay. The levels of MFG-E8 were negatively related to the level of gingival inflammation and increased after both non-surgical and surgical treatment of periodontal disease. These data suggest the potential of MFG-E8 as a novel biomarker of periodontal disease.
# Table of Contents

INTRODUCTION .......................................................................................................................... 5  
  Periodontal Disease ................................................................................................................ 5  
  Gingival Crevicular Fluid ....................................................................................................... 8  
  Luminex xMAP......................................................................................................................... 13  
  MFG-E8 .................................................................................................................................. 16  

AIM ........................................................................................................................................... 19  

MATERIALS & METHODS ......................................................................................................... 20  
  Participants ............................................................................................................................... 20  
  Sample Collection .................................................................................................................. 22  
  GCF Samples ......................................................................................................................... 22  
  MFG-E8 .................................................................................................................................. 23  
  Other Analytes ....................................................................................................................... 23  
  Statistical Analysis ............................................................................................................... 24  

RESULTS .................................................................................................................................. 25  

DISCUSSION ............................................................................................................................... 27  

CONCLUSIONS ......................................................................................................................... 32  

FIGURES .................................................................................................................................. 33  

TABLES .................................................................................................................................... 37  

REFERENCES ......................................................................................................................... 38
Milk Fat Globule-Epidermal Growth Factor-Factor 8 (MFG-E8) as a Novel Biomarker for Periodontal Disease

INTRODUCTION

Periodontal Disease

Dental plaque accumulation can lead to inflammation in the gingival tissues leading to gingivitis. In some susceptible individuals, gingival inflammation may progress and involve the alveolar bone and other tooth supporting structures leading to periodontitis [1].

Gingivitis is characterized by inflammation which is limited to the gingival tissues without affecting the attachment levels of the teeth. The main etiology for this gingival inflammation is the microbial biofilm that accumulates on the dental structures especially with the lack of proper oral hygiene practices [2]. This was proven by the classic 1965 study by Loë [2] where subjects with good biofilm control developed gingivitis 10-21 days after abstaining from oral hygiene practices. Resolution of gingival inflammation was noted in the same subjects within one week of resuming oral hygiene practices which shows a direct relationship between microbial biofilm and gingivitis.

Periodontitis compromises the integrity of the periodontium, i.e., the tooth-supporting structures such as the gingiva, periodontal ligament, and the alveolar bone. The disease is initiated by inflammation caused by dysbiotic bacterial communities forming on subgingival tooth sites [3]. It is broadly classified as being either chronic or aggressive [4]. Aggressive periodontitis differs from chronic periodontitis in the rapid rate of bone and attachment loss is subjects who are generally healthy, as well as having a tendency for familial aggregation [5].
Historically, it was accepted that periodontitis occurs as a continuation of gingival inflammation [6, 7]. In 1986, Loë published the results of a 15-year cohort study of Sri Lankan tea workers. The cohort consisted of a group of male tea workers aged between 14 and 30 years with no access to dental care. At the 15-year follow up, it was found that around 8% of the subjects had “rapidly progressing disease”, or what would be described today as aggressive periodontitis; 81% of the subjects were described as having “moderately progressing disease” while around 11% had no progression of periodontal disease [8]. This led to the conclusion that only a subset of the population are susceptible to periodontal disease progression and that periodontitis does not necessarily occur as a continuation of gingivitis.

Many studies have been done to allow further understanding of the host’s immune response and its role in detecting individuals who are susceptible to periodontal disease progression. Overall, periodontal disease susceptibility is determined by genetic factors that predispose to hyperinflammatory responses or by environmental factors (e.g., diet and stress) and risk-related behavior (e.g., smoking) that can modify the host immune response in a destructive direction. Further understanding of the role of the host’s immune response to periodontal pathogens may allow development of new host modulatory therapies that aim to treat periodontal disease by targeting specific aspects of the host immune system. These therapies differ from the current periodontal treatment modalities which focus on treating the local, mostly microbial etiological factors [9].

Recent microbiome analyses of human dental plaque and animal model-based mechanistic studies collectively suggest that the pathogenesis of periodontitis is not caused by individual bacteria often referred to as “periopathogens” but rather involves polymicrobial
synergy and dysbiosis, that is, an elevation of certain populations in the oral bacterial community that synergistically lead to destructive inflammatory responses and bone loss [10].

Cytokines are key mediators of the host response and play a major role in the pathogenesis of periodontal disease [11]. Cytokines are proteins that are involved in immune regulation and the inflammatory response. Graves in 2008 classified cytokines that are involved in the stimulation of inflammatory reactions as chemokines, innate immune cytokines and acquired immune cytokines [11].

Many pro- and anti-inflammatory cytokines have been studied in relation to periodontal disease. The initial immune response was characterized by the increase in protective anti-inflammatory cytokines such as transforming growth factor-β (TGF-β), interleukin-4 (IL-4) and IL-10 [9, 12]. As periodontal disease progresses, the cytokine profile shows a shift from anti-inflammatory to pro-inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2) and other mediators such as matrix metalloproteinases (MMPs). The increase of pro-inflammatory mediators leads to periodontal tissue breakdown and bone loss leading to apical migration of the dental supporting structures and clinical attachment loss [13].

Periodontitis is typically diagnosed based on clinical and radiographic evaluation of the resulting alveolar bone destruction and clinical attachment loss by manual measuring of probing pocket depths (PPD), bleeding on probing (BOP), and radiographic bone levels [14]. These parameters give information about the history of periodontal infection in an individual but do not necessarily give information about the current activity or future disease progression [15]. The increasing knowledge about the host’s response in various stages of periodontal disease may allow the future development of more accurate diagnostic methods as well as the potential development of new therapeutics that target the host’s immune response.
Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) is a secreted fluid which is naturally found in gingival tissues. It is believed that in healthy gingival tissues, a transudate of interstitial fluid is secreted into the gingival sulcus [16]. As inflammation occurs in gingival tissue, an increased permeability of gingival microvascular structures is seen which leads to the secretion of an inflammatory exudate, which explains the increase GCF volume with increasing gingival inflammation [17-19].

The earliest published reports about the presence of secretions from the gingival fluid are found in the early 1950s studies of gingival pockets by Waerhaug [20, 21]. In 1958, the presence of gingival fluid was found accidentally by Brill and Krasse [22]. In their study, which was initially conducted to examine the gingival microflora in dogs, iodine solution was used to disinfect the teeth before sample collection and it was noted that the iodine rapidly disappeared from the gingival pockets. Also, fluorescein dye was intravenously injected as part of a study and within 30 seconds, they found that the dye was detected in the healthy gingiva of the animals and could be retrieved on filter paper strips [23]. This discovery of gingival fluid secretion led to further studies by Brill. In 1959, Brill and Bjorn showed the presence of gingival fluid secretions in humans with healthy gingival tissues [24]. Brill also showed that the fluid secretion is affected by chewing and brushing and that the fluid is related to vascular permeability.

GCF has been used as a medium to detect various cells and molecules which are related to periodontal pathophysiology including the different biomarkers associated with the periodontal inflammatory process.

Masada found that IL-1 was expressed in the majority of GCF samples they tested [25]. Their samples were taken from subjects with moderate to severe periodontal disease and subjects
with “early-onset periodontitis” or aggressive periodontitis. They also showed significant reduction of IL-1 levels in most sites with aggressive periodontitis after non-surgical periodontal therapy. In 1992 and 1993, Wilton detected IL-1β in GCF in 68.9% of sites with chronic periodontitis in one sample population and in 88.6% of sites with chronic periodontitis tested in another sample population [26, 27].

Hou studied the levels of IL-1β in GCF following phase I therapy. It was found that the GCF levels of IL-1β were reduced at the reevaluation appointment following non-surgical therapy[28].

Geivelis followed 5 subjects on periodontal maintenance for 6 months. GCF samples were taken initially prior to non-surgical therapy followed by samples after 3 months and 6 months [29]. IL-6 in GCF showed positive correlation with inflammation as manifested clinically by increased probing depths and bleeding upon probing. It was also found that IL-6 levels in GCF was increased in sites with progressive periodontal breakdown as compared to inactive sites.

Mogi also reported the levels of IL-6 in GCF in subjects with periodontitis along with levels of IL-1β, β2-microglobulin (B2-MG), interferon-γ (IFN-γ) and transforming growth factor-α (TGF-α) [30]. The sample groups were healthy periodontal sites as controls and sites with mild and severe periodontitis. It was found that the levels of IL-6 increased 3 times in cases of severe periodontitis as compared to healthy controls. The levels of IL-1β and B2-MG were also significantly increased in periodontitis. IFN-γ was increased in periodontitis but the increase was not statistically significant. TGF-α, on the other hand, had a statistically significant decrease in cases with mild and severe periodontitis cases as compared to healthy sites.
Gamonal evaluated GCF IL-1β, IL-8 chemokine, RANTES and IL-10 after provision of periodontal therapy in the previous study. The study enrolled 12 subjects with moderate-severe periodontitis and 6 periodontally healthy subjects as controls. GCF was collected from all subjects at baseline and was also collected 2 months after periodontal therapy in six subjects who received non-surgical periodontal treatment. IL-8, IL-1β and RANTES were detected in GCF from most sites in periodontitis subjects and that all tested cytokines were reduced in subjects who received periodontal therapy. Subjects with periodontitis also showed significantly higher GCF volume when compared to controls and that volume was reduced following periodontal therapy [31].

Vernal used ELISA to study receptor activator for nuclear factor κB ligand (RANKL) in GCF of periodontitis subjects and healthy subjects. They also examined GCF from subjects with progressive periodontal destruction. They found a significantly higher level of RANKL in subjects with periodontitis when compared to controls. Sites with active periodontal disease also showed significantly higher levels of RANKL than inactive sites [32].

In another study published in the same year, Mogi also used ELISA to investigate RANKL and osteoprotegerin (OPG) in GCF samples from subjects with various severities of periodontitis. They found that RANKL was barely expressed in the healthy controls but that its levels increase significantly with the increase in periodontal disease level. The levels of OPG, on the other hand, were increased in healthy states and decreased with periodontal disease. It was found that sites with severe periodontitis had only 12.7% of the OPG level of healthy controls [33].

Bostanci also examined OPG and RANKL in GCF from healthy, gingivitis, aggressive periodontitis and chronic periodontitis of healthy and immunosuppressed populations. They
found a significantly higher RANKL/OPG ratio in all periodontitis groups when compared to gingivitis and healthy sites [34]. They also examined the effect of non-surgical treatment on the levels of OPG and RANKL in GCF in a 4-month follow up period. No significant differences were found between RANKL, OPG and RANKL/OPG ratio at baseline, 2 months, 3 months and 4 months after non-surgical therapy in subjects with chronic and generalized aggressive periodontitis. This led to the conclusion that although RANKL/OPG ratios may be of value in chronic periodontitis, it does not have much value in evaluating successful treatment [35].

In 2005, Vernal examined GCF from sixteen subjects with chronic periodontitis and eight healthy controls. The samples were tested by ELISA for the presence of IL-17. The findings revealed that the levels of IL-17 was significantly higher in periodontitis subjects as compared to healthy controls [36].

Silva followed subjects with moderate-severe chronic periodontitis until disease progression was detected. Two GCF samples were taken from 18 subjects representing an active and an inactive site. It was found that active sites had significantly higher levels of Receptor activator for nuclear factor κB ligand (RANKL), IL-1β and MMP-13 when compared to inactive sites [37].

In a study published in 2010, Offenbacher used an experimental gingivitis model to study the changes in composition of GCF during inflammation and resolution. Twenty-five subjects were enrolled in the study. All subjects underwent 1-week hygiene instruction phase, 3-weeks of experimental gingivitis induction using special acrylic stents that prevent brushing and flossing of two posterior sextants and then a 4-week resolution phase. GCF samples were collected at the beginning and end of the hygiene phase, followed by sample collection weekly for 3 weeks. The subjects were then instructed to use an electric toothbrush for 4 weeks with GCF samples being
collected at 2 weeks and at the end of the study period. It was found that during the experimental gingivitis phase, there was a significant increase in IL-1\(\beta\) and IL-1\(\alpha\) and a decrease in several chemokine and MMP levels. The resolution of experimental gingivitis led to the reversal of these mediators [38]. This shows that inflammatory products in GCF reflect the degree of inflammation in the gingival tissues.

Leppilahti studied MMP levels in GCF from healthy, gingivitis and chronic periodontitis subjects. Their aim was to use GCF biomarkers to identify sites showing attachment loss rather than showing inflammation alone. Fifty-eight samples were analyzed and showed a statistically significant increase in myeloperoxidase (MPO) and MMP-8 in the periodontitis group as compared to both gingivitis and periodontally healthy sites. Azurocidin and MMP-14 were significantly increased in periodontitis sites as compared to health sites and MMP-13 was significantly higher in periodontitis sites when compared to gingivitis sites. The significant difference of MPO, MMP-8 and MMP-13 between sites with gingival inflammation without attachment loss (gingivitis) as compared to sites with attachment loss (periodontitis) may show the potential of using those biomarkers in diagnosing sites with attachment loss [39].

Most of the published research on the use of GCF biomarkers provides data that differentiates periodontal disease status but does not give information about potential of periodontal disease progression. A recently published paper showed the results of a longitudinal study aiming to evaluate the use of GCF biomarkers in predicting periodontal disease progression. 83 subjects were divided into 4 groups, healthy, gingivitis, mild chronic periodontitis and moderate-severe chronic periodontitis. All subjects were monitored for 6 months with GCF sampling done every 2 months. This was followed by a 6-month treatment phase in which subjects received non-surgical periodontal therapy with GCF sampling done ever
2 months as well. It was found that subjects in both periodontitis groups showed significant reduction of MMP-8, MMP-9, C-reactive protein and IL-1β in GCF during the treatment phase as compared to the non-treatment phase. When GCF biomarkers were compared between subjects that were found to be periodontally stable and those with periodontal disease progression it was found that the periodontally-stable subjects had a significantly lower biomarker level as compared to subjects with disease progression [40]. This shows the potential usefulness of utilizing GCF testing methods in identifying patients with susceptibility to periodontal disease progression which may aid in clinical decision making regarding the appropriate mode of therapy.

GCF testing has been shown to reflect the inflammatory status in periodontal tissues during various states of health and disease. GCF collection is a simple, minimally invasive procedure that can be done with little or no discomfort to the patients. Advances in GCF analysis methods may provide new clinical tools that may allow detection of current periodontal inflammation status and to detect patients who maybe susceptible to periodontal breakdown. Additionally, advances in identification of certain biomarker profiles for different disease states may potentially lead researches to develop host modulatory therapies targeting specific biomarkers that are present in active diseased states.

**Luminex xMAP**

The immunological components of GCF and other body fluids have been typically analyzed using different types of immunoassays such as enzyme-linked immunosorbent assays (ELISAs). One disadvantage of using ELISA is the need for a separate analysis for every cytokine which requires a large sample volume in order to perform multiple analyses as well as cost and time consumption.
More recently, multiplexing bead-based immunoassays such as the Luminex xMAP technology have been introduced. These assays allow the analysis of multiple analytes simultaneously using small sample volumes by utilizing carboxylated polystyrene microspheres with fluorescent dyes.

Elshal and McCoy reviewed the studies that compared ELISA to multiplex bead based assays. They concluded that ELISA and multiplex assays do show good correlation between both methods when variables are minimized and similar protocols are followed [41].

Several recent studies utilized the multiplexing bead-based immunoassays to analyze different biomarkers in GCF in different situations. One group of researchers collected 4 GCF samples 1 week post operatively from 15 subjects who underwent periodontal surgery, as well as 4 GCF samples from uninvolved sites in the same subjects. All GCF samples were tested using a multiplexing bead assay for angiopoietin-1 (Ang-1), vascular endothelial growth-factor (VEGF), BMP-2, osteoprotegerin (OPG), tissue inhibitor of metalloprotease-1 (TIMP-1), basic fibroblast growth-factor (bFGF), keratinocyte growth-factor (KGF), and platelet derived growth-factor (PDGF). It was found that at 1-week post periodontal surgery, GCF from surgical sites showed significant increase of Ang-1, VEGF, OPG and TIMP-1. This shows that multiplexing bead based immunoassays have the ability to provide a profile of biomarkers that characterize events that occur as part of periodontal wound healing [42].

Thunell also used multiplex fluorescent bead-based immunoassay to compare cytokine and chemokine levels in GCF samples before and after non-surgical periodontal therapy. Twenty-two cytokines and chemokine were tested but only sixteen were detected by the assay. The detected cytokines includes IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IFN-γ and several chemokines. IL-5, IL-10, IL-12, IL-13, IL-15 and TNF-a were not detected by the assay. At
initial assessment, levels of IL-1α and IL-1β were higher in periodontally involved sites when compared to periodontally healthy sites. IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, IL-12 among with CCL5, RANTES, eotaxin, macrophage chemotactic protein-1, macrophage inflammatory protein-1a and interferon-y were significantly reduced in periodontally involved sites after treatment [43].

Shimada used multiplex fluorescent bead assays to test GCF from patients on periodontal maintenance for 40 biomarkers including interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17A. The assay detected twenty-six biomarkers in all samples. Nine biomarkers, including MMP-3, IL-1β, IL-21, RANTES, showed significantly higher levels in healthy sites when compared to diseased sites [44].

A group of researchers published two studies that used multiplexing bead based assays to analyze GCF in subjects with generalized aggressive periodontitis. Their findings were that subjects with generalized aggressive periodontitis showed significantly higher levels of IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-1β/IL-10 ratio. Another finding of their study was that the different GCF cytokine profiles appear with different composition of biofilm bacterial complexes[45]. Six months after non-surgical therapy of the generalized aggressive periodontitis group, GCF samples were collected again and compared to baseline. It was found that there was significant reduction in GM-CSF, IL-1β and IL-1β/IL-10 ratio in GCF after non-surgical treatment, with and without the administration of systemic antibiotics. It was also noted that GCF of IL-6, which is an pro-inflammatory cytokine, was increased after the same treatment [46].

A more recent study was done on GCF samples from healthy, gingivitis and periodontitis subjects. Multiplexing bead based analysis was also used to detect GCF levels of interferon
(IFN-γ and interleukin (IL)-4, IL-33 and thymic stromal lymphopoietin (TSLP). The significant finding of this study was that IFN-γ was found to be significantly less in healthy sites in the periodontitis group than inflamed sites in the gingivitis group. This indicates that IFN-γ can be used as a site-specific biomarker for inflammation regardless of the patient’s periodontal disease classification [47].

The previous studies have shown the advantage of using Luminex xMAP in detecting multiple mediators in a small amount of fluid with less number of assays. Multiplexing assays seem to have an advantage in analyzing GCF due to the typically limited volume of collected GCF samples especially in periodontally healthy states.

**MFG-E8**

Milk Fat Globule-Epidermal Growth Factor-Factor 8 (MFG-E8) or lactadherin is a secreted glycoprotein found on the milk fat globule membrane [48]. The N-terminal domains of MFG-E8 are similar in structure to epidermal growth factor (EGF) while the C-terminal domains are F5/8 type C which resemble coagulation factors V and VIII [49].

The name MFG-E8 came after the identification of epidermal growth factor-like regions as well as sequences that are similar to coagulation factors V and VII on a protein on the membrane of milk fat globules secreted by murine mammary epithelial cells [50]. They also reported the discovery of this same protein in lactating and non-lactating mice though its level was significantly increased during lactation. Analogous proteins with similar structures were later identified in bovine and guinea pig milk samples, as well [51-54].

In humans, Larocca group identified a protein that was termed BA46 in the milk fat globule membrane of subjects who were in the eighth months of pregnancy and underwent mastectomies due to breast tumors. Their findings have shown that the two C-terminals of BA46
shared 43% with the sequence of coagulation factor V and 38% with coagulation factor VIII. They also found that the area of the C-terminal is 60% identical to the same region in the murine MFG-E8 protein which was described by Stubbs [55].

In 1996, Couto group further studied BA46 and found that it contains a single epidermal growth factor-like domain which has an arginine-glycine-aspartic acid (RGD) tripeptide sequence identical to that found previously on one of the epidermal growth factor-like domains in murine MFG-E8. Their findings suggested that murine MFG-E8 is related to human BA46 even though they do have some differences in structure which maybe related to different functional requirements of mice vs. Humans [56].

Taylor examined the RGD sequence of BA46 in humans, they found that the RGD sequence plays a role in cell adhesion by binding to cellular integrin receptors which led to replacing the name of BA46 to lactadherin [57].

In 1997, Collins group reported the identification of a gene that is homologous to the gene of murine MFG-E8 in human infant brain DNA which they named huMFG-E8 [58]. They noted that this gene contains a sequence that is identical to the partial gene sequence of BA46 which was reported by Larocca which suggests that BA46 and huMFG-E8 might be identical [55, 58].

Further investigations of MFG-E8 have shown that MFG-E8 is not exclusively related to lactation as was initially thought. It was found in mouse fetal gonadal tissue and that it plays a role in gonadogenesis [59]. It was also found that it is secreted by macrophages and plays a role in cell apoptosis [60-62]. The expression of MFG-E8 has also been reported in Langerhans cells, skin dendritic cells [61], epidermal keratinocytes [63], retina and retinal pigment epithelium [64] as well as brain microglial cells [65, 66], pericytes [67] and endometrial cells [68, 69].
Different authors investigated the role of MFG-E8 in different conditions [70]. It was found to play a role in clearing apoptotic cells, as mentioned previously. This was first reported by Hanayama, they found that the C-terminal domains of MFG-E8 binds to apoptotic cells while the RGD motif on the N-terminal binds to phagocytes in preparation to phagocytosis [60, 61]. On the other hand, mice lacking in MFG-E8 showed reduced lymphocyte apoptosis leading to a pathological autoimmune reaction [71-73] as well as reduced apoptosis of atherosclerotic plaques in cases of atherosclerosis [74].

MFG-E8 was also found to play a role in Alzheimer's disease [75, 76], systemic lupus erythematosus [77-79], repair of intestinal epithelium [80], stroke [81], asthma [82], pancreatitis [83], diabetic nephropathy [84], obesity [85], cardiovascular diseases [86] as well as tumor development [87-89], sepsis and ischemia-reperfusion injuries [90-92] and wound healing [92] due to increased angiogenesis [67, 93].

In 2014, a paper was published by Abe et al investigating the role of MFG-E8 in periodontitis in mice. Ligatures were used to induce periodontal inflammation and bone loss [94] in MFG-E8−/− mice as well as wild-type mice which were used as controls. It was found that the level of MFG-E8 mRNA was significantly reduced within the first 24 hours. This was followed by a period of elevation that occurred with the appearance of osteoclasts and multinuclear cells up to the eighth day. Following that, a period of reduced expression was noted (around day 10) following the drop in osteoclast count. Further examination of this relationship between osteoclast levels and MFG-E8 expression revealed that MFG-E8 is expressed by osteoclasts involved in periodontal bone loss. The role of MFG-E8 in osteoclastogenesis was investigated by comparing MFG-E8−/− mice with wild-type mice. It was found that osteoclastogenesis was more efficient in the absence of MFG-E8 and that the application of MFG-E8 to knock-out mice led to
inhibition of osteoclastogenesis. The application of MFG-E8 to human osteoclast progenitors in vitro also inhibited osteoclast differentiation and maturation. In vivo, MFG-E8−/− mice undergoing ligature-induced periodontitis as well as those with naturally occurring periodontitis showed increased number of osteoclasts and periodontal bone loss as compared to controls which led to the conclusion that MFG-E8 negatively regulates periodontal bone loss [95].

Recombinant MFG-E8 was injected mice with ligature-induced periodontitis and was found to inhibit bone loss in both wild-type and MFG-E8−/− groups which may suggest the use of MFG-E8 as a local therapeutic agent in treatment of periodontitis [95].

In summary, the above-described studies have shown that MFG-E8 is expressed in various tissues, including the periodontium, and mediates diverse homeostatic functions including suppression of inflammation, apoptotic cell removal, and regulation of osteoclastogenesis.

**AIM**

The primary aim of this current research was to detect for the first time the presence of MFG-E8 in human GCF of subjects with gingivitis, periodontitis and periodontal health and to determine the role of MFG-E8 as a biomarker to detect periodontal disease activity. The secondary aim was to test the usefulness of the magnetic multiplexing assays in analyzing multiple inflammatory mediators in small GCF samples and to relate the MFG-E8 levels to those of cytokines previously shown to play a role in periodontitis.
MATERIALS & METHODS

Participants

A group of patients presenting to the Penn Dental Medicine Graduate Periodontal Clinic, The University of Pennsylvania, Philadelphia, PA were recruited to participate in this cross-sectional study from January 2013 to June 2014. The subjects enrolled were grouped as healthy (H), gingivitis (G), moderate periodontitis (Pm), severe periodontitis (Ps) or localized aggressive periodontitis (LAP).

The main inclusion criteria were adult subjects with good general health and with at least 10 teeth in the functional dentition excluding third molars and who had never received periodontal treatment at the time of initial clinical examination. All subjects were required to be able to read and understand the written consent form. For patients with periodontitis, at least two quadrants had to involve deep probing depths (PD), clinical attachment loss (CAL) and detectable radiographic bone loss.

Subjects with the following conditions were excluded:

1. Smoking, drug or alcohol abuse.
2. Uncontrolled diabetes.
3. Previous head and neck radiotherapy.
4. History of chemotherapy in the previous 12 months.
5. Immunocompromised subjects or subjects suffering from systemic diseases that significantly affect the periodontium.
6. Subjects taking medications known to affect the periodontium (e.g. Phenytoin, calcium channel blockers, cyclosporine…etc).
7. Pregnant or lactating females.
8. Subjects requiring prophylactic antibiotics.


10. Subjects using systemic antibiotics within three months prior to enrollment.

11. Subjects who currently have, or history of (within three months), of the following diseases: severe cardiovascular, pulmonary or liver diseases, end stage renal disease, active malignancy, cerebral vascular disease, HIV, TB, hepatitis or other active infectious diseases.

All subjects who volunteered to participate underwent routine periodontal examination and were classified according to the Armitage Classification System [4] as either having plaque-induced gingivitis, chronic moderate periodontitis, chronic severe periodontitis, localized aggressive periodontitis or being periodontally healthy.

Subjects with PD < 3 mm, no signs of gingival inflammation, CAL, bleeding on probing (BOP) or radiographic bone loss were placed in the healthy group. Subjects with PD < 3 mm, with signs of gingival inflammation or BOP but without CAL or radiographic bone loss were placed in the gingivitis group. The Moderate Periodontitis group included subjects with CAL of 3-4 mm and moderate radiographic bone loss. Subjects with CAL of 5 mm or more with severe radiographic bone loss were placed in the severe periodontitis group. Subjects with minimal amount of gingival inflammation and BOP with CAL and radiographic bone loss that was limited to the first molars and incisors were placed in the aggressive periodontitis group.

This study was approved by the IRB of the University of Pennsylvania (IRB #817153). The protocol was explained to the subjects and verbal and written consent was taken in accordance with the Declaration of Helsinki.
Sample Collection

All samples were collected by one investigator (F. M.) after reviewing the medical history and performing clinical and radiographic periodontal examination and establishing a periodontal diagnosis. PD and CAL were measured at 6 sites for every tooth using a manual probe (UNC 15, Hu-Friedy, Chicago, IL, USA). Five teeth were selected from each subject with at least one anterior tooth and two posterior teeth from two different quadrants. The teeth were dried and isolated with cotton rolls and the samples were obtained from the surface with the deepest PD. Samples were collected using absorbent paper strips (Periopaper, ProFlow Inc., Amityville, NY, USA) which were placed in the selected sites until mild resistance was felt and kept in place for 30 seconds [96, 97]. Samples with blood or saliva contamination were discarded. The individual paper strips were subsequently transferred into polypropylene tubes (Eppendorf, Hamburg, Germany) and were frozen (-80°C) until they were ready to be analyzed.

GCF Samples

A total of 46 subjects were included in the analysis with a total of 230 sites. The site distribution was as follows: 35 sites (7 subjects) in the Healthy group, 35 sites (7 subjects) in the Gingivitis group, 60 sites (12 subjects) in the moderate periodontitis group, 70 sites (14 subjects) in the severe periodontitis group and 30 sites (6 subjects) in the aggressive periodontitis group. Thirty samples (5 subjects) from the severe periodontitis group were reexamined at reevaluation appointment after non-surgical periodontal treatment consisting of scaling and root planing and home care oral hygiene instructions as well as after pocket reduction surgery (Fig 1). At the time of analysis, the contents of the GCF samples were eluted from the paper strips using phosphate buffered solution. The level of MFG-E8 and other cytokines were analyzed using Luminex®
xMAP® multiplexing bead-based immunoassays utilizing magnetic carboxylated polystyrene microspheres (MagPlex™).

**MFG-E8**

MFG-E8 was quantified with a Human Premixed Multi-Analyte Kit (R&D Systems, Minneapolis, MN, USA). The assay was done according to manufacturer’s instructions. The diluted magnetic micro particle cocktail was added to the wells of a 96-well magnetic plate. Samples and standards were added in duplicate to the magnetic beads and incubated for 2 hours at room temperature with shaking on a plate shaker. The wells were then washed three times with the supplied Wash Buffer using a magnetic plate holder. Diluted Biotin Antibody Cocktail was then added to each well and the plate was incubated at room temperature on a plate shaker for 1 hour. After that, the wash procedure was repeated and diluted Streptavidin-PE was added to each well. The plate was sealed and incubated for 1 hour on a shaker at room temperature. The plate was then washed and the microparticles were resuspended in Wash Buffer and incubated on a shaker for 2 minutes. The plate was then ready to be read by the Milliplex Analyzer (EMD Millipore, Darmstadt, Germany).

**Other Analytes**

All samples were also analyzed using HCYTMAG-60K-PX30 Human Cytokine/Chemokine Magnetic Bead Panel Kit (EMD Millipore, Darmstadt, Germany) to detect levels of Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-17, TNF-α and TNF-β. The assay was performed according to the manufacturer’s instructions. The diluted Wash Buffer was added to the wells of a 96-well plate and the plate was sealed and shaken on a plate shaker for 10 minutes at room temperature. The plate was then decanted and dried. Standards and Controls were added in
duplicate to the specified wells followed by PBS. Diluted Assay Buffer was added to the sample wells followed by the samples which were also added in duplicate. After that, the premixed beads were added to each well and the plate was sealed and incubated on a plate shaker overnight at 4°C. At the following day, the plate contents were removed using a magnetic plate holder to retain the magnetic beads and the plate was washed twice using the Wash Buffer. Detection antibodies were placed into each well and the plate was sealed and incubated with agitation on a plate shaker for 1 hour at room temperature. After incubation, Streptavidin-Phycoerythrin was added to each well and the plate was incubated with agitation for 30 minutes at room temperature. The washing procedure was repeated twice. The beads were resuspended by the addition of Sheath Fluid and agitation for 5 minutes and the plate was run by the Milliplex Analyzer (EMD Millipore, Darmstadt, Germany).

The samples were also processed a third time using Human Bone Magnetic Bead Panel (EMD Millipore, Darmstadt, Germany) to detect RANKL, OPG and OPN following the same procedure as the human cytokine/chemokine kit.

**Statistical Analysis**

Data were analyzed using Graphpad Prism software. Comparison between groups was done using Kruskal-Wallis test with Dunn's multiple comparison test for each detected analyte. Correlation between MFG-E8 and PPD was done using Spearman's rank-order correlation. In the treatment subgroup, differences after nonsurgical treatment were checked using Wilcoxon signed ranks test that was repeated for each analyte. *p*-value of 5% was considered significant.
RESULTS

The current investigations shows the results of a human GCF analysis in subjects classified as periodontally healthy, gingivitis, moderate and severe periodontitis as well as localized aggressive periodontitis. Figure 1 shows the distribution of sites among all groups. 45.65% of the subjects were male and 54.35% were female with a mean age of 53.23 and a standard deviation of 4.33. The distribution of age and gender among groups can be found in Table 1. Additionally, table 1 shows the probing pocket depth (PPD) measurements in all groups at time of initial examination. Table 2 shows the levels of the analytes which were detected in this study. These include MFG-E8, OPG, RANKL, IL-1α, IL-1β, IL-6 and IL-17A. Table 3 shows the reduction in (PPD) in the treated severe periodontitis subgroup while Table 4 includes the cytokine levels of the analyses were detected in the treated severe periodontitis subgroup (MFG-E8, RANKL, IL-6 and IL-17A).

Figure 2 shows the levels of detected analytes in all subject groups as well as differences in PPD. No significant difference was found in the levels of MFG-E8 in gingivitis and in health ($p > 0.9999$) but the levels in both groups were significantly higher than all periodontitis groups ($p < 0.0001$). The moderate periodontitis group showed significantly higher levels of MFG-E8 than both the severe periodontitis group and the aggressive periodontitis group ($p < 0.0005$) but no significant difference was found between levels in severe periodontitis and sites with localized aggressive periodontitis ($p > 0.9999$). Figure 3 shows the scatter plot of the correlation between PPD and MFG-E8 in all sites at initial examination. Spearman’s rank correlation showed significant negative correlation between PPD and GCF MFG-E8 levels ($r = -0.7101$, $p < 0.0001$).
When MFG-E8 was examined after non-surgical and surgical therapy in the severe periodontitis group, it was found that the level of MFG-E8 in GCF increases with decreasing probing pocket depths. The level of MFG-E8 was significantly higher after non-surgical therapy and further increased significantly after surgical therapy as periodontal pockets were eliminated (\( p < 0.0001 \)) as seen in Figure 4.

The level of OPG was found to follow a trend similar to MFG-E8 levels. It was found to be significantly higher in health and gingivitis as compared to periodontitis groups (\( p < 0.05 \)). Moderate periodontitis had significantly higher levels of OPG when compared to severe (\( P = 0.0026 \)) and aggressive periodontitis (\( p < 0.0001 \)). No significant differences were found between OPG levels in severe and aggressive periodontitis (\( p = 0.5604 \)). RANKL, on the other hand, showed an opposing trend with significantly higher levels in the periodontitis groups as compared to health and gingivitis (\( P < 0.0001 \)). No significant differences were found between the health and gingivitis group (\( p > 0.9999 \)) or between all three periodontitis groups (\( p > 0.9999 \)). The level of RANKL was found to be reduced as probing depths were reduced. It was found to significantly reduce following non-surgical and surgical therapy (\( p < 0.0001 \)).

The level of IL-1\( \alpha \) was also found to be significantly reduced in health and gingivitis as compared with periodontitis groups (\( p < 0.0450 \)) with no significant differences among all three periodontitis groups and no significant difference between health and gingivitis (\( p > 0.9999 \)). IL-1\( \beta \) was significantly lower in health and gingivitis groups as compared to the periodontitis groups (\( p < 0.0001 \)). No significant difference was found between health and gingivitis group (\( p > 0.9999 \)) or among the periodontitis groups (\( p < 0.167 \)).

IL-6 was barely detectable in health and gingivitis groups with no significant differences between both groups but was significantly increased in periodontitis groups (\( p < 0.0001 \)). The
The moderate periodontitis group had significantly lower levels of IL-6 as compared to the severe periodontitis group \((p = 0.0007)\) and the localized aggressive periodontitis group \((p < 0.0001)\). No significant difference was found between the severe and localized aggressive periodontitis groups \((p = 0.8767)\). IL-6 was also significantly reduced after surgical and non-surgical therapy \((p < 0.0001)\).

IL-17A was not detected in health and gingivitis but it was detected in all periodontitis groups with the highest level found in the localized aggressive periodontitis group. The level of IL-17A in the aggressive periodontitis groups was significantly higher than all other test groups \((p < 0.0001\) for healthy, gingivitis and moderate periodontitis. \(P = 0.0194\) for severe periodontitis). Its level in the severe periodontitis group was significantly higher than the moderate periodontitis group \((p = 0.0003)\) and both the health and gingivitis groups \((p < 0.0001)\). Furthermore, the level of IL-17A in the moderate periodontitis group was significantly higher than the level in health and gingivitis \((p < 0.0001)\). IL-17A was found to be significantly reduced following non-surgical and surgical therapy \((p < 0.0001)\).

**DISCUSSION**

The results of the current study have shown that the secreted glycoprotein MFG-E8 can be detected in human gingival crevicular fluid in different states of health and disease using magnetic bead-based multiplexing assays. It was also found that the levels of MFG-E8 in human GCF were higher in cases of health and gingivitis than in periodontal disease, where they are inversely related to the severity of the disease. It was also shown to be decreased in areas affected with localized aggressive periodontitis.
In sites with severe periodontitis that were examined after non-surgical and surgical periodontal treatment, it was found that total levels of MFG-E8 have increased at the re-evaluation visit following non-surgical therapy as compared to the initial values. The levels of MFG-E8 further increased after surgical therapy which was inversely related to the decrease in probing pocket depth.

These findings show that the levels of MFG-E8 decrease with increasing inflammation which is consistent with the findings of several authors in different inflammatory conditions such as sepsis [98-100], Alzheimer's disease [75, 76], atherosclerosis [74] and others [70]. MFG-E8 is produced by different cell-types in various tissues including epithelial cells, monocytes, macrophages, dendritic cells, fibroblasts, osteoblast, and osteoclasts. In general, the expression of MFG-E8 decreases under inflammatory conditions [70] In this regard, in sepsis, LPS inhibits the expression of MFG-E8 by macrophages [99]. Similarly, microbial LPS translocated into the periodontal lesions may inhibit the production of MFG-E8 by macrophages or other cell types that are commonly seen in the gingival corium subjacent to the epithelium that lines the gingival crevice. This would result in less MFG-E8 to reach the GCF via the gingival plexus of blood vessels in the gingival corium, a notion that is consistent with the data of this study. On the other hand, inflammation-induced osteoclastogenesis is accompanied by increased MFG-E8 expression in the generated osteoclasts [95]. However, since the net effect of inflammation on MFG-E8 is a decrease of its levels in the GCF, it is possible that the osteoclast-derived MFG-E8 represents a small fraction of its overall production in the periodontium or it is primarily consumed locally by the osteoclasts in an autocrine manner, as recently shown [95]. Another possibility might be that the osteoclast-derived proteins may not reach the GCF as readily as proteins by other cell types that are closer to the gingival crevice or periodontal pockets (for
instance, passive diffusion of proteins into the crevice should be facilitated by the proximity of their cellular source). In summary, periodontitis results in decreased levels of MFG-E8 in the GCF, whereas successful periodontal treatment or periodontal health are associated with relatively higher GCF levels of MFG-E8. The data of this study therefore suggest that MFG-E8 can be considered as a novel biomarker for periodontal disease.

Receptor activator of nuclear factor-κB ligand (RANKL) which is expressed by osteoblasts and other stromal cells plays a role in osteoclast differentiation thus leading to increased bone resorption [101]. Osteoprotegerin (OPG) blocks the activation of osteoclasts by preventing the activation of RANK on osteoclast progenitor cells by RANKL leading to decreased bone resorption. The interplay of OPG and RANKL plays a role in regulating periodontal bone loss [102]. The RANKL and OPG findings of the current study are consistent with what was shown in previous studies in that the level on RANKL is up regulated in human GCF from periodontitis sites while OPG was down regulated [32-34, 37, 42]. Moreover, the level of RANKL in the current study was found to be reduced at re-evaluation as compared to initial values and was further reduced after surgical therapy.

Overall, the investigation of different cytokines which play a role in periodontal disease, showed that the levels of pro-inflammatory and pro-osteoclastogenic cytokines IL-1α, IL-1β, IL-17A, RANKL were up regulated with increasing periodontal disease severity while the levels of homeostatic molecules, OPG and MFG-E8, were down regulated.

Levels of IL-1 were found to be lower in cases of health and gingivitis and were increased in moderate periodontitis, severe periodontitis and localized aggressive periodontitis. That is in agreement with the findings of pervious authors [25-27, 30, 37, 38, 40, 43, 45]. On the
other hand, another study found a significantly higher levels of IL-1β in healthy sites when compared to disease sites using a multiplex bead-based assay [44].

IL-6 is a pro-inflammatory cytokine that is released by osteoblastic cells and interacts with other factors to induce bone resorption [11, 103, 104]. The human GCF levels of IL-6 in the current study were almost undetected in healthy and gingivitis sites while their levels were significantly increased in moderate and severe chronic periodontitis as well as in localized aggressive periodontitis.

Following surgical and non-surgical treatment, it was found that the levels of IL-6 were reduced. This was also noted by Thunell [43] and Reis [105] where multiplex assays showed a significant reduction in IL-6 GCF levels in diseased sites after non-surgical therapy. Emingil also showed similar findings following treatment with Subantimicrobial-dose doxycycline. They found significant reduction in IL-6 levels up to nine months in sites with probing depths >7 mm [106]. These findings are inconsistent with the findings of de Lima Oliveira in generalized aggressive periodontitis cases [46].

IL-17 is a pro-inflammatory cytokine that plays a role in many systemic inflammatory diseases such as rheumatoid arthritis, asthma and allergic diseases [107]. It exacerbates periodontal disease by increasing the production of inflammatory mediators from the gingival fibroblasts [108]. Moreover, IL-17 induces the expression of RANKL by osteoblasts [109]. The level of IL-17 in the current study was not detected in health and gingivitis sites. It was however detected in both chronic and aggressive periodontitis sites, consistent with the findings of Vernal et al [36].

IL-17 was reduced following non-surgical and surgical treatment. Similarly, the study by Emingil showed significantly lower levels of IL-17 following treatment with scaling and root
planing with subantimicrobial-dose doxycycline when compared to scaling and root planing alone.

Although MFG-E8 is an anti-inflammatory protein [70] it is uncertain whether the down regulation of MFG-E8 contributes to periodontal tissue destruction as a result of defective regulation of the inflammation. Alternatively, or additionally, the inhibition of MFG-E8 production in periodontitis may result in less efficient phagocytic uptake of apoptotic cells, thereby contributing to necrosis and enhanced local inflammation. Moreover, Abe et al showed that MFG-E8 knockout mice had increased osteoclastogenesis leading to increased periodontal bone loss [95]. Although osteoclasts produce MFG-E8, apparently the level of production is not sufficient to effectively restrain osteoclastogenesis and pathologic bone resorption in periodontitis. Therefore, local administration of MFG-E8 may be protective in periodontitis as suggested by an intervention study in mice, where local microinjection of MFG-E8 protected mice against periodontal bone loss. Further research is required to confirm if MFG-E8 has similar protective effects in higher animal species and in humans. This may potentially open the door for the use of MFG-E8 as a local adjunctive therapy against inflammatory periodontal bone loss.

In the current study, multiple mediators were detected by analyzing the diluted fluid from a single paper strip by using 3 different kits. The use of multiplex bead-based immunoassays enabled the detection of multiple mediators from a single GCF strip. This offers a large advantage over the use of traditional methods such as ELISA.
CONCLUSIONS

The results of the current study have shown for the first time the presence of MFG-E8 in human GCF collected from healthy, gingivitis and periodontitis subjects by using a magnetic bead-based immunoassay.

The levels of MFG-E8 were negatively related to the level of gingival inflammation and were found to increase after surgical and non-surgical treatment of periodontal disease. This may indicate its value as a potential biomarker for periodontal inflammation. This may pave the way for future research in this field and the potential for development of therapeutic agents.

Multiplexing bead-based immunoassays are capable of detecting multiple analytes in a single assay leading to findings that are consistent with other studies.
FIGURES

**Figure 1:** Flowchart showing distribution of subjects and sites in each group. GCF testing was done at baseline for all groups. Repeated testing was done after non-surgical treatment and surgical treatment for a subgroup (30 sites) from the Chronic Severe Periodontitis group.
Figure 2: Levels of different analytes and probing pocket depths (PPD) among groups. (A) MFG-E8, (B) IL-17A, (C) IL-6, (D) RANKL, (E) OPG, (F) IL-1β, (G) IL-1α. (H) PPD. Pm: Moderate Periodontitis, Ps: Severe Periodontitis, LAP: Localized Aggressive Periodontitis, G: Gingivitis, H: Healthy. * p < 0.05, ** p < 0.0005.
Figure 3: Scatter plot of correlation between PPD and MFG-E8 in all subjects at initial examination. Significant negative correlation found between PPD and MFG-E8 ($r$-coefficient = -0.7101, $p$-value < 0.0001).
**Figure 4:** Change of levels of different analytes and probing pocket depths (PPD) in the treated severe periodontitis subgroup at initial examination, re-assessment and post-operation. (A) PPD, (B) MFG-E8, (C) IL-17A, (D) IL-6, (E) RANKL. * $p < 0.05$, ** $p < 0.0005$. 
TABLES

<table>
<thead>
<tr>
<th></th>
<th>Moderate Periodontitis</th>
<th>Severe Periodontitis</th>
<th>Aggressive Periodontitis</th>
<th>Gingivitis</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MFG-E8</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>27.00</td>
<td>12.38</td>
<td>10.60</td>
<td>43.82</td>
<td>27.78</td>
</tr>
<tr>
<td>SD</td>
<td>5.26</td>
<td>5.88</td>
<td>5.32</td>
<td>5.67</td>
<td>7.51</td>
</tr>
<tr>
<td><strong>OPG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>120.93</td>
<td>93.69</td>
<td>75.65</td>
<td>175.68</td>
<td>162.10</td>
</tr>
<tr>
<td>SD</td>
<td>37.50</td>
<td>29.72</td>
<td>36.69</td>
<td>53.84</td>
<td>55.36</td>
</tr>
<tr>
<td><strong>RANKL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>134.95</td>
<td>143.73</td>
<td>140.07</td>
<td>149.29</td>
<td>67.67</td>
</tr>
<tr>
<td>SD</td>
<td>23.09</td>
<td>35.58</td>
<td>32.68</td>
<td>30.27</td>
<td>16.41</td>
</tr>
<tr>
<td><strong>IL-1α</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>476.01</td>
<td>525.16</td>
<td>503.67</td>
<td>799.76</td>
<td>34.00</td>
</tr>
<tr>
<td>SD</td>
<td>324.55</td>
<td>342.00</td>
<td>266.34</td>
<td>138.28</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>513.31</td>
<td>597.81</td>
<td>651.68</td>
<td>149.29</td>
<td>0.03</td>
</tr>
<tr>
<td>SD</td>
<td>134.73</td>
<td>133.52</td>
<td>179.22</td>
<td>30.27</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>587.36</td>
<td>754.01</td>
<td>865.53</td>
<td>149.29</td>
<td>0.03</td>
</tr>
<tr>
<td>SD</td>
<td>150.24</td>
<td>146.52</td>
<td>170.66</td>
<td>30.27</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>IL-17A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>15.93</td>
<td>24.11</td>
<td>34.20</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>SD</td>
<td>6.22</td>
<td>4.85</td>
<td>6.22</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Table 1:** Mean level of MFG-E8, OPG, RANKL, IL-1α, IL-1β, IL-6 and IL-17A among groups at time of initial GCF collection.

<table>
<thead>
<tr>
<th></th>
<th>Moderate Periodontitis</th>
<th>Severe Periodontitis</th>
<th>Aggressive Periodontitis</th>
<th>Gingivitis</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PPD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>4.13</td>
<td>5.60</td>
<td>6.70</td>
<td>2.57</td>
<td>2.09</td>
</tr>
<tr>
<td>SD</td>
<td>1.24</td>
<td>1.55</td>
<td>1.58</td>
<td>0.78</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>53.32</td>
<td>52.35</td>
<td>19.32</td>
<td>56.78</td>
<td>49.88</td>
</tr>
<tr>
<td>SD</td>
<td>6.28</td>
<td>5.66</td>
<td>3.33</td>
<td>4.23</td>
<td>6.57</td>
</tr>
<tr>
<td><strong>Gender (%)</strong></td>
<td>Male Female</td>
<td>Male Female</td>
<td>Male Female</td>
<td>Male Female</td>
<td>Male Female</td>
</tr>
<tr>
<td>Mean</td>
<td>58.3%</td>
<td>42.9%</td>
<td>33.3%</td>
<td>57.1%</td>
<td>28.6%</td>
</tr>
<tr>
<td>SD</td>
<td>41.7%</td>
<td>57.1%</td>
<td>66.7%</td>
<td>42.9%</td>
<td>71.4%</td>
</tr>
</tbody>
</table>

**Table 2:** Mean probing pocket depth (PPD), age and gender distribution among groups at time of initial GCF collection.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Reevaluation</th>
<th>Post Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PPD</strong></td>
<td>6.03</td>
<td>3.97</td>
<td>2.63</td>
</tr>
<tr>
<td>SD</td>
<td>1.61</td>
<td>1.07</td>
<td>0.67</td>
</tr>
</tbody>
</table>

**Table 3:** Mean probing pocket depth (PPD) in severe periodontitis treatment subgroup at time of initial GCF collection, 4-week re-evaluation appointment after non-surgical therapy and 4-week postoperative appointment after pocket reduction surgery.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Reevaluation</th>
<th>Post Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MFG-E8</strong></td>
<td>11.31</td>
<td>17.03</td>
<td>27.78</td>
</tr>
<tr>
<td>SD</td>
<td>3.42</td>
<td>5.47</td>
<td>7.51</td>
</tr>
<tr>
<td><strong>RANKL</strong></td>
<td>149.29</td>
<td>107.92</td>
<td>67.67</td>
</tr>
<tr>
<td>SD</td>
<td>30.27</td>
<td>23.61</td>
<td>16.41</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>799.76</td>
<td>323.20</td>
<td>152.70</td>
</tr>
<tr>
<td>SD</td>
<td>138.28</td>
<td>178.82</td>
<td>69.42</td>
</tr>
<tr>
<td><strong>IL-17A</strong></td>
<td>25.64</td>
<td>21.64</td>
<td>12.16</td>
</tr>
<tr>
<td>SD</td>
<td>5.15</td>
<td>5.55</td>
<td>4.93</td>
</tr>
</tbody>
</table>

**Table 4:** Mean levels of MFG-E8, RANKL, IL-6, IL-17A in severe periodontitis treatment subgroup at time of initial GCF collection, 4-week re-evaluation appointment after non-surgical therapy and 4-week postoperative appointment after pocket reduction surgery.
REFERENCES


