Immune Regulation and Tissue Remodeling during Gingivitis and Periodontitis

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Abstract

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Immune and tissue homeostasis are crucial to maintain periodontal health and to prevent further tissue destruction. Orchestrating crosstalk among immune cells and tissues, mediators modulate equilibrium between tissue and immune homeostasis. Once homeostasis is disrupted, progression of periodontal disease could lead to irreversible tissue destruction, resulting in tooth loss. Recent findings suggested tissue remodeling might be initiated as early as 4 days after plaque accumulation. Comprehensive analyses of this tissue homeostasis will benefit the understanding the pathogenesis, diagnosis, and the treatment of periodontal diseases.

This thesis investigated clinical and molecular changes of gingivitis in individuals in terms of periodontal health, experimental gingivitis, naturally occurring gingivitis, and periodontitis. A systematic review was first conducted to summarize the profiles of immune and tissue regulation during the induction of experimental gingivitis. We further analyzed gingival crevicular fluid samples in either experimental or naturally occurring gingivitis to compare their differences in immune regulation,

angiogenesis, and tissue remodeling. Another cross-sectional study was also conducted to investigate the clinical and mediator profiles among inflammatory sites in patient populations with gingivitis only and severe (stage III grade B) periodontitis, including gingivitis sites in gingivitis only patients, gingivitis sites in periodontitis patients, and periodontitis sites in periodontitis patients. Commercially available bead-based multiplex immunoassays were used to analyze the gingival crevicular fluid samples.

Our results demonstrated that the induction of experimental gingivitis increased the expression of MPO, IL-1α and IL-1β, along with the decreases of MIP-1β and MCP-1/CCL2. The involvement of angiogenesis and tissue remodeling was limited during experimental gingivitis. Among 74 investigated mediators, previously published experimental gingivitis studies did not always show consistent outcomes in most of the mediator expression levels. Several factors were found to contribute to these inconsistencies, including stress, age, systemic status, and individual variability. Comparing experimental and naturally occurring gingivitis, the former exhibited greater level of IL-1β and MPO; whereas the latter had greater expression of angiogenin, C3a, MMP-13, BMP-2, OPG, and RANKL. Among naturally occurring diseases, gingivitis sites in gingivitis only patients shared several similarities with the gingivitis sites in periodontitis patients, except less plaque index, lower level of CCL2 and CCL3. Within individuals with periodontitis, gingivitis sites showed less expression of CCL5, GM-CSF, IL-6, SDF-1α, MMP-2, MMP-7, MMP-12, and similar level of mediators associated with bone metabolism. In addition, most of the investigated mediators were significantly greater in the group of periodontitis compared with the group of gingivitis only.

With the limitations, this thesis revealed that the characteristics of gingivitis varied in the individuals with different states of periodontal health and diseases. Experimental gingivitis represented a self-limiting and acute inflammatory lesion with limited involvement of angiogenesis and tissue remodeling. Naturally occurring gingivitis was a chronic lesion with involvement of diverse immune regulation, including neutrophils, monocytes, and macrophages. It also displays more activities in angiogenesis and tissue remodeling compared with experimental gingivitis. Compared with periodontitis, furthermore, gingivitis exhibited less complexity in immune and tissue regulations; whereas periodontitis

exhibits more diversity in immune regulation and greater activities in tissue turnover. The gingivitis sites in periodontitis population showed the transitional profiles between gingivitis and periodontitis.

To our knowledge, this thesis is the first study summarizing the features of experimental gingivitis and providing the most comprehensive comparisons between experimental and naturally occurring gingivitis in terms of immune regulation and tissue remodeling. These findings revealed the limitations of these gingivitis models and their indications. It also provides a foundation for personalized periodontal therapy in diagnosis, treatments, and prevention of periodontal diseases. Further investigation is required to explore the interaction of these biomarkers and the comparison between experimental gingivitis and naturally occurring gingivitis in disease resolution.

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Dedication

Dedicated to my parents, husband, little boy, and brother for their unconditional love and endless support

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Chapter 1. Background and literature review

1.1 Plaque-induced gingivitis and periodontitis

Periodontal diseases are one of the most commonly seen diseases in humans (Addy and Adriaens 1998). Induced by the plaque accumulation on a susceptible host, plaque-induced gingivitis and periodontitis represent the mild and severe form of oral diseases. Gingivitis features a reversible inflammation limited to soft tissue (Trombelli, Farina et al. 2018), whereas periodontitis is irreversible destruction of both soft and hard tissues (Caton, Armitage et al. 2018). Understanding the immune regulation and tissue remodeling in both diseases and the transitional phase in between the two significantly benefits the diagnosis, prognosis and the treatment of periodontal diseases.

1.1.1 Plaque-induced gingivitis

Clinical gingival inflammation is a well-defined site-specific condition. However, the diagnosis of gingivitis requires a complete evaluation of the patient. Based on the full-mouth evaluation of all sites, gingivitis is defined by the presence of bleeding on probing (BOP) with a patient presenting with a BOP score of ≥ 10%. Generalized gingivitis is then identified if the full-mouth BOP score is ≥ 30% (Trombelli, Farina et al. 2018). Depending on the definitions and the study populations, the prevalence of gingivitis in previous literature ranges from 16.2 to91% (Mamai-Homata, Polychronopoulou et al. 2010, Funieru, Klinger et al. 2017). A high prevalence

of approximately 93.9% in an American adult population was reported along with a significant association between consistent gingivitis and periodontitis (Li, Lee et al. 2010).

Clinically, gingivitis is considered reversible in terms of inflammation and tissue alteration once the biofilm is fully removed (Trombelli, Farina et al. 2018). The tissue change during gingivitis is usually subtle and painless. Most of the patients and, sometimes, even clinicians underestimate the early stage of gingivitis. However, gingivitis serves as the precursor of periodontitis and thus holds a particular clinical significance during the transition from periodontal healthy to a disease status.

Histologically, four stages have been proposed in disease pathogenesis. They are initial, early, established, and advanced lesions. The initial lesion features vessel vasculitis enhancing the migration of leukocytes to the junctional epithelium and gingival sulcus, followed by the early lesion. Early lesion is clinically considered the gingivitis status, with the presence of lymphoid cells accumulation and further loss of the collagen fiber network along with the proliferation of junctional epithelium. The predominance of plasma cell within the affected connective tissue highlights the presence of established lesions. At this stage, junctional epithelium proliferates and extends apically, closer to the underneath bone. If left untreated, the lesion could extend into alveolar bone and cause irreversible bony destruction, called the "advanced lesion" or, clinically, "periodontitis." (Page and Schroeder 1976).

With the advancement of molecular biology, specific biomarkers have been detected in gingival crevicular fluid (GCF), saliva, and dental plaque and examined to better comprehend the knowledge about gingivitis (Trombelli, Farina et al. 2018). In the comparison with healthy controls, patients with gingivitis have greater levels of alkaline phosphatase (ALP) (Perozini, Chibebe et al. 2010), and matrix metalloproteinases (MMP), such as MMP-8 and MMP -9

(Ramseier, Kinney et al. 2009). Cytokines associated with inflammation, such as interleukin (IL)-1β, IL-8 (Ertugrul, Sahin et al. 2013) and TNF-α (Gokul 2012, Ertugrul, Sahin et al. 2013) were also found to be increased in gingivitis patients versus healthy controls. Complement proteins in GCF were also elevated when periodontium transitions from healthy to disease status (Huynh, Veith et al. 2015). Taken all together, these data demonstrate that both innate and adaptive immunity contribute to the gingival inflammation during gingivitis. Evidence of tissue remodeling was also noticed even at the very early stage of gingivitis (Bamashmous, Kotsakis et al. 2021).

Most of the knowledge concerning gingivitis has been acquired from either experimental or naturally occurring gingivitis induced by plaque accumulation. A comprehensive analysis or direct comparisons of gingivitis biomarkers as they relate to the clinical status of periodontium remain scarce due to the heterogeneity of sampling procedure, disease definitions, and lab analysis protocols (Trombelli, Farina et al. 2018). More details of experimental gingivitis are summarized in section 1.1.2.

1.1.2 The experimental gingivitis model

The experimental gingivitis (EG) model is a popular approach to investigate the sequence of changes in microbial flora and gingival tissue in response to plaque accumulation during gingivitis. To eliminate the impact of confounding factors, systemic healthy volunteers with periodontal health are the most common patient population to be enrolled in the EG studies. In general, the EG model consists of three phases: pre-induction phase, induction phase and resolution phase. In the pre-induction phase, volunteers (usually systemically and periodontally healthy) are recruited and receive the first prophylaxis to eliminate plaque-induced inflammation. Two weeks later, the volunteers undergo another periodontal examination to ensure the removal

of gingival inflammation and are officially enrolled in the EG study, this examination and treatment serves as the baseline of the EG induction phase. The period of EG induction varies from several days to up to 4 weeks (Lamster, Hartley et al. 1985, Persson, DeRouen et al. 1990, Giannopoulou, Cappuyns et al. 2003). During EG, oral hygiene is refrained to rapidly accumulate plaque and induce gingival inflammation. At the end of the EG induction, the volunteers receive another prophylaxis and resume the oral hygiene, followed by the resolution phase until the end of the EG study.

In 1965, Loe and colleagues conducted the first EG model (Löe 1965) in human and subsequently other modifications have been introduced for different purposes (Heasman, Offenbacher et al. 1993, Lang, Sander et al. 2002, Offenbacher, Barros et al. 2010). In the original EG model, dental students were recruited and given instruction to abstain from any type of home care until gingival inflammation was induced. EG was later resolved by reinstitution of oral hygiene procedures during resolution phase (Löe 1965). In the 2000s, researchers introduced a localized stent-induced biofilm overgrowth model to reduce the discomfort of the participants during the gingivitis induction (Offenbacher, Barros et al. 2010). In this partial-mouth design, a customized stent is fabricated for each volunteer, resembling an acrylic mouthguard and extending to cover approximately 2mm over gingival margins. The volunteer is then asked to wear the stent every time when brushing or flossing to retain most of the plaque at the test sites with the protection of the stent (Offenbacher, Barros et al. 2010). These modifications allows comparisons of local changes in microbiome and inflammatory response between the abovementioned test sides and controls with routine cleaning.

The EG model is a powerful tool to demonstrate the role of biofilm and host immune responses on the initiation of gingival inflammation (Theilade, Wright et al. 1966). Along with

the increase of gingival inflammation and plaque accumulation, a shift of microbial composition was found during EG induction, including an increase of gram-negative flora, especially the motile vibrios and spirochetes (Syed and Loesche 1978). The shifts in the bacterial community during plaque growth was further confirmed later by the 16S rRNA sequencing and the diversity also increased significantly at aged plaque (Kistler, Booth et al. 2013). Host immune responses also reflect the changes of inflammatory status, such as the increasing level of IL-1β (Johnson, Reinhardt et al. 1997, Deinzer, Förster et al. 1999), PGE_2(Johnson, Reinhardt et al. 1997), and leukotriene B4 (Heasman, Offenbacher et al. 1993) throughout the time of EG induction. In addition to changes in immune regulation, tissue responses also alter during gingival inflammation, e.g. the increased expression of MMP-8 and MMP-9 (Offenbacher, Barros et al. 2010, Salvi, Franco et al. 2010). Indeed, the advent of new technology of biomarkers analysis facilitates the investigation on the changes of these mediators, allowing the identification of individual variations against bacterial challenges (Offenbacher, Barros et al. 2010, Bamashmous, Kotsakis et al. 2021). These investigations resulted in greatly enhanced the ability to test therapeutic products but also increased the understanding of periodontal inflammation.

To further understand the clinical implications of EG on the disease pathogenesis, several deficits need to be addressed. Due to the heterogeneity of analysis and protocols in the different EG studies, a comprehensive analysis of EG in terms of immune regulation and tissue remodeling is currently lacking. Also, limited data is available comparing the immune regulation and tissue remodeling during EG to naturally occurring gingivitis. Using the original model refraining from full-mouth oral hygiene, EG showed higher plaque accumulation and concentration of IL-1 ß in GCF samples than naturally occurring gingivitis (Trombelli, Scapoli et al. 2010, Farina, Guarnelli et al. 2012). There is no doubt that EG models allow a thorough

investigation on the shift pattern of microbial compositions, inflammatory mediators, and clinical changes during plaque accumulation. However, the differences in the host inflammatory response between EG and naturally occurring gingivitis have rarely been investigated. There is a significant gap in our knowledge concerning the potential variation in the inflammatory response when EG and naturally gingivitis are compared.

1.1.3 Disease transition from gingivitis to periodontitis

Disease transition from gingivitis to periodontitis is a crucial stage for disease pathogenesis. From EG studies, it was believed that an equilibrium between the host response and bacterial challenges could be achieved in the status of gingivitis to prevent irreversible tissue destruction. With the periodic exacerbations of the inflammation process, a linear progression is anticipated in the gingival inflammatory lesions when the destructive process affects the Sharpey's fibers, resulting in periodontitis (Listgarten 1986). From the setting of naturally occurring periodontal diseases, however, previous studies found that different patterns of disease activities. Within a year of observation on a total of 1155 sites with gingival inflammation, most (956 sites) of the sites (82.8%) show no significant change. Twenty-two sites had progression. Twenty-eight sites showed exacerbation and spontaneous remission; whereas 133 sites had spontaneously shallower in probing depths (Goodson, Tanner et al. 1982). For the onset and the extent of destructive periodontal diseases, several theories have been proposed, including linear, random burst, and asynchronous multiple burst models (Socransky, Haffajee et al. 1984, Jeffcoat and Reddy 1991). Interestingly, these studies all showed that not all sites of gingivitis progress to periodontitis.

Little information for humans is available to investigate the transition from gingivitis to periodontitis because it is difficult to establish the time point of onset. The concept of individual susceptibility has been repeatedly discussed since some individuals appear to have less risk of disease progression, called "non-susceptible patients." In contrast to susceptible hosts, non-susceptible patients seem to have a protective response by immune regulation via neutrophils and cytokine expression to reduce the impacts of bacterial challenges on the degree of gingival inflammation and associated tissue destruction (Listgarten, Schifter et al. 1985, Page, Offenbacher et al. 1997, Bamashmous, Kotsakis et al. 2021).

Advancements in technologies have facilitated a deeper understanding of the immune responses during the transition from gingivitis to periodontitis. During this transition, the acute inflammation from the initial phase of gingivitis turns into a chronic inflammatory lesion, leading to the development and activation of immune-defense mechanisms, involving pattern recognition and toll-like receptors, nuclear factor-κB signaling, and mitogen-activated protein kinase signaling (Kurgan and Kantarci 2018). The expression of these mediators represents attempts by the host to achieve full restoration of homeostasis in the tissue. Inadequate resolution and failure to return tissue to homeostasis results in the chronicity and extension of the inflammatory damage, leading to irreversible tissue loss characteristic of periodontitis (Van Dyke 2008, Wong, Gallant-Behm et al. 2009).

Histologically, an lesion in the stage transitioning from gingivitis (early lesion) to periodontitis (advanced lesion) is called "established lesion" (Page and Schroeder 1976). The predominant cells in an established lesion are macrophages, plasma cells, and lymphocytes. Degradation of collagen persists from the early stage (i.e., gingivitis) with the proliferation of inflammatory infiltrates (Page, Offenbacher et al. 1997, Hans and Hans 2011). Loosely adherent

pocket epithelium then replaces the junctional epithelium to allow the migration of bacteria towards the bottom of the periodontal pockets, leading to heavy inflammatory cell infiltration and further tissue destruction later in the established lesion (Kinane and Lappin 2001, Loesche and Grossman 2001, Kurgan and Kantarci 2018). Clinically, the periodontium at the stage of established lesions remains intact without bony destruction. The presence of bleeding on probing and changes in color/texture represent the characteristics of gingivitis. It is believed that the gingival inflammation could still be reversed at this point and treated without evolving into periodontitis (Kurgan and Kantarci 2018).

1.1.4. Plaque-induced periodontitis

As a microbially-associated and host-mediated inflammation, periodontitis is characterized by the irreversible tissue destruction and loss of periodontal attachment, leading to tooth loss if left untreated (Caton, Armitage et al. 2018). It is a multifactorial disease associated with systemic diseases, smoking, gender, ethnicity, genetics, diet, and lifestyle (Kornman 2020). The latest National Health and Nutrition Examination Survey (2009–2014) estimated that 42% of dentate adults in the United State with age of ≥30 years old had periodontitis (Eke, Thornton-Evans et al. 2018). Most of the patients present with mild-to moderate periodontitis and only 7.8% of the patient population suffer from severe periodontitis (Baelum, Fejerskov et al. 1986, Loe, Anerud et al. 1986, Eke, Thornton-Evans et al. 2018).

Clinically, periodontitis is defined by the presence of deep probing depth and clinical attachment loss. Additional elements to classify the periodontitis include severity (such as, radiographic bone loss and tooth loss), complexity of management (such as pattern of bone loss,

furcation involvement, masticatory and occlusal consideration), the extent of diseases, and the rate of progression (Tonetti, Greenwell et al. 2018). With these criteria, American Academy of Periodontology and European Federation of Periodontology proposed the staging and grading system in 2018, as the current classification of periodontitis (Caton, Armitage et al. 2018). Smoking and uncontrolled diabetes are considered as additional risk factors, with that those patients "smoking ≥10 cigarettes/day or diabetic patients with HbA1c ≥7.0%" are modified to grade C to highlight the potential risk of rapid rate of disease progression (Tonetti, Greenwell et al. 2018).

"Bacterial dental plaque on a susceptible host" is the main etiology of periodontitis. The role of bacteria has been widely investigated in the past century (Belibasakis, Belstrøm et al. 2023). Theilade (Theilade 1986) suggested the "non-specific" plaque hypothesis," that the virulence properties of the dental plaque were attributed to the overall increases of oral species and therefore plaque elimination could control the periodontal inflammation. Meanwhile, the "specific" plaque hypothesis was proposed with the advancement of methodological progress in lab analysis that specific indigenous bacterial species were responsible for the periodontal disease (Loesche 1979, Socransky, Haffajee et al. 1998). The "ecological" plaque hypothesis was later introduced to highlight the importance of homeostatic balance between the host and the microbiota (Marsh 1994). This hypothesis was further framed in the concept of "polymicrobial synergy and dysbiosis" and "keystone pathogen" that *P. gingivalis* serves as the puppet-master orchestrating other bacterial members of the biofilm community and collectively develops a disease-provoking microbiota for periodontal inflammation and disease progression (Hajishengallis, Darveau et al. 2012, Hajishengallis and Lamont 2012). Like a "chicken and egg" debate, another theory called "inflammation-mediated-polymicrobial-emergence and dysbiotic-

exacerbation" model (Van Dyke, Bartold et al. 2020) was recently proposed to complement the abovementioned AAP/EFP Classification of Periodontal Diseases (Caton, Armitage et al. 2018). In this model, gingivitis is considered the inflammation associated with an overgrowth of commensal plaque bacteria. Periodontitis initiates when the dysbiosis is triggered with the increase of polymicrobial diversity, followed by dysbiosis exacerbation mediated by inflammation. At the late stage of periodontitis, the polymicrobial diversity decreases. Indeed, the continuity of inflammation drive the disease progression and it is believed that microbial specificity (pathogenicity) involves in this process only at the late stage of periodontal diseases (Van Dyke, Bartold et al. 2020).

Host immune, both innate and adaptive systems, also play roles which contribute to the development of periodontitis (Becerra-Ruiz, Guerrero-Velázquez et al. 2022). With the presence of bacteria, the innate immune response is initiated through stimulating toll- like receptors located on the membranes of various hematopoietic and non-hematopoietic cells, such as gingival epithelial cells, fibroblasts, dendritic cells, and macrophages (Liu, Du et al. 2013, Li, Chen et al. 2014). Cytokines and chemokines are consequently produced via the activation of the mitogen-activated protein kinase pathway, recruiting non-resident leukocytes to the site of infection. The accumulation of neutrophils releases neutrophil extracellular traps to bind and destroy the pathogens, also causing the degradation of tissue in periodontium (Nicu, Rijkschroeff et al. 2018). Mast cells and macrophages are also activated, producing pro-inflammatory cytokines (TNF-α, IL-6) and metalloproteinases to evolve the disease by maintaining the inflammation and further degrading periodontal tissues (Yang, Zhu et al. 2018, Shahsavari, Azizi MaZreaH et al. 2020). Binding the pathogens, natural killer cells also induce dendritic cells to mature. Releasing TNF-α and IL-2, the dendritic cells acts as messengers between innate and

adaptive systems by traveling to the lymph nodes to present the antigen to T and B lymphocytes (Di Benedetto, Gigante et al. 2013). More cytokines then are released by different classes of T lymphocytes (Sommer, Dalia et al. 2019), orchestrating the networks of cells and cytokines for disease progression.

Recognizing the contribution of both the microbiota and the host immune response on the periodontium, disease pathogenesis of periodontitis has been also evolving since 1960s. The discussion started with the role of a microbial challenge to the disease initiation and progression (Löe 1965, Lindhe, Hamp et al. 1973). The host immune-inflammatory response was later included in the concept (Loe, Anerud et al. 1978, Loe, Anerud et al. 1986). In 1997, Page and Kornman proposed the landmark model of the pathogenesis of human periodontitis. In addition to the abovementioned elements, this model included additional contributing factors from environment and tissue metabolism. It also highlighted the acquired and genetic risk factors to address the complexity and non-linear progression of periodontitis (Page and Kornman 1997). Inheriting the concept of a non-linear model and contributing factors, in 2008, Kornmann proposed another biologic system model. It is a hierarchical model using multiple levels to provide a framework to present the interaction among individual immune responses (the lower level), tissue metabolisms, and clinical presentation (the top level) (Kornman 2008).

The ultimate goal of understanding gingivitis and periodontitis is to define the interaction of host immune regulation and tissue metabolism against the bacterial challenges in the oral cavity with the respect to each set of environmental and genetic conditions. This will fundamentally benefit the clinician in diagnosis and the selection of treatment strategies. It also helps the patient to find the best personalized periodontal therapy to manage periodontitis or even prevent further disease progression.

1.2 Mediators of immune regulation and tissue remodeling in gingival crevicular fluid

The advent of ELISA technology to monitor the host inflammatory response has greatly enhanced the ability to diagnose disease. For example, biomarkers in oral fluids have been used to detectand monitor periodontal diseases (Taba, Kinney et al. 2005, Barros, Williams et al. 2016, Arias-Bujanda, Regueira-Iglesias et al. 2020). Indeed, in the gingival crevicular fluid (GCF), the biomarker concentrations fluctuate depending on the health/disease status of the periodontium. Along with inflammation, a mixture of cytokines, proteinases, and cells are released into the GCF. Therefore, GCF is considered a non-invasive tool to detect subclinical changes of disease status, including tissue metabolism and immune regulation (Barros, Williams et al. 2016).

1.2.1 Mediators of immune regulation

Defined as soluble small proteins (~5-20kDa), cytokines contribute to cell signaling and communication to control immune and inflammatory responses via binding to definite receptors on specific cells. Based on their functions, cytokines are categorized as lymphokines (lymphocyte-produced cytokines), tumor necrosis factors (pro-inflammatory activity), chemokines (promoting chemotaxis), interleukins (regulating the communication between leukocytes), and interferons (involving innate immunity) (Ramadan, Hariyani et al. 2020).

Serving as surveillance and first responders of the initial immune response in the periodontium, polymorphonuclear neutrophils (PMNs) are found in the superficial layers of the epithelium and the base of the gingival sulcus in healthy periodontium, migrating into gingival

crevice in the presence of periodontal diseases guided through chemotaxis (Khoury, Glogauer et al. 2020). Chemokines, also known as chemotactic cytokines, are responsible for the activation and migration of PMN's and other inflammatory cells. Based on their ligand structures, they are divided into 4 subfamilies: CXC, CC, C, and CX3C (Zlotnik and Yoshie 2000). Their receptors, CXCR and CCR, are expressed in different types of immune cells (Graves 2008, de Carvalho Fraga, Alves et al. 2013). Previous studies have shown changes in chemokine expression during periodontal inflammation. In CC chemokines, the level of CCL2/monocyte chemoattractant protein-1 (MCP-1) and CCL5/ RANTES elevate during periodontal inflammation, regulating microbial signals in periodontal tissues (Rath-Deschner, Memmert et al. 2020). CXC chemokines, including CXCL5/ENA-78 and CXCL8/IL-8, are also found in greater concentration in the patients with periodontal diseases, and thus are considered as potential indicators for the inflammatory process (Lappin, Murad et al. 2011, Bamashmous, Kotsakis et al. 2021). CX3CL1 is the only member in CXC3 family, also known as fractalkine, which is recently highlighted for its role on reflecting and regulating the inflammation of periodontium (Balci, Cekici et al. 2021).

Interleukins (ILs) are another group of cytokines released mainly by leukocytes and other inflammatory cells, such as lymphocytes, monocytes, and macrophages. Some ILs are considered pro-inflammatory, including IL-1 α, IL-1 –β, IL-6, IL-12 and IL-17, with the abundance of evidence demonstrating a relationship between their increase and periodontal diseases (Reinhardt, Masada et al. 1993, Tsai, Tsai et al. 2005, Sánchez-Hernández, Zamora-Perez et al. 2011, Goldbach-Mansky 2012, Papathanasiou, Conti et al. 2020). On the other hand, anti-inflammatory cytokines, such as IL-1ra, IL-4, and IL-10, downregulate and control inflammation (Lappin, MacLeod et al. 2001, Pradeep, Roopa et al. 2008, Papathanasiou, Conti et

al. 2020). Indeed, periodontal therapy benefits in reducing the level of pro-inflammatory cytokines along with the decrease of inflammation, indicating that the level of cytokines reflect the disease status (Toker, Poyraz et al. 2008, Reis, Da Costa et al. 2014).

Other pro-inflammatory cytokines expressed in periodontal lesions include tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), macrophage migration inhibitory factor (MIF), and granulocyte-macrophage colony stimulating factor (GM-CSF). Targeting different immune cells, they play vital roles in the regulation of innate immunity (Jäger, Ringhoffer et al. 1996, Calandra and Roger 2003, Šedý, Bekiaris et al. 2015). A recent study showed the level of GM-CSF varies from different stages of periodontitis (Dikilitas, Karaaslan et al. 2022). In a mouse model, Darveau's lab also found the MIF contributes to the maintenance of oral homeostasis via regulating the expression of TLR4 and IL-8/CXCL8, showing the crosstalk of these cytokines and their role in immune regulation of periodontium (Kantrong, Chang et al. 2022).

1.2.2 Mediators of angiogenesis

Angiogenesis is crucial in maintaining periodontal health and tissue repair/ regeneration. Formatting new capillaries from pre-existing blood vessels, the presence of angiogenesis also represents the chronicity of an inflammatory lesion (Carlile, Harada et al. 2001). Similar to the concept that immune homeostasis is maintained by a balance between pro-inflammatory and anti-inflammatory mediators, angiogenesis is a result of a balance between positive and negative regulators, affecting the function of endothelial cells, such as proliferation, migration, and activation (Naldini and Carraro 2005). Several growth factors and cytokines regulate the process, including angiogenin, angiopoietin-1, angiostatin, IL-12, stromal cell-derived factor 1(SDF-

1/CXCL12), vascular endothelial growth factor (VEGF), and intercellular adhesion molecule-1 (ICAM-1).

Among those promoting angiogenesis, VEGF is the most widely investigated mediator for its role in the periodontium. It promotes expansion of the vascular network and contributes to the disease progression, especially during the transition from gingivitis to periodontitis (Johnson, Serio et al. 1999, Suthin, Matsushita et al. 2003, Artese, Piattelli et al. 2010). Angiopoietin-1 also has a protective role on angiogenesis since it stabilizes the vessels after VEGF-mediated development of primary vascular plexus (Kiss and Saharinen 2019). Angiogenin is another major mediator of angiogenesis and increases in response to hypoxia in periodontium (Janjić, Bauer et al. 2019). In regenerative medicine, recombinant angiogenin have been found to promote wound healing and bone regeneration (Shi, Han et al. 2008, Kim, Kim et al. 2015).

In contrast, IL-12 has a strong impact on angiogenesis inhibition (Voest, Kenyon et al. 1995) through the modulation of angiostatin (Albini, Brigati et al. 2009) and interferon-inducible protein 10 (Sgadari, Angiolillo et al. 1996). Interestingly, angiopoitin-1 can counteract the angiogenic activity stimulated by VEGF. An *in vitro* study showed that Angiopotin-1 could decrease VEGF-induced inflammation via suppressing the endothelial adhesiveness through the reduction of adhesion molecules, such as ICAM-1, vascular cell adhesion molecule-1, and E-selectin (Kim, Moon et al. 2001). In gingival tissue, the reduction of angiopoietin-1 concentration may promote gingival inflammation via increasing more VEGF release (Lester, Bain et al. 2009). The crosstalk between these mediators has been discussed in tumor control and regenerative dentistry (Nguyen, Vrabel et al. 2020, Baru, Nutu et al. 2021). However, the cross talk between these mediators remains unclear with respect to its contribution to periodontal pathogenesis.

1.2.3 Mediators of tissue remodeling

Irreversible tissue destruction defines periodontitis, and results from chronic periodontal inflammation. Recent findings in Darveau's lab suggest tissue remodeling and turnover could initiate as early as the 3-4 days after plaque accumulation (Bamashmous, Kotsakis et al. 2021). Similar to immune regulation in the periodontium, chronic periodontal inflammation disrupts tissue homeostasis, enhances collagen degradation, and uncouples bone remodeling (a process consisting of bone resorption and formation), leading to a net bone resorption (Graves, Oates et al. 2011, Hathaway-Schrader and Novince 2021).

Matrix metalloproteinases (MMPs), also known as matrix metallopeptidases or matrixins, have been widely investigated for their role on oral diseases (Sapna, Gokul et al. 2014). Based on their structures, MMPs are divided into 6 groups: collagenases (MMP-1, 8, 13, 18), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10, and 11), matrilysins (MMP-7 and 26), membrane-type MMPs (MMP-14, 15, 16, 17, 24, 25), and others (Verma and Hansch 2007). Collagenases initiate matrix degradation, followed by further breakdown by gelatinases and stromelysins (Domeij, Yucel-Lindberg et al. 2002). These MMPs orchestrate the tissue homeostasis, which are further regulated by genes, precursor activation, differences in substrate specificity, and MMP inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) (Nagase 1999). As the main collagenase in gingival connective tissue, MMP-8 is anti-inflammatory and responsible for the majority of collagenolytic activity in GCF, especially in chronic periodontitis (Sorsa, Gursoy et al. 2016). MMP-8, 9, and 13 are considered as indicators of periodontal disease progression (Hernández, Dutzan et al. 2011), since MMP-13 has a strong impact on collagenolysis and bone resorption via regulating MMP-9 activation and mediating RANKL release (Golub, Lee et al.

1997, Hernández Ríos, Sorsa et al. 2009, Nannuru, Futakuchi et al. 2010). Another collagenase, MMP-1, is predominant in patients with aggressive periodontitis (Tüter, Kurtiş et al. 2002). The latent form of MMP-1 could be activated by MMP-3. Interestingly, several pro-inflammatory cytokines (ex. IL-1β, TNF-α, and IL-17) upregulate both MMP-1 and MMP-3 in fibroblasts via COX-2, MAP-kinases and tyrosine kinases pathways in human fibroblasts (Domeij, Yucel-Lindberg et al. 2002, Beklen, Ainola et al. 2007) to promote soft tissue breakdown. This further confirms the cytokine regulation on matrix degradation. Conversely, TIMPs inhibit proteinase production and block autocatalytic MMP activation (Shibata, Takiguchi et al. 1999). Clinically, the elevation of TIMPs and decrease of MMPs could both be found after active periodontal therapy for periodontitis patients (Tüter, Kurtiş et al. 2005, Gursoy, Könönen et al. 2010, Marcaccini, Meschiari et al. 2010). The balance of MMP and TIMP, i.e., the ratio of MMP/TIMP, is therefore suggested as an indicator for disease monitoring and the treatment efficacy.

The balance of mediators is also crucial in bone metabolism. A good example is the interaction among receptor activator of nuclear factor kappa-B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG). Released from osteoblast lineage cells, RANKL stimulates osteoclastic activity and bone resorption. As a receptor of RANKL, RANK on the surface of osteoclast progenitor cells binds RANKL promotes osteoclasts differentiation and activation. On the other hand, the decoy receptor of RANKL, OPG competes for binding sites with RANK to inhibit osteoclastic activity, which are upregulated by the inflammation (Boyle, Simonet et al. 2003). Similar to matrix degradation, multiple cytokines are involved in the regulation (Graves, Oates et al. 2011). In animal models, pro-inflammatory cytokines released by Th1 cells (ex. IL-1β, TNF-α, and IFN-γ) upregulate RANKL expression, leading to bone

resorption (Assuma, Oates et al. 1998, Takayanagi, Ogasawara et al. 2000, Delima, Oates et al. 2001, Delima, Karatzas et al. 2002). Counteracts of Th2 cells released cytokines, such as IL-4 and IL-10, inhibits bone-resorbing activity via reducing RANKL expression and increasing OPG release (Mangashetti, Khapli et al. 2005, Claudino, Trombone et al. 2008, Fujii, Kitaura et al. 2012).

Taken all together, periodontal inflammation is caused by the interaction between bacteria and the host's immune system, both innate and adaptive, through cells and signaling pathways. Disruption of immune balance leads to an imbalance in the mediators released by immune cells, which sequentially enhances matrix degradation and increases osteoclastic activities. In the periodontium, tissue homeostasis is maintained and regulated by mediators such as MMPs and the RANK/RANKL/OPG system. An imbalance in immune and tissue mediators exacerbates the disease progression from health to gingivitis and potentially to periodontitis. Understanding the mediators involved in these transitions is crucial for comprehending the initiation and progression of periodontal diseases and avails in their detection, monitoring, and treatment.

Chapter 2. Immune and Tissue Regulation during Human Experimental Gingivitis

2.1 Introduction

Plaque-induced gingivitis is one of the most common oral diseases. To investigate immune responses in gingival tissue against bacterial challenges, the setting of experimental gingivitis (EG) was introduced in 1960s (Theilade, Wright et al. 1966) which facilitated the accumulation of dental plaque within a short period in systemic healthy volunteers by refraining their oral hygiene. Another modification used in recent years is a partial mouth experimental design that the volunteers abstain from brushing or flossing in one or two sextants by wearing individualized acrylic stents during the EG induction phase (Offenbacher, Barros et al. 2010). These methods have been popular and widely used. They have significantly increased our knowledge concerning microbial ecological succession during plaque accumulation as well provided an avenue to test therapeutic products during reversible gingival inflammation.

Derived from the periodontium, gingival crevicular fluid (GCF) is a serum transudate which is often employed to determine the status of inflammation and tissue remodeling. GCF has been therefore broadly used in previous literature as a research fluid to investigate underlying inflammatory and tissue alterations among different severities of periodontal diseases. It provides a non-invasive testing for the presence of periodontal diseases and also contributes better knowledge of bacterial profiles and immune responses at the beginning of gingival inflammation. .

Despite the broad utilization of EG model and GCF analysis in the field of periodontics, the features of EG in immune regulation and tissue remodeling have not been fully characterized. The lack of knowledge on EG features makes it difficult to set up the estimate outcomes and prevents researchers to further utilize the model. The factors affecting EG outcomes also remain unclear. This systematic review investigated the changes of immune regulation and tissue remodeling mediators in GCF before and during EG.

2.2 Materials and methods

This systematic review was performed following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Liberati, Altman et al. 2009, Moher, Shamseer et al. 2015). This systematic review has been registered on INPLASY (registration number: INPLASY2023120050). Ethical approval was not required for this systematic review.

2.2.1 Search strategy

2.2.1.1 Focused Questions

Based on the PICO principle,

1. Participants: healthy patients with periodontal health
2. Interventions: induction of EG via oral hygiene refrain
3. Comparisons: healthy periodontium (day 0)
4. Outcomes of interests and the study design: the primary outcome is the alteration of GCF mediators and the secondary outcomes are the changes in clinical parameters, including gingival index (GI) and plaque index (PI) during EG.

2.2.1.2 Study Selection Criteria

1. Studies had to be published in English.

2. Studies had to be randomized controlled trials (RCT) and clinical controlled trials (CCT) evaluating the immune regulation and tissue remodeling mediators in GCF before and after EG via oral hygiene refrain
3. Studies enrolled ≥ 10 human participants with age more or equal to 18.
4. Studies had to report the changes of immune regulation and tissue remodeling mediators
5. The length of induction phase of EG had to be more or equal to 14 days
6. In vitro studies, case reports, animal studies, retrospective studies, narrative review, unpublished data, communications, or expert opinions were excluded.
7. The study with any intervention, such as mouth rinse/brushing/medication, performed during EG induction phase were excluded.

2.2.1.3 Searching Source

A search was conducted for studies published from January 1960 to June 2023 in several electronic databases, including MEDLINE (PubMed), EMBASE, Web of Science, and ClinicalTrials.gov., using specific key terms. Moreover, the additional search was performed in the following journals: Journal of Periodontology, Journal of Clinical Periodontology, Journal of Periodontal Research, and Journal of Dental Research. Finally, the reference lists of identified articles were screened to find additional articles that might fit the selection criteria.

2.2.1.4 Data Extraction

The titles and abstracts of all retrieved articles were independently screened by two examiners. The full texts of potentially qualifying articles were reviewed. Any disagreement between the two authors was resolved by discussion. If the identified studies had multiple groups of subjects, only those groups fitting the selection criteria described above were included. Data were independently extracted by the two examiners with a specially designed form on Covidence

platform and the accuracy was confirmed by a third reviewer. The authors of potentially qualifying articles were contacted if there was unclear information that needed clarification.

The following information was extracted: bibliometric information (author, publication year), demography of volunteer population (age, gender, number of volunteers, inclusion of smokers, and diabetes mellitus (DM)), EG design, duration of EG induction, and the outcomes of immune regulatory and tissue remodeling mediator at the baseline and the end of EG.

2.2.1.5 Search Strategy

- PubMed: ("experimental gingivitis"[tiab:~2] OR "experimentally gingivitis"[tiab:~2]) NOT (Animals[Mesh] NOT Humans[Mesh])
- Embase: (experimental* NEAR/2 gingivitis) NOT ('animal'/exp NOT 'human'/exp)
- Web of Science: (TS=(experimental* NEAR/3 gingivitis)) AND DT=(Article OR Early Access OR Correction)

2.2.2 Quality assessment

The Version 2 of the Cochrane risk-of-bias tool for randomized trials (RoB2) (Sterne, Savovic et al. 2019) and Risk Of Bias In Non-randomized Studies - of Interventions (ROBINS-I)(Sterne, Hernan et al. 2016) tools were used to assess quality of RCT and CCT respectively. The quality assessment was conducted by two examiners independently and the agreement between the two authors was evaluated using the kappa statistic.

2.2.3 Grading the body of evidence

Quality-of-evidence (risk of bias in reported outcomes, inconsistency of outcomes among studies, indirect reporting of outcomes, lack of precision with reported outcomes, and potential publication bias) assessment was based on the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system (Guyatt, Oxman et al. 2008, Guyatt, Oxman et

al. 2011). The GRADE system classifies the quality of evidence in four levels: high, moderate, low, and very low. The quality of evidence is rated based on 5 elements, including risk of bias, inconsistency, indirectness, imprecision, and publication bias. Studies were independently evaluated by two examiners and agreement was reached by discussion, as needed (GRADE system assessment).

2.2.4 Data synthesis

Extracted data were summarized in evidence tables to detect differences in study characteristics and quantify the body of evidence. Heterogeneity of both the design and data of the studies precluded a meta-analysis from being performed.

2.3 Results

One hundred and forty six articles were selected for full-text review after screening 745 abstracts and tiles. Figure 1 is the PRISMA flow chart. One hundred and sixteen studies were excluded. The excluded articles and the reason(s) for their exclusion can be found in table 1. A total of 30 studies with 594 patients were included in this systematic review. Characteristics of selected papers were described and summarized in table 2.

2.3.1 Characteristics of selected papers

Three selected articles are RCT (Deinzer, Waschul et al. 2004, Scott, Milward et al. 2012, Bamashmous, Kotsakis et al. 2021) and the rest are CCTs (Table 2). Twenty-one studies (Lamster, Hartley et al. 1985, Chapple, Socransky et al. 1996, Johnson, Reinhardt et al. 1997, Preshaw, Geatch et al. 1998, Deinzer, Förster et al. 1999, Fransson, Mooney et al. 1999, Gonzáles, Herrmann et al. 2001, Herrmann, Gonzáles et al. 2001, Tsalikis, Parapanisiou et al. 2002, Wright, Chapple et al. 2003, Deinzer, Weik et al. 2007, Nonnenmacher, Helms et al. 2009,

Ozdemir, Ozcan et al. 2009, Grant, Creese et al. 2010, Tsalikis 2010, Scott, Milward et al. 2012, Dommisch, Skora et al. 2019, Nascimento, Møller et al. 2020, Bamashmous, Kotsakis et al. 2021, Leite, Nascimento et al. 2022, Roberts, Yonel et al. 2022) included young (18-35 years old) and healthy patient populations. Seven articles (Fransson, Mooney et al. 1999, Tsalikis, Parapanisiou et al. 2002, Nonnenmacher, Helms et al. 2009, Offenbacher, Barros et al. 2010, Salvi, Aglietta et al. 2012, Scott, Milward et al. 2012, Meyer, Giannopoulou et al. 2017) included patient with age of 35 or more. Four selected articles(Fransson, Mooney et al. 1999, Tsalikis, Parapanisiou et al. 2002, Nonnenmacher, Helms et al. 2009, Tsalikis 2010) included both young and old groups for comparison (see details in section 3.4) Five (Persson, DeRouen et al. 1990, Kinane, Adonogianaki et al. 1991, Deinzer, Kottmann et al. 2000, Waschul, Herforth et al. 2003, Deinzer, Waschul et al. 2004) articles did not include the information of the age of patient population but mention they were medical students, indicating they may be young and well-educated. Only 1 study specified that they enrolled patients with type 1 diabetes (Salvi, Franco et al. 2010).

Fourteen studies (Persson, DeRouen et al. 1990, Kinane, Adonogianaki et al. 1991, Fransson, Mooney et al. 1999, Gonzáles, Herrmann et al. 2001, Herrmann, Gonzáles et al. 2001, Tsalikis, Parapanisiou et al. 2002, Deinzer, Weik et al. 2007, Nonnenmacher, Helms et al. 2009, Ozdemir, Ozcan et al. 2009, Salvi, Franco et al. 2010, Tsalikis 2010, Meyer, Giannopoulou et al. 2017, Nascimento, Møller et al. 2020, Leite, Nascimento et al. 2022) were conducted in a full-mouth design, refraining patients from any types of oral hygiene for the entire periods of EG induction. The rest of studies used the partial mouth design, having the participants wear an individualized acrylic stent while routine brushing and flossing to prevent any plaque disturbance for the duration of the study. Twenty selected studies induced the EG for 21 days; whereas the

durations of induction phase were 14, 18 and 21 days in 3 (Persson, DeRouen et al. 1990, Nonnenmacher, Helms et al. 2009, Ozdemir, Ozcan et al. 2009), 2(Gonzáles, Herrmann et al. 2001, Herrmann, Gonzáles et al. 2001), and 5 (Lamster, Hartley et al. 1985, Johnson, Reinhardt et al. 1997, Waschul, Herforth et al. 2003, Deinzer, Waschul et al. 2004, Deinzer, Weik et al. 2007) studies.

A total of 74 biomarkers and clinical parameters in GCF samples were anlyzed in these selected articles. Among them, 32 biomarkers and clinical parameters were measured repeately in different studies, including PI, GI, GCF volume, myeloperoxidase (MPO), macrophage migration inhibitory factor (MIF), macrophage inflammatory protein-1 β (MIP-1β), monocyte chemoattractant protein-1 (MCP-1/CCL2), CC chemokine ligand 20 (CCL20), CXC chemokine epithelial cell-derived neutrophil activating peptide (ENA-78/CXCL5), Fractalkine/CX3CL1, Prostaglandin E2 (PGE2), interleukin (IL)-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, tumor necrosis Factor alpha (TNF-α), interferon gamma (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF), fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-8, MMP-9, lactoferrin, alkaline phosphatase (ALP), α 2 macroglobulin, α 1-antitrypsin, and elastase. Among all selected articles, thirteen studies (Johnson, Reinhardt et al. 1997, Preshaw, Geatch et al. 1998, Fransson, Mooney et al. 1999, Gonzáles, Herrmann et al. 2001, Tsalikis, Parapanisiou et al. 2002, Deinzer, Waschul et al. 2004, Ozdemir, Ozcan et al. 2009, Offenbacher, Barros et al. 2010, Tsalikis 2010, Meyer, Giannopoulou et al. 2017, Nascimento, Møller et al. 2020, Leite, Nascimento et al. 2022, Roberts, Yonel et al. 2022) provided the value of each biomarker and the rest of the studies presented their data in graphs, instead of numbers.

Figure 1. PRISMA flow chart

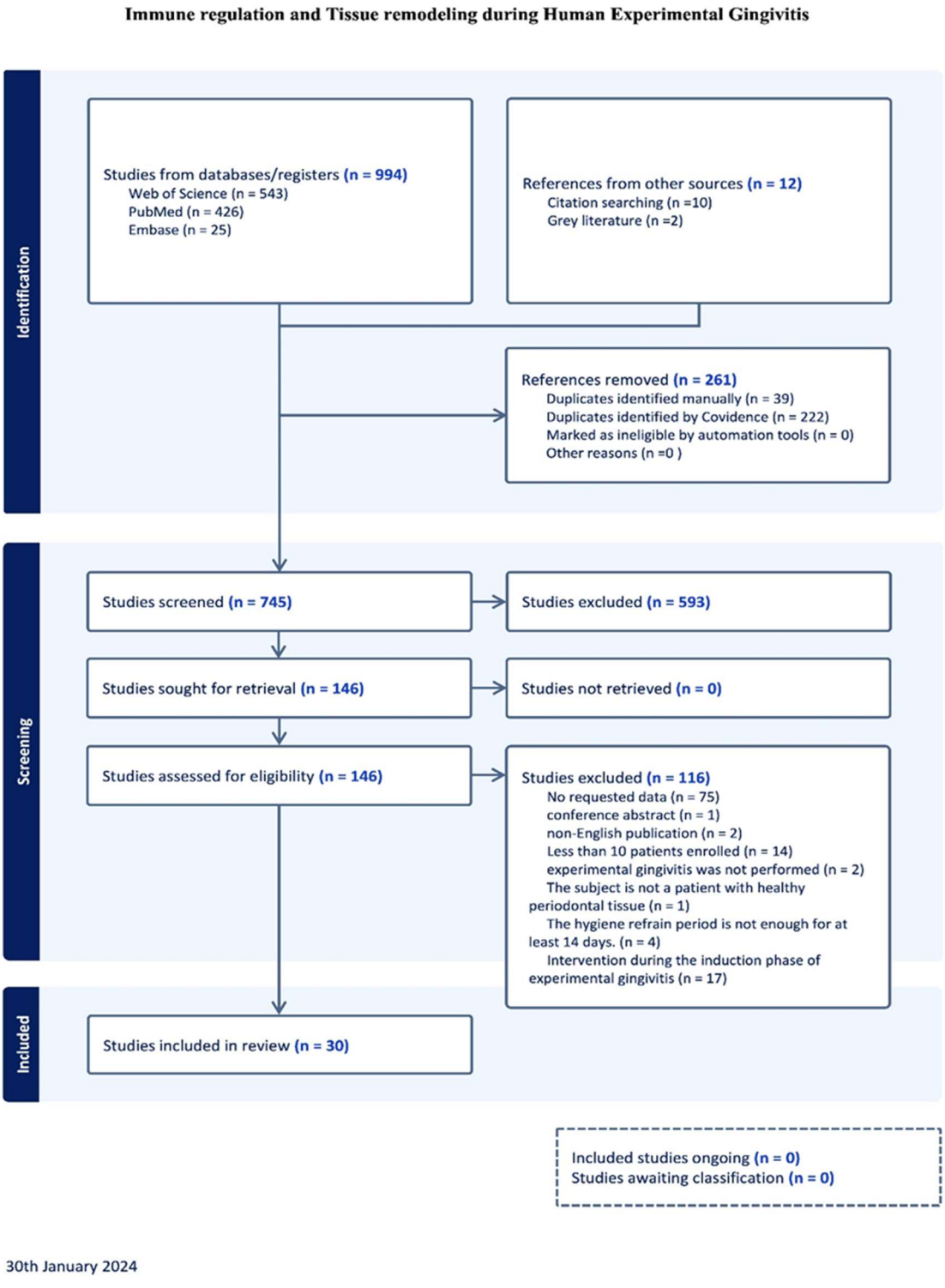

Table 1. Excluding articles and reasons

Intervention during the induction phase of experimental gingivitis	Gerber et al. 1979, Pack 1986, Jones et al. 1990, Heasman et al. 1993, Saxton et al. 1993, Mickels et al. 2001, Lang et al. 2002, Rosin et al. 2002, Sharma et al. 2003, Hasturk et al. 2004, Arweiler et al. 2011, Hallström et al. 2013, , Lee et al. 2015, Kinane et al. 2015, Pulikkotil et al. 2015, Pancer et al. 2016, Teng et al. 2016
Less than 10 patients enrolled	Payne et al. 1975, Tzamouranis et al. 1979, Condacci et al. 1982, Lamster et al. 1985, Kunimatsu et al. 1990a and 1990b, Kinane et al. 1992, McHenry et al. 1992, Raberdurlacher et al 1994, Kunimatsu et al. 1995, Rüdiger et al. 2002, Aboodi et al. 2015,
Non-English publication	Pfister et al. 1984, Patters et al. 1989, Sokolova et al. 2019,
No requested data	Loe et al. 1965, Friedman and Klinkhamer 1971, Robertson and Grupe 1972, Lehner et al. 1974, Lang et al. 1976, Bosman and Powell 1977, Hugoson 1978, Loesche and Syed 1978, Vogel and Deasy 1978, Baehni et al 1979, Seymour et al. 1983, Bonfil et al. 1985, Bergström and Preber 1986, Wynne et al. 1986, Henry et al. 1987, Bergstrom et al. 1988, Danielsen et al 1989, Heasman et al 1989, Danielsen et al 1990, Moughal et al. 1992, Nylander et al 1993, Schenck et al. 1993, Van der Weijden et al. 1994, Van der Weijden et al. 1995, Daly and Highfield1996, Fransson et al. 1996, Zee et al. 1996, Lie et al. 1998a, Lie et al. 1998b, Biesbrock and Yeh 2000, Zee et al. 2000, Kowolik et al. 2001, Preshaw et al. 2001, Putt et al. 2001, Gemmell et al. 2002, Lie et al. 2002, Jepsen et al. 2003 (JCP), Van der Weijden et al. 2002, Jepsen et al. 2003 (EJPS), Trombelli et al. 2003, Trombelli et al. 2004 II and III), Zhou et al. 2004, Rosema et al. 2005, Rosin et al. 2005, Salvi et al. 2005, Scapoli et al. 2005, Trombelli et al. 2005, Versteeg et al. 2005, Scapoli et al. 2007, Trombelli et al. 2008, Lorenz et al. 2009, Rodrigues et al. 2009, Salvi et al. 2009, Wahaidi et al. 2009, Trombelli et al. 2010, Preisser 2011, Slawik et al. 2011, Farina et al. 2012, Bostanci et al. 2013, Branco et al. 2015, Dommisch et al. 2015, Hirschfeld et al. 2015, Klukowska et al. 2015, Prodan et al. 2016, , Van der Weijden et al. 2016, Wellappuli et al. 2018, Zeza et al. 2018, Silbereisen et al. 2019, Aghazada et al. 2020, Aljuboury et al. 2020, Alshehri et al 2020
The hygiene refrain period lasts less than 14 days	Helldén and Kahnberg 1973, Giannopoulou et al. 2003, Que et al. 2004
Conference abstract	Flötra et al. 1969
The subject is not a patient with healthy periodontal tissue	Thurre et al. 1984
Wrong experimental design	Zachrisson 1969, Giannobile et al. 1993

Table 2. Demographic information and Experimental Designs of Selected Articles

Author/ Year	Study Type	Group	# of Pts	Age (years old)	Gender	EG Design	Duration of EG induction (days)	Smoking (Y/N)	Diabetes (Y/N)
Lamster et al 1985(Lamster, Hartley et al. 1985)	CCT		12	24.9 (mean)	3 F 9 M	Partial	28	N/A	N
Persson et al 1990 (Persson, DeRouen et al. 1990)	CCT		16	N/A	6F 10M	Full	14	N/A	N/A
Kinane et al 1991 (Kinane, Adonogianaki et al. 1991)	CCT		12	N/A	N/A	Full	21	N/A	N
Chapple et al 1996 (Chapple, Socransky et al. 1996)	CCT		10	19.3	10M	Partial	21	N/A	N
Johnson et al 1997 (Johnson, Reinhardt et al. 1997)	CCT		10	33.6 ±2.0	5F 5M	Partial	28	Y	N/A
Preshaw et al 1998 (Preshaw, Geatch et al. 1998)	CCT		10	21 (mean)	5F 5M	Partial	21	N	N
Deinzer et al 1999 (Deinzer, Förster et al. 1999)	CCT	Stress (+)	13	21-26	4F 9M	Partial	21	Y	N
		Stress (-)	13	21-26	4F 9M	Partial	21	Y	N
Fransson et al 1999 (Fransson, Mooney	CCT	Young	10	20-25	N/A	Full	21	N/A	N
		old	10	65-80	N/A	Full	21	N/A	N

et al. 1999)									
Deinzer et al 2000 (Deinzer, Kottmann et al. 2000)	CCT	Stress (+)	18	N/A (medical student)	13F 5M	Partial	21	N	N
		Stress (-)	21	N/A (medical student)	13F 8M	Partial	21	N	N
Gonzales et al 2001 (Gonzáles, Herrmann et al. 2001)	CCT		12	28 (mean)	12M	Full	18	Y	N/A
Herrmann et al 2001 (Herrman n, Gonzáles et al. 2001)	CCT		12	22-25	12M	Full	18	N/A	N
Tsalikis et al 2002 (Tsalikis, Parapani siou et al. 2002)	CCT	Young	5	20-22	2F 3M	Full	21	N	N/A
		Old	5	61-65	2F 3M	Full	21	N	N/A
Waschul et al. 2003 (Waschul, Herforth et al. 2003)	CCT	Stress (+)	13	N/A (Student)	6F 7M	Partial	28	N	N
		Stress (-)	14	N/A (Student)	6F 8M	Partial	28	N	N
Wright et al 2003 (Wright, Chapple et al. 2003)	CCT		10	19-28	5F 5M	Partial	21	N/A	N
Deinzer et al 2004 (Deinzer, Waschul et al. 2004)	RCT		14	N/A (medical student)	6F 8M	Partial	28	Y	N
Deinzer et al 2007 (Deinzer, Weik et al. 2007)	CCT		26	25.3± 5.5	13F 13M	Full but clean frontal teeth every other weeks	28	Y	N/A

Ozdemir et al 2009 (Ozdemir, Ozcan et al. 2009)	CCT		12	19-21	N/A	Full	14	N	N/A
Nonnenmacher et al 2009 (Nonnenmacher, Helms et al. 2009)	CCT	Young	12	25.1± 3.0	3F 9M	Full	14	N/A	N/A
		Old	9	58.7± 9.0	4F 5M	Full	14	N/A	N/A
Grant et al 2010 (Grant, Creese et al. 2010)	CCT		10	19-28 y/o	6F 4M	Partial	21	N	N
Offenbacher et al 2010 (Offenbacher, Barros et al. 2010)	CCT		25	44.0 ±12.7	20F 5M	Partial	21	N	N
Salvi et al 2010 (Salvi, Franco et al. 2010)	CCT	Type 1 diabetes	9	25.6 ± 5.8	N/A	Full	21	N/A	Y
		control	9	24.8 ± 5.7	N/A	Full	21	N/A	Y
Tsalikis 2010 (Tsalikis 2010)	CCT	Young	5	20-22	2F 3M	Full	21	N	N
		Adults	5	61-65	2F 3M	Full	21	N	N
Salvi et al 2012 (Salvi, Aglietta et al. 2012)	CCT		15	58.7± 10.9	7F 8M	Partial	21	Y	N/A
Scott et al 2012 (Scott, Milward et al. 2012)	RCT		56	29.8± 9.8	42F 14M	Partial	21	N	N
Meyer et al 2017 (Meyer, Giannopoulou et al. 2017)	CCT		21	77±5.7	10F 10M	Full	21	N	Y
Dommisch et al 2019 (Dommisch, Skora	CCT		20	21-24	11F 9M	Partial	21	N	N

et al. 2019)									
Nascimento et al 2020 (Nascimento, Møller et al. 2020)	CCT		42	23.8 ± 3.6	27F 15M	Full	21	N	N
Bamashmous et al 2021 (Bamashmous, Kotsakis et al. 2021)	RCT		21	18-35	10F 11M	Partial	21	N/A	N
Leite et al. 2022 (Leite, Nascimento et al. 2022)	CCT		42	23.8 ± 3.6	27F 15M	Full	21	N	N
Roberts et al 2022 (Roberts, Yonel et al. 2022)	CCT		15	22 ± 3	9F 6M	Partial	21	N	N

*EG= experimental gingivitis, CCT= clinical controlled trial, RCT= randomized controlled trial, N/A= not available, Pts= participants, F= female, M= male, Partial= partial-mouth oral hygiene refrain via asking volunteers wearing stents while performing home care during EG induction; Full= full-mouth oral hygiene refrain via asking volunteers stop any types of home care during EG induction, Y= yes, N= no.

2.3.2 Expression of mediators associated with immune regulation and tissue remodeling

Table 3 summarized the major biomarkers and outcomes of selected studies. Clinically, PI and GI increased along with the EG induction as well as the GCF volumes. Compared with the baseline (day 0), the EG induction also increased MPO, CXCL2, C5C5a, IL-1, IL-1α, IL-1β, TGF-β, lactoferrin, ALP, human beta-defensin-2, α 2 macroglobulin, transferrin, ß-Glucuronidase, Arylsulfatase, collagenase, aspartate aminotransferase, Cathepsin G, Substance P, elastase, IgG1, IgG2, IgG3, IgG4, albumin, sCD163, s100A9 and s100A9/s100A8.

During EG induction, in contrast, several biomoarkers decreased along with gingival inflammation. They included GCP-2/CXCL6, CXCL16, MIP-1/CCL3, MIP-1α, MIP-1β, MCP-1/CCL2, RANTES/CCL5, MPIF-1/CCL23, C3a, IL-12, MMP-1, MMP-3, and Serpin-E1.

Interestingly, inconsistent results were found among studies on the level of MIF (Nonnenmacher, Helms et al. 2009, Bamashmous, Kotsakis et al. 2021), CCL20 (Dommisch, Skora et al. 2019, Bamashmous, Kotsakis et al. 2021), ENA-78/CXCL5 (Offenbacher, Barros et al. 2010, Roberts, Yonel et al. 2022), Fractalkine/CX3CL1 (Bamashmous, Kotsakis et al. 2021, Roberts, Yonel et al. 2022), PGE2 (Persson, DeRouen et al. 1990, Nonnenmacher, Helms et al. 2009), IL-2 (Offenbacher, Barros et al. 2010, Leite, Nascimento et al. 2022), IL-8 (Deinzer, Weik et al. 2007, Offenbacher, Barros et al. 2010, Salvi, Franco et al. 2010, Tsalikis 2010, Meyer, Giannopoulou et al. 2017, Bamashmous, Kotsakis et al. 2021, Leite, Nascimento et al. 2022, Roberts, Yonel et al. 2022), TNF-α (Offenbacher, Barros et al. 2010, Tsalikis 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022, Roberts, Yonel et al. 2022), IFN-γ (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022), MMP-8 (Offenbacher, Barros et al. 2010, Salvi, Franco et al. 2010, Salvi, Aglietta et al. 2012), MMP-9 (Offenbacher, Barros et al. 2010, Salvi, Franco et al. 2010), and α 1-

antitrypsin (Kinane, Adonogianaki et al. 1991, Scott, Milward et al. 2012) when comparing before and after EG induction (see section 3.2 for more comparisons).

Other biomarkers, such as IL-5, G-CSF, FGF, VEGF, IL-1ra, IL-4, IL-6 (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017), IL-10, IL-12, IL-13, IL-17, GM-CSF, MMP-7, MMP-13, CRP, Complement-D, Adiponectin, Resistin, thrombopoietin, and s100A8 were also measured but their levels showed no significant difference between before and after EG induction.

Table 3. Overview of clinical and biomarkers results of the selected articles

Author/Year	Investigated Clinical Parameters and Biomarkers	Major reported outcomes during EG induction (i.e., from day 0 to the end of EG induction)
Lamster et al 1985 (Lamster, Hartley et al. 1985)	PI, GI, GCF volume, ß-Glucuronidase, Arylsulfatase, Collagenase	Increased PI, GI, GCF volume, Increased level of ß-Glucuronidase, arylsulfatase, collagenase
Persson et al 1990 (Persson, DeRouen et al. 1990)	GI, aspartate aminotransferase	Increased GI Increased level of aspartate aminotransferase
Kinane et al 1991 (Kinane, Adonogianaki et al. 1991)	PI, GI, GCF volume, IL-1, Alpha 2 macroglobulin, Alpha 1-antitrypsin, Transferrin	Increased PI, GI, GCF volume Increased level of IL-1, Alpha 2 macroglobulin, and Alpha 1-antitrypsin, Transferrin
Chapple et al 1996 (Chapple, Socransky et al. 1996)	PI, GI, GCF volume, Alkaline Phosphatase	Increased PI, GI, GCF volume Increased level of Alkaline Phosphatase,
Johnson et al 1997 (Johnson, Reinhardt et al. 1997)	PI, GI, GCF volume, PGE2, IL-1 β	Increased PI, GI, GCF volume Increased level of IL-1 β and PGE2
Preshaw et al 1998 (Preshaw, Geatch et al. 1998)	PGE2	Decreased level of PGE2
Deinzer et al 1999 (Deinzer, Förster et al. 1999)	PI, GI, GCF volume, IL-1 β	All parameters and level of biomarkers increased in both groups (PI, GI, GCF Volume, IL-1 β)
Fransson et al 1999 (Fransson, Mooney et al. 1999)	GCF volume, Lactoferrin, α 2 macroglobulin, IgG1, IgG2, IgG3, IgG4, albumin	All parameters and level of biomarkers increased in both groups (GCF Volume, Lactoferrin, α 2 macroglobulin, IgG1, IgG2, IgG3, IgG4, albumin). A significantly larger GCF volume and total increase of α 2 macroglobulin were observed in old compared to young subject during EG induction. At day 21, the level of α 2 macroglobulin and IgG3 were significantly larger in the old than young group.
Deinzer et al 2000 (Deinzer, Kottmann et al. 2000)	IL-1 β	Increase level of IL-1 β in both groups. The level of IL-1 β was significantly increased in the group with stress compared with controls.
Gonzales et al 2001 (Gonzáles, Herrmann et al. 2001)	PI, IL-1 β, Elastase	Increase PI and the level of IL-1 β No significant difference in levels of Elastase
Herrmann et al 2001 (Herrmann, Gonzáles et al. 2001)	PI, GCF volume	Increase PI and GCF volume

Tsalikis et al 2002 (Tsalikis, Parapanisiou et al. 2002)	PI, GI, GCF volume, IL-1 α, IL-1 β	Increased PI, GI, GCF volume in both groups during EG induction. Significantly greater PI, GI, and GCF volume were observed in old compared to young subject during EG induction. Increase level of IL-1 α and IL-1 β in both groups during EG induction. A significantly greater level of IL-1 β was observed in old compared to young subject during EG induction.
Waschul et al. 2003 (Waschul, Herforth et al. 2003)	PI, bleeding on probing, IL-1 β, IL-1ra	Increase PI, bleeding on probing and the level of IL-1 β in both groups. The increase of IL-1 β expression was faster in male compared with female group without stress. Compared with non-stress group, female groups with stress showed greater increase of IL-1 β level than non-stress female controls. No significant difference in levels of IL-1ra during EG induction between stress or gender groups.
Wright et al 2003 (Wright, Chapple et al. 2003)	PI, GI, GCF volume, TGF-β, Alkaline Phosphatase	Increased PI, GI, GCF volume Increased level of TGF-β, Alkaline Phosphatase
Deinzer et al 2004 (Deinzer, Waschul et al. 2004)	PI, PGE2	Increased PI No significant difference in levels of PGE2
Deinzer et al 2007 (Deinzer, Weik et al. 2007)	PI, number of sites with bleeding, IL-1 β, IL-8	Increased PI and number of sites with bleeding Increased level of IL-1 β and the levels of IL-8 decreased during EG reduction
Nonnenmacher et al 2009 (Nonnenmacher, Helms et al. 2009)	PI, GI, MIF, PGE2	Increased PI, GI. The young group had less PI. The level of PGE2 increased in young group but decreased in old group. MIF concentration slightly increased during EG induction in the young group and young participants had statistically higher concentrations of MIF than old participants.
Ozdemir et al 2009 (Ozdemir, Ozcan et al. 2009)	PI, GI, lactoferrin	Increased PI, GI. Increase levels of lactoferrin
Grant et al 2010 (Grant, Creese et al. 2010)	PI, GI, GCF volume	Increased PI, GI, GCF volume
Offenbacher et al 2010 (Offenbacher, Barros et al. 2010)	PI, GI, IL-1α, IL-1 β, IL-1 ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, MIP-1 β, MCP-1/CCL2, RANTES/CCL5, ENA-78/CXCL5, Serpin-E1, MIP-1 α, PGE2, TNF-α, IFN-γ, GM-CSF, G-CSF, FGF, VEGF, MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, CRP, Resistin, Thrombopoietin	Increased PI, GI. Increased levels of IL-1α and IL-1 β Decreased levels of MIP-1 β, MCP-1/CCL2, RANTES/CCL5, ENA-78/CXCL5, MMP-1, MMP-3, and Serpin-E1. There is no significant difference in levels of MIP-1 α, PGE2, IL-1 ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, TNF- α, IFN-γ, GM-CSF, G-CSF, FGF, VEGF, MMP-7, MMP-8, MMP-9, MMP-13, CRP, Resistin, Thrombopoietin, Complement-D, and Adiponectin. The level of IL-5 was undetectable.

Salvi et al 2010 (Salvi, Franco et al. 2010)	PI, GI, IL-1 β, IL-8, MMP-8, MMP-9	Increased PI, GI. Increased levels of IL-1 β, MMP-8, MMP-9 in both groups. No significant difference in levels of IL-8. IL-1b levels in T1DM patients were elevated compared with healthy individuals
Tsalikis 2010 (Tsalikis 2010)	PI, GI, GCF volume, IL-6, IL-8, TNF-α	Increased PI, GI, GCF volume. Increased level of IL-6 in young group and TNF-α in adults group during EG induction. There is no significant different found on IL-8 expression before and after EG induction. At the end of EG induction, young group had significantly greater level of IL-6 and lower level of TNF-α than adults groups.
Salvi et al 2012 (Salvi, Aglietta et al. 2012)	PI, GI, IL-1 β, MMP-8	Increased PI, GI, Increased level of IL-1 β and MMP-8
Scott et al 2012 (Scott, Milward et al. 2012)	PI, MGI, GCF volume, IL-1 β, alpha1-antitrypsin, Substance P, Cathepsin G, Elastase	Increased PI, MGI, and GCF volume Increased levels of IL-1 β, Substance P, Cathepsin G, and Elastase. There is no significant difference in levels of alpha1-antitrypsin before and after EG induction
Meyer et al 2017 (Meyer, Giannopoulou et al. 2017)	PI, GI, MIP-1 β, IL-1 β, IL-1ra, IL-6, IL-8, IL-17, TNF-α, IFN-γ, GM-CSF, FGF, VEGF	Increased PI, GI. The levels of MIP-1 β and IL-1 β increased and the levels of TNF-α, IFN-γ, and GM-CSF decreased during EG induction. No significant difference found in levels of IL-1ra, IL-6, IL-8, IL-17, FGF, and VEGF
Dommisch et al 2019 (Dommisch, Skora et al. 2019)	PI, GCF volume, CCL20, Human-β-defensin-2	Increased PI and GCF volume. Increased levels of CCL20 and Human-β-defensin-2
Nascimento et al 2020 (Nascimento, Møller et al. 2020)	PI, GI, sCD163	Increased PI, GI. The levels of sCD163 slightly increased over the EG induction.
Bamashmous et al 2021 (Bamashmous, Kotsakis et al. 2021)	PI, GI, GCF volume, MPO, MIF, GCP-2/CXCL6, MIP-1/CCL3, MCP-1/CCL2, CCL23, Fractalkine/CX3CL1, IL-1 β, IL-8	Increased PI, GI and GCF volume. Increased levels of MPO, MIF, IL-1 Beta Decreased levels of GCP-2/CXCL6, MIP-1/CCL3, MCP-1/CCL2, MPFIF-1/CCL23, Fractalkine/CX3CL1, IL-8
Leite et al. 2022 (Leite, Nascimento et al. 2022)	PI, GI, GCF volume, IL-1 β, Il-2. IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, TNF-α, IFN-γ	Increased PI, GI and GCF volume. GBTM analysis revealed two cytokine profiles, “non-organized response” (IL-4, IL-6, IL-8, IL-12, and IL-13) and “organized response” (IL-2, IL-10, and TNF-α). Among the “slow” responders, neither cytokine profile was associated with gingivitis. In contrast, a “fast” response was associated with a higher “non-organized response” factor and a lower “organized response” factor.

Roberts et al 2022 (Roberts, Yonel et al. 2022)	GCF volume, MPO, ENA-78/CXCL5, Fractalkine/CX3CL1, CXCL2, C5C5a, C3a, IL-1 β, IL-8, TNF-α, S100A8, S100A9	Increased GCF volume. At the test side, the level of C5C5a, IL-1 β, MPO, S100A9 and the ratio of S100A9/S100A8 increased while the level of IL-8 decreased during EG induction. At the end of EG induction, the test sides showed greater level of IL-1 β, s100A9, and the ratio of S100A9/S100A8 than controls.

*PI= plaque index, GI= gingival index, MGI= modified gingival index, GCF= gingival crevicular fluid, IL= interleukin, MMP= matrix metalloproteinases, PGE2= Prostaglandin E2, TGF= Transforming Growth Factor, TNF= tumor necrosis factor, IFN= Interferons, GM-CSF= Granulocyte-macrophage colony-stimulating factor, FGF= Fibroblast growth factor, VEGF= Vascular endothelial growth factor, MPO = Myeloperoxidase, MIF= Macrophage migration inhibitory factor

2.3.3 Outcomes of repeated measured biomarkers associated with immune regulation and tissue Remodeling

Due to the heterogeneity of data in graph/ value and units, the meta-analysis was not able to be performed. We then summarized the repeatedly investigated biomarkers in different studies and categorized them in immune regulatory mediators (table 4), inflammation associated mediators (table 5 and 6), and tissue remodeling mediators (table 7).

Nine articles evaluated the changes of immune regulatory mediators before and after EG induction (table 2.4). The biomarkers included MPO, PGE2, MIF, MIP-1β, MCP-1/CCL2, ENA-78/CXCL5, CCL20, and Fractalkine/CX3CL1. Along with EG induction, the EG sites showed an increase of MPO and the decrease of MIP-1β and MCP-1/CCL2, indicating the recruitment of neutrophils along with the decreased chemotaxis and migration of monocytes and macrophages at EG sites. It is also noteworthy that inconsistent changes among selected studies were found in the expression of PGE2, MIF, CCL20, ENA-78/CXCL5, and Fractalkine/CX3CL1. Regardless of consistency in their results, the magnitude of cytokine changes among these studies showed remarkable differences.

A total of sixteen articles investigated the changes of inflammation associated mediators during EG induction, including proinflammatory (table 2.5) and anti-inflammatory mediators (table 2.6). The inflammatory mediators included IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-17, TNF-α, IFN-γ and GM-CSF. The anti-inflammatory mediators were IL-1ra, IL-4, and IL-10. IL-1β was the most widely investigated biomarkers for the inflammation during EG and the results were consistently increased among the 15 articles, along with the increase of IL-1α. The levels of GM-CSF were decreased but showed no significantly difference (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017) during EG induction. A similar trend was found in the

expression of anti-inflammatory cytokines, IL-1ra, IL-4 and IL-10, indicating that some proinflammatory cytokines may be activated by the EG but not the anti-inflammatory cytokines. Regardless of the statistical significance, the inconsistent trends of IL-2 and IFN-γ expression were observed among 3 selected studies (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022). Three studies (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022) showed a trend of decrease in TNF-α expression and another study showed a slightly increase(Roberts, Yonel et al. 2022). None of these changes reached to statistical significance. However, remarkable increases of TNF-α expression and IL-8 were found in the old group of the EG study published by Tsalikis and coworkers (Tsalikis 2010). Also, the decrease of IL-8 expression was reported in 5 studies (Deinzer, Weik et al. 2007, Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022, Roberts, Yonel et al. 2022); and another study showed no statistically significant difference (Salvi, Franco et al. 2010) before and after EG induction.

Six tissue-remodeling mediators, i.e., FGF, VEGF, MMP-8, MMP-9, ALP, and elastase were repeatedly evaluated among 8 articles (table 2.7). The results showed that EG sites had elevated level of ALP and elastase. Two studies from the same research group DM= diabetes mellites ; FGF= fibroblast growth factors; VEGF= vascular endothelial growth factor; MMP= matrix metalloproteinase; ALP = alkaline phosphatase (Salvi, Franco et al. 2010, Salvi, Aglietta et al. 2012) also reported the increased expression of MMP-8 and MMP-9 during EG, despite another EG study(Offenbacher, Barros et al. 2010) using partial-mouth design in old patient population failed to show the significance in their differences before and after EG induction. The levels of FGF and VEGF showed insignificant changes during EG, indicating EG may have limited evidence of angiogenesis.

Table 4. Summary of repeated measurements in immune regulatory mediators

Authors and years (unit)	Group	MPO	PGE2	Cytokine					
				MIF	MIP-1β	MCP-1/ CCL2	CCL20	ENA-78/ CXCL5	Fractalkine/ CX3CL1
Johnson et al 1997 (Johnson, Reinhardt et al. 1997)			increase						
Preshaw et al 1998 (Preshaw, Geatch et al. 1998)(ng/ml)			Day 0: 53.4 ± 4.5 Day 21: 40.5 ± 7.2						
Deinzer et al 2004 (Deinzer, Waschul et al. 2004)			NSSD						
Nonnenmacher et al 2009 (Nonnenmacher, Helms et al. 2009)	Young		increase	increase					
	Old		decrease	decrease					
Offenbacher et al 2010 (Offenbacher, Barros et al. 2010) (log ng/ml)			NSSD		Day 0: 1.82 ± 0.08 Day 21: 0.82 ± 0.15	Day 0: 1.12 ± 0.05 Day 21: 0.98 ± 0.07		Day 0: 2.42 ± 0.08 Day 21: 2.20 ± 0.07	
Meyer et al 2017 (Meyer, Giannopoulou et al. 2017)(pg/ml)					Day 0: 17.8 Day 21: 4.4				
Dommisch et al 2019 (Dommisch, Skora et al. 2019)							increase		
Bamashmous et al 2021 (Bamashmous, Kotsakis et al. 2021)		increase		increase		decrease	decrease		decrease
Roberts et al 2022 (Roberts, Yonel et al. 2022)(pg/30s)		Day 0: 12.72 ± 14.56 Day 21: 21.92 ± 14.75						Day 0: 0.017 ± 0.021 Day 21: 0.02 ± 0.006	Day 0: 0.29 ± 0.39 Day 21: 7.42 ± 4.82

MPO= myeloperoxidase, PGE2= prostaglandin E2; MIF= macrophage migration inhibitory factor; MIP-1β = macrophage inflammatory protein-1 β; MCP-1/CCL2= monocyte chemoattractant protein-1; CCL 20 = CC chemokine ligand 20; ENA-78/CXCL5 = CXC chemokine epithelial cell-derived neutrophil activating peptide, NSSD= No statistically significant difference.

Table 5. Summary of repeated measurements in pro-inflammatory mediators

Authors and years (unit)	Group	Pro-inflammatory								
		IL-1α	IL-1β	IL-2	IL-6	IL-8	IL-17	TNF-α	IFN-γ	GM-CSF
Johnson et al 1997 (Johnson, Reinhardt et al. 1997)			increase							
Deinzer et al 1999 (Deinzer, Förster et al. 1999)	Stress (+)		increase							
	Stress (-)		increase							
Deinzer et al 2000 (Deinzer, Kottmann et al. 2000)	Stress (+)		increase							
	Stress (-)		increase							
Gonzales et al 2001 (Gonzáles, Herrmann et al. 2001)(ng/ml)			Day 0: 229.25 Day 18: 526.13							
Tsalikis et al 2002 (Tsalikis, Parapanisiou et al. 2002)(ng/ml)	Young	Day 0: 4.62 ± 0.70 Day 21: 43.05 ±10.41	Day 0: 8.26 ± 1.31 Day 21: 9.31 ± 2.89							
	Old	Day 0: 6.18 ±1.11 Day 21: 46.55 ±15.27	Day 0: 9.73 ±1.59 Day 21: 29.12 ± 7.89							
Waschul et al. 2003 (Waschul, Herforth et al. 2003)	Stress (+)		increase							
	Stress (-)		increase							
Deinzer et al 2007 (Deinzer, Weik et al. 2007)			increase			decrease				

Offenbacher et al 2010 (Offenbacher, Barros et al. 2010)(log ng/ml)		Day 0: 3.10± 0.13 Day 21: 3.59 ± 0.07	Day 0: 2.83 ± 0.11 Day 21: 3.24 ± 0.08	Day 0: 0.85 ± 0.13 Day 21: 1.08 ± 0.12	Day 0: 0.20 ± 0.07 Day 21: 0.21 ± 0.08	Day 0: 3.39 ± 0.06 Day 21: 3.08 ± 0.06	Day 0: 0.08 ± 0.04 Day 21: 0.04 ± 0.03	Day 0: 0.47 ± 0.08 Day 21: 0.28 ± 0.09	Day 0: 0.19 ± 0.08 Day 21: 0.27 ± 0.07	Day 0: 0.38 ± 0.08 Day 21: 0.31 ± 0.07
Salvi et al 2010 (Salvi, Franco et al. 2010)	Type I DM		increase			NSSD				
	Ctrls		increase			NSSD				
Tsalikis 2010 (Tsalikis 2010)	Young				Day 0: 4.05 ± 2.9 Day21: 5.99± 2.78	Day 0: 12.88 ± 9.88 Day 21: 12.5 ±4.6		Day 0: 4.69±4.37 Day 21: 3.84±3.71		
	old				Day 0: 4.65 ± 3.67 Day 21: 3.91 ± 2.35	Day 0: 10.47 ± 10.77 Day 21: 11.78± 5.33		Day 0: 4.52 ±5 .79 Day 21: 7.56 ± 3.78		
Salvi et al 2012 (Salvi, Aglietta et al. 2012)			increase							
Scott et al 2012 (Scott, Milward et al. 2012)			increase							
Meyer et al 2017 (Meyer, Giannopoulou et al. 2017)(pg/ml)			Day 0: 87.3 Day 21: 375.1		Day 0: 4.7 Day 21: 1.1	Day 0: 429.3 Day 21: 263.5 (SSD)	Day 0: 20.3 Day 21: 13.3	Day 0: 11.1 Day 21: 9.6	Day 0: 123.2 Day 21: 92.3	Day 0: 496.1 Day 21: 259.5
Bamashmous et al 2021 (Bamashmous, Kotsakis et al. 2021)			increase			decrease				
Leite et al. 2022 (Leite, Nascimento et al. 2022)(pg/ml)			Day 0: 68.00 ± 36.7 Day 21: 241.33 ± 151.9	Day 0: 5.53 ± 6.1 Day 21: 3.20 ± 2.2	Day 0: 3.19 ± 2.9 Day 21: 1.66 ± 1.4	Day 0: 1215.57 ± 311.6 Day 21: 663.3 ± 273.3		Day 0: 1.83 ± 2.1 Day 21: 0.89 ± 1.0	Day 0: 3.30 ± 1.1 Day 21: 3.84 ± 6.6	
Roberts et al 2022 (Roberts, Yonel et al. 2022)(pg/30s)			Day 0: 0.04 ± 0.05 Day 21: 0.28 ± 0.2			Day 0: 0.21 ± 0.32 Day 21: 0.11 ± 0.05		Day 0: 0.003 ± 0.002 Day 21: 0.004 ± 0.002		

IL= interleukin, DM= diabetes mellitus, Ctrl= controls, TNF-α= tumor necrosis Factor alpha, IFN-γ = interferon gamma; GM-CSF= granulocyte-macrophage colony stimulating factor; NSSD= no statistically significant difference.

Table 6. Summary of repeated measurements in anti-Inflammatory mediators

Authors and years (unit)	Anti-inflammatory		
	IL-1ra	**IL-4**	**IL-10**
Offenbacher et al 2010 (Offenbacher, Barros et al. 2010)(log ng/ml)	Day 0: 4.84 ±0.04 Day 21: 4.89 ± 0.04	Day 0: 0.72 ± 0.13 Day 21: 0.71 ± 0.13	Day 0: 0.38 ± 0.08 Day 21: 0.32 ± 0.06
Meyer et al 2017 (Meyer, Giannopoulou et al. 2017)(pg/ml)	Day 0: 45.6 Day 21: 66.6		
Leite et al. 2022 (Leite, Nascimento et al. 2022)(pg/ml)		Day 0: 0.21 ± 0.1 Day 21: 0.18 ± 0.0	Day 0: 149 ± 1.99 Day 21: 0.59 ± 0.67

IL= interleukin, DM= diabetes mellitus, Ctrl= controls, TNF-α= tumor necrosis Factor alpha, IFN-γ = interferon gamma; GM-CSF= granulocyte-macrophage colony stimulating factor; NSSD= no statistically significant difference.

Table 7. Summary of repeated measurements in tissue remodeling

Authors and years (unit)	group	Tissue Remodeling					
		FGF	VEGF	MMP-8	MMP-9	ALP	elastase
Chapple et al 1996 (Chapple, Socransky et al. 1996)						increase	
Gonzales et al 2001 (Gonzáles, Herrmann et al. 2001)(ng/ml)							Day 0: 480.80 Day 18: 1444.30
Wright et al 2003 (Wright, Chapple et al. 2003)						increase	
Offenbacher et al 2010 (Offenbacher, Barros et al. 2010)(log ng/ml)		Day 0: 1.23 ± 0.13 Day 21: 1.49 ± 0.08	Day 0: 2.13 ± 0.06 Day 21: 2.16 ± 0.04	Day 0: 4.93 ± 0.08 Day 21: 5.16 ± 0.07	Day 0: 5.50 ± 0.07 Day 21: 5.54 ± 0.07		
Salvi et al 2010 (Salvi, Franco et al. 2010)	Type 1 DM			increase	increase		
	controls			increase	increase		
Salvi et al 2012 (Salvi, Aglietta et al. 2012)				increase			
Scott et al 2012 (Scott, Milward et al. 2012)							increase
Meyer et al 2017 (Meyer, Giannopoulou et al. 2017)(pg/ml)		Day 0: 31.7 Day 21: 30.8	Day 0: 391.0 Day 21: 317.8				

DM= diabetes mellites ; FGF= fibroblast growth factors; VEGF= vascular endothelial growth factor; MMP= matrix metalloproteinase; ALP = alkaline phosphatase

2.3.4 The influences of patient factors

We next examined the impact of patient factors, including stress, DM, age, and smoking on the changes of these biomarkers during EG induction. Six studies (Johnson, Reinhardt et al. 1997, Deinzer, Förster et al. 1999, Gonzáles, Herrmann et al. 2001, Deinzer, Waschul et al. 2004, Deinzer, Weik et al. 2007, Salvi, Aglietta et al. 2012) specified their enrollment of current smokers. However, the data between smokers and non-smokers were not analyzed separately. Therefore, the role of smoking on the changes of these biomarkers during EG are not available.

Three articles (Deinzer, Förster et al. 1999, Deinzer, Kottmann et al. 2000, Waschul, Herforth et al. 2003) directly compared these analytes between the populations with or without stress. All of them were conducted in student populations using partial mouth design. Along with EG induction, the PI and gingival inflammatory index increased (Deinzer, Förster et al. 1999, Waschul, Herforth et al. 2003). PI showed no significant different between the groups, but males under stress may have greater sites of bleeding on probing at the end of EG compared with the females stress group or controls in either genders (Waschul, Herforth et al. 2003). During EG induction, the group with stress had greater level of IL-1β compared with controls at the sites with EG (Deinzer, Förster et al. 1999, Deinzer, Kottmann et al. 2000) but the level of IL-1ra did not show significant difference between genders or stress groups (Waschul, Herforth et al. 2003).

Four selected articles (Fransson, Mooney et al. 1999, Tsalikis, Parapanisiou et al. 2002, Nonnenmacher, Helms et al. 2009, Tsalikis 2010) directly compared the immune and tissue remodeling between young and old groups. All of them induced EG using full-mouth oral hygiene refrain design. The young group enrolled participants aged 20-25 years old and the old group enrolled those with more than 55 years old in average. During the induction of EG, the young group had significantly less increase of PI and GI compared with the old group (Tsalikis,

Parapanisiou et al. 2002, Nonnenmacher, Helms et al. 2009, Tsalikis 2010) as well as the GCF volume (Fransson, Mooney et al. 1999, Tsalikis, Parapanisiou et al. 2002). At the end of EG induction, interestingly, both the levels of MIF (Nonnenmacher, Helms et al. 2009), PGE2 (Nonnenmacher, Helms et al. 2009) and IL-6 (Tsalikis 2010) were increased in the young group whereas decreased in the old group. The young group also had less expression of TNF-α (Tsalikis 2010), IL-1β (Tsalikis, Parapanisiou et al. 2002), α 2-macroglobulin (Fransson, Mooney et al. 1999) and IgG3 (Fransson, Mooney et al. 1999) than the old group during EG induction. However, the level of IL-1α (Tsalikis, Parapanisiou et al. 2002) and IL-8 (Tsalikis 2010) did not show significant difference between the old and young groups.

Direct comparison between patients with or without DM was only investigated in 1 study (Salvi, Franco et al. 2010), using full-mouth design which enrolled patients between 18-31 years old. The DM patients were defined with a confirmed diagnosis of type1DM had undergone insulin therapy for ≥12 months and had a mean glycosylated hemoglobin level ≤9.5% in the previous 12 months. Both groups had increased level of PI and GI during the EG induction without statistically significant difference between groups. Both groups also had elevated level of IL-1β, MMP-8, and MMP-9 before and after EG induction. The comparison between the groups showed a greater level of IL-1β in DM group than controls at the end of EG induction.

2.3.5 Quality assessment and heterogeneity evaluation, Quality of evidence

The table of quality assessment can be found in table 8. The kappa coefficients for the agreement in these two risks of bias assessments between the two authors (K.C and Y.H) were 0.90 and 0.88, respectively. All 3 RCTs demonstrated “some concern” for the overall risk of bias. Among 27 NRCTs, The overall “low” risk of bias was determined in 10 selected NRCTs, and other 17 studies had moderate risk of bias. Based on GRADE guidelines, the features of immune

regulation and tissue remodeling responses during EG presented in this systematic review had "moderate" evidence quality.

2.4 Discussion

In the past 60 years, EG has been a widely used tool to investigate gingival immune responses and tissue changes in response to rapid plaque accumulation. It also provides a useful and reversible human model to investigate the efficacy of therapeutic products without causing permanent destruction. The current systematic review summarized the features of EG, including the increase of gingival inflammation and neutrophil recruitment, decrease of monocyte and macrophage migration, and limited involvement in angiogenesis. Our current analysis also suggests that patient factors, including age and stress, could potentially affect the outcomes of EG, leading to inconsistent results in mediator expression. To the authors' knowledge, this is the first paper systematically summarizing the features of EG and investigating potential factors contributing to the inconsistent outcomes.

Plaque-induced gingivitis is an inflammatory response, which consists of an innate system of cellular and humoral responses following bacterial triggers (Chapple, Mealey et al. 2018). Instead of duration, acute and chronic inflammation are defined based on the nature of the inflammatory cells appearing in tissues (Ward 2010). Along with the margination and chemotaxis of neutrophils, the acute inflammatory response induces a series of tissue responses not only to eliminate invading organisms but also to repair injured tissue. Ideally, the acute lesion is self-limited so tissues could return to homeostasis following the complete resolution of inflammatory infiltrates and clearance of cellular debris (Medzhitov 2010, Ward 2010).

Table 8. Risk of bias assessment

ROB2 for randomized controlled trials								
Study	**Randomization**	**Identification and recruitment**	**Deviations from intended interventions**	**Missing data**	**Measurement of the outcome**	**selection of the reported result**	**Overall bias**	
Deinzer 2004	Some Concerns	Low	Some Concerns	Low	Some concerns	Low	Some Concerns	
Scott et al 2012	Low	Low	Some Concerns	Low	Some concerns	Low	Some concerns	
Bamashmous et al 2021	Low	Low	Some Concerns	Low	Some concerns	Some concerns	Some concerns	
ROBINS-I for non-randomized controlled trials								
Study	**Confounding Bias**	**selection of participants**	**classification of interventions**	**deviations from intended interventions**	**Missing data**	**measurement of outcomes**	**selection of the reported result**	**Overall bias**
Lamster et al 1985	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Persson et al 1990	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Kinane et al 1991	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Chapple et al 1996	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Johnson et al 1997	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Preshaw et al 1998	Moderate	Low	Low	Low	Low	Low	Low	Low
Deinzer et al 1999	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Fransson et al 1999	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Deinzer et al 2000	Moderate	Low	Low	Low	Low	Low	Low	Low
Gonzales et al 2001	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Herrmann et al 2001	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Tsalikis et al 2002	Moderate	Low	Low	Low	Low	Low	Low	Low
Waschul et al. 2003	Moderate	Low	Low	Low	Low	Low	Low	Low
Wright et al 2003	Moderate	Low	Low	Low	Low	Low	Low	Moderate

Deinzer et al 2007	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Ozdemir et al 2009	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Nonnenmacher et al 2009	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Grant et al 2010	Moderate	Low	Low	Low	Low	Low	Low	Low
Offenbacher et al 2010	Moderate	Low	Low	Low	Low	Low	Low	Low
Salvi et al 2010	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Tsalikis 2010	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Salvi et al 2012	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Meyer et al 2017	Moderate	Low	Low	Low	Low	Low	Low	Moderate
Dommisch et al 2019	Moderate	Low	Low	Low	Low	Low	Low	Low
Nascimento et al 2020	Moderate	Low	Low	Low	Low	Low	Low	Low
Leite et al. 2022	Moderate	Low	Low	Low	Low	Low	Low	Low
Roberts et al 2022	Moderate	Low	Low	Low	Low	Low	Low	Low

The current findings of EG report a neutrophil-dominated inflammatory lesion with little evidence of angiogenesis and monocyte/macrophage involvement. As a key component of tissue repair, in contrast, well-programed inflammation-associated angiogenesis in chronic lesions is controlled by tissue macrophages and angiogenic mediators, such as VEGF and FGF (Szekanecz and Koch 2007). With lack of monocyte and macrophage involvement, EG seems to minimally activate the anti-inflammatory and tissue repair systems, indicating a tight immune regulation of acute inflammation to avoid its excessive perturbations which cause defective healing (Soliman and Barreda 2022) and highlighting its feature as a self-limiting acute lesion. These features allow EG to serve as an excellent inflammatory model to investigate the initial immune responses of the periodontium against bacterial challenges without leading to permanent tissue destruction. On the other hand, the disease progression from gingivitis to periodontitis (a non-healed chronic inflammatory lesion) is always of the interest for clinicians in the development of therapeutic strategies. However, the self-limiting features of EG fails to represent the transitional process when the inflammation persists and exceeds the threshold of tissue homeostasis, leading to replacement of neutrophils by chronic inflammatory cells and transitions to a chronic lesions. Indeed, previous studies have shown the differences between EG and naturally occurring gingivitis (Trombelli, Scapoli et al. 2010, Farina, Guarnelli et al. 2012). Therefore, EG studies are not the same as naturally occurring gingivitis and caution should be employed when trying to extrapolate lessons from EG to naturally occurring gingivitis.

The current findings also suggested that the features of EG may not be always consistent among all EG studies. Several factors may contribute to the inconsistent outcomes among selected articles, including age of volunteer population, stress and the history of systematic disease (such as DM). The impact of smoking on EG outcomes was not able to be identified in

this review since all included EG studies did not report separate results from smokers and non-smokers. An EG study recruiting smokers only, however, reported an induction of inflammation in smokers by decreasing anti-inflammatory cytokines and Th2 cytokines. Th2 cytokines (IL-2, IL-12, IFN-γ and IL-1ra) were increased following the onset of clinical gingivitis and even after resolution of clinical inflammation, indicating the immune responses of gingival tissue was altered by smoking (Matthews, Joshi et al. 2013). Comparing with non-smokers, another EG study also showed the distinct expression pattern for key immune-regulatory cytokines in smokers, including greater expression of IL-8, IL-17, IFN-γ and less expression of IL-4, 6, 10, IL-1β. These demographic factors, therefore, should always be taken into consideration when interpreting EG outcomes.

Other potential contributing factors to the variation in the human immune response include the volunteer's inflammatory responder types (IRT) and the experimental design of EG induction, in terms of full-mouth or partial-mouth (with personalized stent fabrication) oral hygiene refraining. The individual variation against bacterial challenges during EG induction has been reported previously (Trombelli, Scapoli et al. 2004, Offenbacher, Barros et al. 2010, Bamashmous, Kotsakis et al. 2021). In response to intense plaque accumulation, in brief, volunteers present distinct IRTs: high, low, and slow IRTs. The high IRT has a rapid plaque growth rate along with high levels of inflammation; whereas the low-IRT volunteer has lower plaque growth and low level of inflammation. Slow-IRT, on the other hands, presented similar level of plaque growth rate and inflammatory level with high-IRT in a delayed pattern. The current data which displays a large standard deviation may reflect the individual variations of the volunteer populations. In the present review, interestingly, the inconsistent trend of IL-2 and IFN-γ expression were observed among 3 selected studies:(Offenbacher, Barros et al. 2010,

Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022) that Offenbacher and colleagues (Offenbacher, Barros et al. 2010) reported an increased expression of IL-2 and IFN-γ during EG induction whereas the other two studies showed decreased expression of IL-2 (Leite, Nascimento et al. 2022) and IFN-γ (Meyer, Giannopoulou et al. 2017). In addition to abovementioned factors (age and IRTs) contributing to the inconsistent results among these articles, Offenbacher and colleagues (Offenbacher, Barros et al. 2010) applied partial mouth design with the use of personalized stents while the other two studies (Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022) refrained full-mouth oral hygiene during EG induction. Recently, the presence of a nonlocalized effect on distant tooth sites within the human oral cavity during partial-mouth EG induction was proposed (Kerns, Bamashmous et al. 2023). The variation in healthy control sites, in terms of magnitude and time, relied on the volunteer's IRTs,(Kerns, Bamashmous et al. 2023) implying the presence of personalized stents and IRTs contribute to the diverse results of certain immune-regulatory mediators shown among different EG studies. Since the amount and rates of plaque accumulation affect the gingival inflammation (Offenbacher, Barros et al. 2010), further investigation is necessary to evaluate the impacts of EG designs on expression pattern of immune and tissue remodeling mediators.

Limitations encountered when performing the current systematic review include great heterogeneity with patient populations, sample processing, and data outputs. The investigated mediators among selected articles are widely spread. It is noteworthy that the standardization/normalization protocols of samples were done differently. For example, 30 second protocol was performed in some selected studies (Bamashmous, Kotsakis et al. 2021, Roberts, Yonel et al. 2022) to ensure the GCF collection strips/ paper points were inserted in the sulcus for 30 seconds; while a 2-minute protocol was performed in another group (Cionca,

Hashim et al. 2016, Meyer, Giannopoulou et al. 2017). Also, different lab protocols and statistical analysis were applied among studies since the results were not normally distributed (Offenbacher, Barros et al. 2010). Using bead-based multiplex immunoassays, protein standardization is recommended in the manual since it optimizes the assay to ensure that the multiplex plate is not getting oversaturated and losing sensitivity. However, some selected studies did not specify if the protein standardization was performed in their analysis. This heterogeneity in sample collection and analysis not only makes it difficult to compare the results among EG studies, but also highlights that the field lacks a well-accepted protocol for standardizing samples.

In summary, the results suggested that EG induces gingival inflammation by increasing neutrophil recruitment as an acute inflammatory lesion. EG induction decreases the migration of monocytes/macrophages and also shows limited involvement in angiogenesis and tissue remodeling. Multiple factors may affect the expression of immune regulation and tissue remodeling during EG induction and lead to inconsistent results, including age, diabetes, stress, and individual variation in response to inflammation.

Chapter 3. Comparison between Experimental, Naturally Occurring Gingivitis, and Periodontal Health

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"Yung-Ting Hsu, Ana Chang, Diane Daubert, Frank Roberts, Dandan Chen, Harsh M. Trivedi, Juliana Gomez, Rich Darveau. Inflammation and Tissue Remodeling During Gingivitis- A Comparison between Experimental and Naturally Occurring Gingivitis."

3.1 Introduction

Plaque-induced gingivitis is one of the most common oral diseases. If left uncontrolled, it may progress to periodontitis and lead to loss of periodontium and teeth (Kurgan and Kantarci 2018). Histologically, several stages of gingival inflammation show transition from health to disease: initial, early, established and advanced lesions (Page and Schroeder 1976). Starting from acute inflammation, the initial lesion of gingivitis shows accumulation of lymphoid cells subjacent to the junctional epithelium. As inflammation develops in the later phase of gingival inflammation, macrophages and plasma cells accumulate in the connective tissue; whereas neutrophil migration through the epithelium decreases. Cytopathic alterations in resident fibroblasts occurs in the late phase of gingivitis along with further loss of the collagen fiber network (Page, Offenbacher et al. 1997). In short, gingivitis displays distinct immune regulatory mechanisms in early and late phase of inflammation, leading to leukocyte infiltration with differing proportion and patterns of tissue remodeling.

The setting of experimental gingivitis (EG), refraining participants from oral hygiene is a common approach to investigate immune responses in gingival tissues against the challenges of microbial flora. This method has been a popular and convenient way to study immune regulation during gingival inflammation and to test therapeutic effects of oral hygiene products.

Researchers have made abundant efforts to investigate both EG and naturally-occurring gingivitis (NG) (Trombelli, Scapoli et al. 2010, Farina, Guarnelli et al. 2012). However, a thorough picture of NG with respect to the host response is not available. Moreover, comprehensive comparisons of EG and NG have not been performed. Therefore, the aim of this study is to evaluate the immune regulation and tissue remodeling responses during EG and NG. This work provides a comprehensive analysis of 39 mediators involved in disease progression of both EG and NG, pointing out differences and similarities.

3.2 Materials and methods

This study consists of three groups, (1) periodontal health (H), (2) EG from a previous study (see section 3.2.1), and (3) NG from a study conducted here at the University of Washington (see section 3.2.2). Gingival crevicular fluid (GCF) samples were used from the abovementioned two human studies to compare inflammatory mediators between H, EG, and NG. Twenty-six healthy participants were recruited for an EG protocol. Data from Day 0 represents H (control) and data at Day 21 represents EG. Another 26 participants, age-matched to the EG group with NG, were recruited for a total of 52 participants. These studies were approved by the human subjects ethics board of University of Toronto and Washington (UT REB Protocol #30044, 29410 & 32899, UW IRB# STUDY00012410). They were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013.

3.2.1 Human experimental gingivitis

An Institutional Review Board (IRB)-approved human EG study conducted at the University of Toronto (REB Protocol #30044, 29410 & 32899) provided the samples for the group of individuals subjected to EG.(Fine, Barbour et al. 2023) In brief, a human EG study was conducted in participants who were both systemically and periodontal healthy. Twenty-six systematically and periodontally healthy volunteers (aged from 20-55 y/o) were recruited, including 13 males and 13 females. These participants were examined and received prophylaxis two weeks before the induction of EG to ensure plaque and inflammation free periodontium. For EG induction, participants refrained from all oral hygiene for three weeks, followed by a second prophylaxis, and a three-week recovery period. GCF samples were collected from two surfaces of six index teeth using sterile paper strips for 30 seconds (Periopaper; Oraflow Inc, Smithtown, NY, USA) into the gingival crevice and stored at -80^{o}C until processing. Clinical and mediator

data from the baseline visit (Day 0) represents H, and the results obtained at Day 21 (the end of EG induction) represent EG.

3.2.2 Naturally occurring gingivitis

Participants were recruited in an independent IRB-approved study (IRB# STUDY00012410) at the University of Washington School of Dentistry (UWSOD). Twenty-six patients age-matched with H and EG groups were included in this comparison. Inclusion criteria included: (1) systematically healthy, (2) have more than three teeth with gingivitis following the definition of the 2018 AAP/EFP disease classification (Chapple, Mealey et al. 2018) without any history of periodontitis. Exclusion criteria included: (1) use of antibiotics within 30 days, (2) patients with diabetes, (3) refusal to sign the informed consent or unable to communicate in English, (4) history of periodontitis or have received active periodontal therapy such as scaling and root planning, and periodontal surgery, amongst others.

A single, calibrated researcher (YH) recruited, examined, and collected NG biospecimens. The calibration process was performed in five volunteers not involving in this study prior to the beginning of the study. The same quadrant was probed for two times seperately from 1-3 weeks. The inter-examiner agreement was achieved to ≥ 80% before the beginning of the study. Patient demographic information and health status were recorded as well as periodontal health indices including clinical attachment level, probing depth, bleeding on probing, visible plaque index (Silness and Loe 1964), and gingival index (Loe and Silness 1963) (GI) around tested teeth. GCF samples were collected at 6 surfaces of 6 selected teeth with NG using the protocol of 30 seconds and stored at -80^{o}C until processing.

3.2.3 Analysis of Gingival crevicular fluid

All GCF samples underwent an assay of 39 mediators of inflammatory/immune responses and tissue remodeling using commercially available bead-based multiplex immunoassays (Procartaplex 39-plex Panel, Thermo Fisher Scientific, Waltham, MA, USA) at UWSOD (AMC and YH). These mediators include angiogenin, angiopoietin-1, angiostatin, BMP-2, C-peptide, C3a, Cortisol, CRP, ENA-78/CXCL5, CX3CL1, GCP-2/CXCL6, GM-CSF, ICAM-1, Interleukin (IL)-1beta, IL-12p70, IL-17A/CTLA-8, IL-6, IL-8, insulin, LOX-1, MCP-1/CCL2, MIF, MIP-1 alpha/CCL3, matrix metalloproteinase (MMP)-1, -2, -3, -7, -8, -9, -12, -13, MPO, OPN, OPG, RANKL, RANTES, SDF-1alpha, TIMP-1 VEGF-A.

The GCF samples were prepared using 200 µl of 0.01M PBS to each tube and rotating for one hour at 4^{o}C. An aliquot of 10µl per sample was transferred for protein analysis (VMax microplate reader; GMI, Ramsey, MN, USA). Based on the protein concentration, the GCF samples were diluted with 0.01M PBS to normalize samples to 10µg/ml. Bead based multiplex analysis was then performed on duplicated, standardized GCF samples (i.e., samples diluted to 10µg/ml) using the bead-based multiplex immunoassays according to manufacturer's protocol. The data was obtained using a flow-cytometry-based array reader (Bio-Plex 200 reader, Bio-Rad Laboratories, Hercules, CA, USA), by acquiring the signal from the fluorescent dye within each bead for assay identification along with the fluorescent signal from the reporter for quantification. We than anlyzed the data with a software (Bio-Plex Manager Software V6 (Bio-Rad Laboratories, Hercules, CA, USA). The concentrations of different mediators were calculated based on the respective standard curve for each chemokine with 5-parameter logistic (5PL) equation. Mediator data were reported in mean value with the unit of pg/ml. The intra-plate variability is 7.75% in average (6.33-9.64%) and the inter-plate variability is 5.02%.

3.2.4 Statistical analysis

Statistical analysis was performed using a statistical analytic software (JMP® software version, Pro 17, JMP Statistical Discovery LLC., Cary, NC, USA). Clinical and mediator data were summarized into single average scores for test and control per person at each time point and were reported using boxplots showing medians and interquartile ranges. The out of range data of each analytes were removed from statistical analysis. The information of numbers of detected analytes and the limit of detection were included in table 9. The differences in GI and mediator expression among groups were determined at a 95% confidence level ($p \leq 0.05$) by a two-way ANOVA with a post-hoc Tukey's test. A p-value < 0.05 was set to indicate statistical significance.

Table 9. Information of each analytes

	Number of Analytes	limit of detection
Angiogenin	78	0.01-600
Angiopoietin-1	78	7.08-145600
Angiostatin	78	0.19-5150
BMP-2	73	1.16-303000
c-peptide	78	1.13-295700
C3a	78	0.54-142800
CRP	76	0.11-28800
ENA-78/CXCL5	70	0.11-29100
Fractalkine/CX3CL1	73	0.02-5750
CXCL6	58	0.02-6200
GM_CSF	58	0.19-49900
ICAM_1	78	1.91-501200
IL-1B	78	0.03-7000
IL_12p70	77	0.12-32300
IL_17A	77	0.04-11100
IL_6	73	0.16-42000
IL_8	78	0.04-9250
LOX_1	78	0.02-6300
CCL2	76	0.08-20000
MIF	78	0-1100
CCL3	68	0.04-9600
MMP_1	77	0.11-28800
MMP_12	78	0.1-25100
MMP_13	74	0.12-32500
MMP_2	67	0.12-32700
MMP_3	78	0.03-7100
MMP_7	77	0.08-21200
MMP_8	78	0.39-101700
MMP_9	78	0.02-4400
MPO	78	1.31-344100
OPN	78	0.28-73300
OPG	73	0.01-2800
RANKL	76	0.12-30500
CCL5/RANTES	77	0.01-3500
SDF_1_alpha	53	0.24-63400
TIMP_1	78	044-116300
VEGF_A	78	0.08-20000

3.3 Results

A total of 52 patients were included in these two studies. The EG study included 26 patients (13 females and 13 males) with an average age of 32.33± 8.76 years old. The NG study consisted of 8 males and 18 females with an average age of 31.88± 9.41 years old. The age among groups were not significantly different ($p>0.05$). The EG group had the greatest GI, followed by NG, and H group, which had the lowest GI (all $p<0.001$). Table 10 reported the changes of both GI and PI during EG. Table 11 summarized the results of GI, total protein and mediator expression among health, EG and NG groups.

3.3.1 Migration of immune cells

Comparing each group, Figure 2 displays the expression levels of mediators associated with migration of different types of immune cells, including neutrophils, macrophages, and leukocytes. The EG group demonstrated significantly higher expression levels of myeloperoxidase (MPO) when compared to H (Figure 2A). In contrast, the NG group demonstrated significantly lower MPO values than either of the other two groups ($p<0.001$ NG vs. EG, $p<0.01$ NG vs. H). In addition, the NG group demonstrated significantly increased expression levels of MIF ($p<0.05$ for both comparisons, Figure 2B) and Fractalkine/CX3CL1 ($p< 0.001$ for both comparisons, Figure 2C) than H and EG. The levels of CCL5/RANTES was significantly decreased (($p<0.01$ NG vs. EG, $p<0.001$ NG vs. H, (Figure 2D) when compared to either the controls or the EG groups. These data reveal significant differences in immune cell migration mediators between EG and NG groups.

Significantly decreased levels of ENA-78/CXCL5 ($p<0.05$, Figure 2E) were also observed in the NG group when compared to the H group, although this difference was not significant when compared to the EG group ($p>0.05$). Finally, there was a significant decrease in IL-8

($p<0.01$, Figure 2F) in the EG group when compared to the H control. This decrease was also observed in the NG group ($p<0.001$, Figure 2F). The expression of ICAM-1, CXCL6 (for neutrophils), CCL2 (for monocytes and basophils), granulocyte-macrophage colony stimulating factor (GM-GSF, for macrophage), and SDF-1alpha (for leukocytes) displayed similar trends described above showing a decrease of neutrophil-related mediators and an increase of macrophage-associated mediators in NG group. However, the differences among groups were not statistically significant ($p>0.05$).

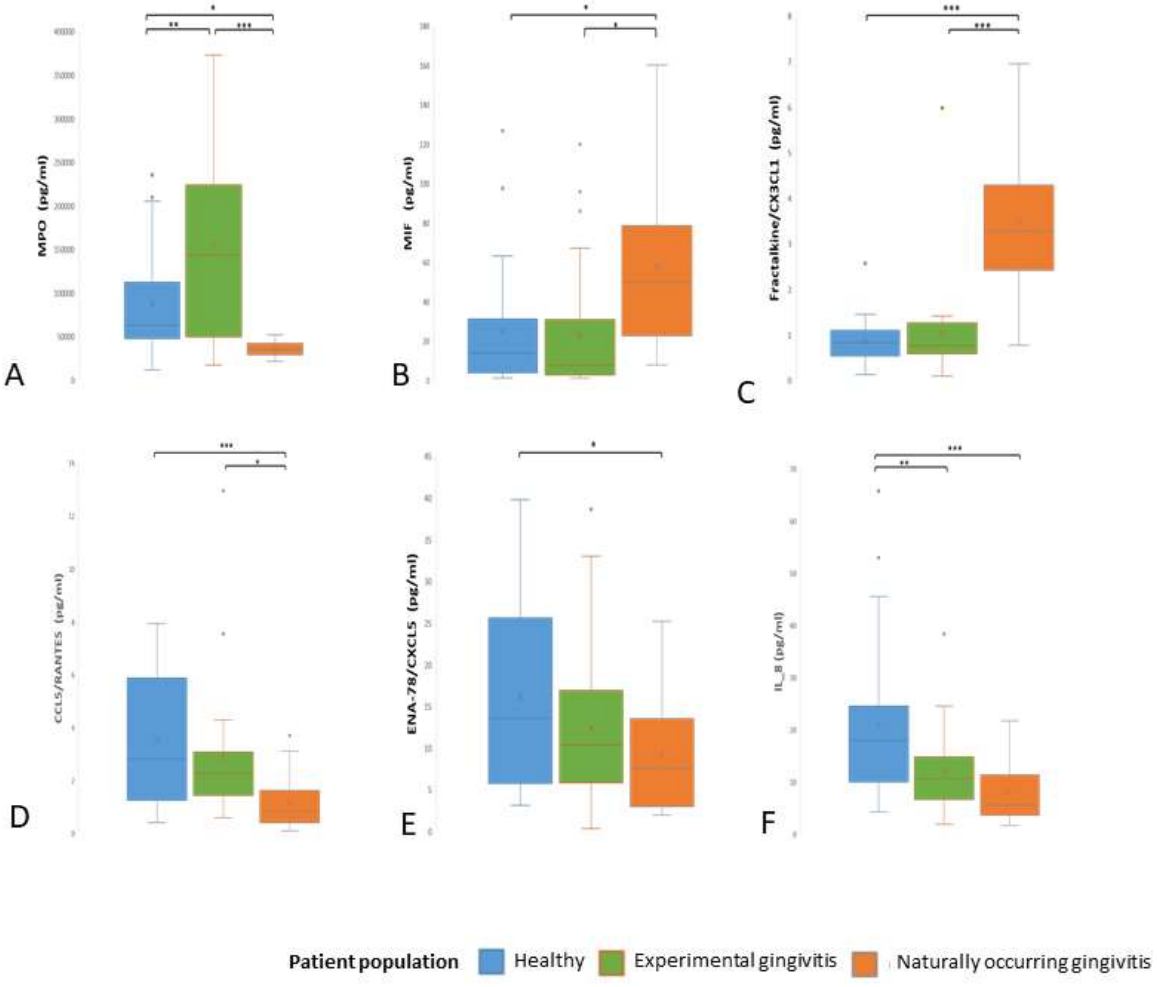
Table 10. Changes of clinical indices for experimental gingivitis group (n=26)

Time Point		Gingival Index	Plaque Index
Baseline	**Day 0**	0.19± 0.40	0.24± 0.43
Induction Phase	**Day 7**	1.01± 0.76	0.50± 0.50
	Day 14	1.23± 0.75	0.65± 0.48
End of Induction Phase	**Day 21**	1.69± 0.62	0.81± 0.39
Resolution Phase	**Day 28**	1.92± 0.38	0.95± 0.22
	Day 35	1.38± 0.62	0.34± 0.47
End of Resolution Phase	**Day 42**	1.08± 0.73	0.34± 0.47

Table 11. Comparison of clinical parameters and mediator expression among health, experimental and naturally occurring gingivitis groups

Group	Healthy (n=26)	Experimental gingivitis (n=26)	Naturally occurring gingivitis (n=26)
Protein concentration of standardized mediators (pg/ml)			
Angiogenin	0.84 ± 0.42	0.91 ± 0.68	1.37 ± 0.67
Angiopoietin-1	23.58 ± 17.44	19.64 ± 20.74	10.35 ± 8.43
Angiostatin	48.89 ± 22.40	37.88 ±23.27	46.09 ± 33.38
BMP-2	48.56 ± 39.81	49.35 ± 64.58	477.51 ± 293.72
c-peptide	53.89 ± 24.58	53.42 ± 25.02	119.68 ± 24.32
C3a	41.32 ± 26.84	27.38 ± 12.74	114.36 ± 72.15
CRP	29.68 ± 42.38	12.55 ± 9.83	7.68 ± 6.66
ENA-78/CXCL5	16.03 ± 11.37	12.33 ± 9.36	6.70 ± 1.50
Fractalkine/CX3CL1	0.86 ± 0.50	1.04 ± 1.14	3.47 ± 1.52
CXCL6	0.86 ± 0.14	0.95 ± 0.85	0.62 ± 0.46
GM_CSF	9.34 ± 10.07	12.03± 16.88	11.40 ± 6.69
ICAM_1	654.07± 197.62	601.05± 206.70	630.74± 252.46
IL-1B	18.76 ± 8.33	23.68± 13.70	9.68± 5.12
IL_12p70	8.23 ± 4.31	10.29 ± 9.15	5.35 ± 2.82
IL_17A	6.25 ± 2.54	8.12± 6.74	5.41± 2.75
IL_6	15.80 ± 13.00	17.37 ± 20.58	12.80 ± 8.07
IL_8	21.01± 14.90	12.13± 7.80	7.99± 5.63
LOX_1	60.40± 37.01	46.83± 34.48	36.79 ± 25.13
CCL2	8.02± 3.45	8.70± 5.83	8.06± 7.04
MIF	25.09± 31.37	22.84 ± 32.60	57.64 ± 41.51
CCL3	3.78 ± 1.67	4.33± 2.42	1.56± 0.31
MMP_1	14.67± 10.27	12.47± 9.14	5.89± 4.86
MMP_12	18.32± 8.15	17.40 ± 9.88	24.88 ± 15.71
MMP_13	21.73 ± 7.22	20.21 ± 9.17	150.10 ± 96.19
MMP_2	10.32± 7.84	9.55± 8.93	8.83 ± 6.71
MMP_3	20.22 ± 9.18	21.27 ± 15.25	3.26 ± 1.37
MMP_7	8.84 ± 5.24	13.34 ± 8.14	7.55± 2.90
MMP_8	1021.97± 518.87	926.37 ± 589.51	441.36 ± 255.63
MMP_9	1052.96 ± 1376.66	406.75 ± 388.33	823.68 ± 549.92
MPO	87853.26 ± 60371.56	154331.38 ± 108765.60	35495.59± 8514.58
OPN	150.71± 55.27	150.92± 70.70	199.26 ± 63.55
OPG	7.47± 4.82	10.24± 9.17	0.66± 0.33
RANKL	8.68± 4.56	8.87± 7.65	46.68± 8.16
CCL5/RANTES	3.50± 2.51	2.90± 2.54	1.19± 0.98
SDF_1_alpha	51.07± 58.93	53.74± 54.05	69.25± 74.49
TIMP_1	582.27± 263.64	544.93± 336.18	535.98 ± 366.73
VEGF_A	23.43± 11.90	24.09 ± 18.22	22.46 ± 9.71
Total protein (ug/ml)	289.38± 131.09	289.67 ± 121.49	491.48 ± 262.80
GI	0.19± 0.40	1.69 ± 0.62	1.18 ± 0.32

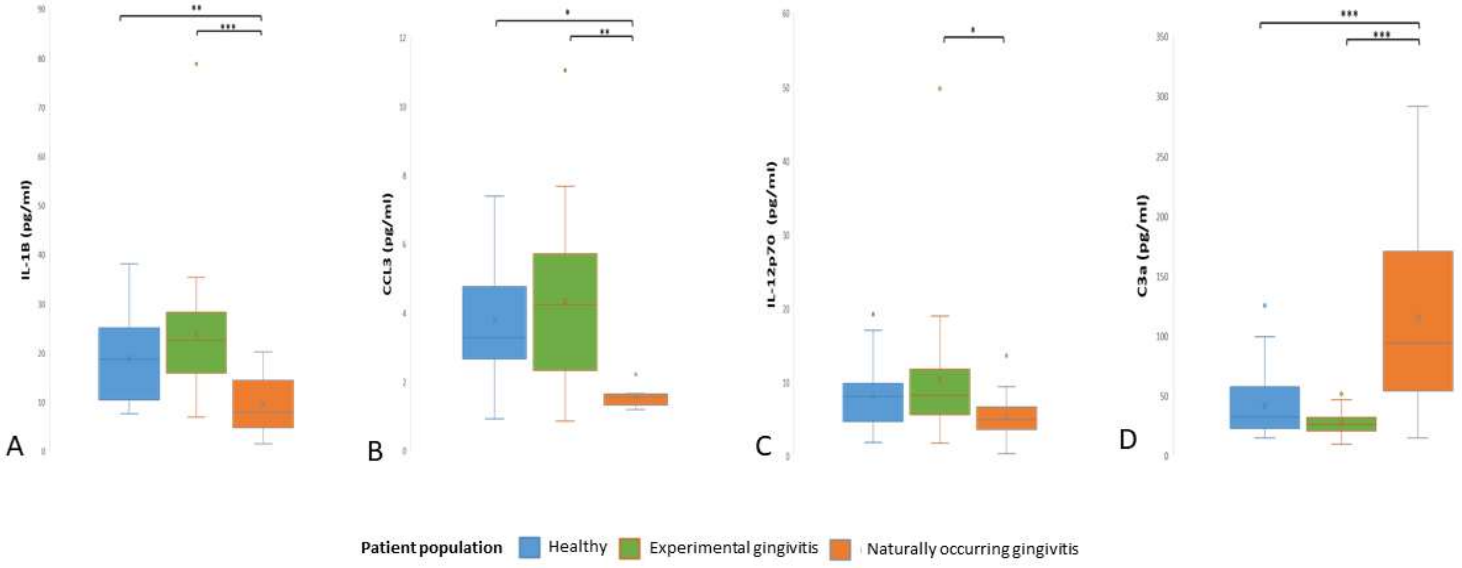
Figure 2. Migration of immune cells in healthy, experimental and naturally occurring gingivitis groups. The expression level of (A) MPO (B) MIF (C) Fractalkine/CX3CL1 (D) CCL5/RANTES (E) ENA-78/CXCL5 (F) IL-8 comparing healthy, experimental, and naturally occurring gingivitis group (n=26 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. Experimental gingivitis group showed greater expression of MPO than healthy and naturally occurring gingivitis groups (figure A). Naturally occurring gingivitis had greater expression of MIF and Fractalkine/CX3CL1 (figure B-C) and lower release of CCL5/RANTES, ENA-78/CXCL5, and IL-8 (figure D-F) than healthy and experimental gingivitis groups.



3.3.2. Pro-inflammatory mediator profile

Additional differences between EG and NG groups were found in select pro-inflammatory mediators, Figure 3A and 3B showed significant differences between EG, NG, and H groups. Both IL-1β and CCL3 were significantly lower in the NG group when compared to either the H or EG groups (IL-1β: $p<0.01$ NG vs. H and $p<0.001$ NG vs. EG; and CCL3: $p<0.05$ NG vs. H and $p<0.01$ NG vs. EG). This is similar to. A third pro-inflammatory mediator, IL-12p70, which regulates innate responses and determines the type of adaptive immune response was also significantly reduced in NG compared to EG ($p<0.05$ NG vs. EG, Figure 3C). In contrast, C3a a peptide mediator of inflammation significantly increased when compared to either the H or EG groups ($p<0.001$ both comparisons) during NG (Figure 3D). Other pro-inflammatory cytokines, such as IL-6 and GM-CSF, did not show significant difference in the level of expression among three groups ($p>0.05$)

Figure 3. Changes of pro-inflammatory mediators during experimental and naturally occurring gingivitis. The expression level of (A) IL-1β (B) CCL3 (C) IL-12p70 (D) C3a comparing healthy, experimental, and naturally occurring gingivitis group (n=26 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. (A-B) Naturally occurring gingivitis showed less level of pro-inflammatory cytokine (IL-1β and CCL3) than healthy and experimental gingivitis groups. (C) The level of IL-12p70 was slightly greater in the experimental gingivitis group than naturally occurring gingivitis. (D) Naturally occurring gingivitis showed greater level of anti-inflammatory mediator (C3a) than healthy and experimental gingivitis groups.



3.3.3 Patterns in tissue remodeling and angiogenesis

Next, the pattern of angiogenesis and soft tissue remodeling among the H, EG, NG groups were determined. Figure 4 shows the concentration of angiogenic and MMPs markers. The NG group had an increased level of angiogenin than H ($p<0.01$) and EG ($p<0.05$, Figure 4A). In contrast the level of angiopoietin-1, angiostatin, and vascular endothelial growth factor (VEGF) among three groups did not show significant differences ($p>0.05$).

With respect to the expression levels of the different MMPs, EG and NG each displayed a unique pattern which was significantly different than H. For example, NG displayed significantly lower values of MMP-1, 3 and 8 and a higher value for MMP-13 when compared to either H or EG (Figure 4B-D). Significantly less expression of these MMPs was observed in NG compared with the H, such as MMP-1 ($p<0.01$, Fig 4B), MMP-3 ($p<0.001$, Figure 4C), MMP-8 ($p<0.01$, Figure 4D); whereas the level of MMP-13 was greater in NG than H or EG ($p<0.001$ for both comparisons, Figure 4E). Compared with EG, NG expressed lower-level MMPs, MMP-1 ($p<0.05$, Figure 4B), MMP-3 ($p<0.001$, Figure 4C), MMP-7 ($p<0.05$, Figure 4F), and MMP-8 ($P<0.01$, Figure 4D). EG displayed statistically higher values of MMP-7 and lower values of MMP-9 when compared to the H ($p<0.05$ in both mediators, Figure 4F and 4G). This data demonstrate that both EG and NG may undergo soft tissue remodeling mediators, however the remodeling of NG was significantly different from EG.

3.3.4 Expression of Bone remodeling mediators

We further evaluated the mediators associated with bone metabolism among H, EG, and NG groups. Figure 5 showed the level of mediators involved in the regulation of bone remodeling using BMP-2, OPN, RANKL, and OPG. EG showed similar level of all four mediators compared

with H controls ($p>0.05$). In contrast, NG had a greater level of BMP-2 ($p<0.001$ for both comparisons, Figure 5A) than either H or EG groups. The level of OPN in NG was increased compared with either of the other two groups ($p<0.05$ for both comparisons, Figure 5B). Compared with either H or EG group, NG had significantly increased level of RANKL($p<0.001$ for both comparisons, Figure 5C) and decreased expression of OPG ($p<0.01$ NG vs. H and $p<0.001$ NG vs. EG, Figure 5D). Interestingly, the level of TIMP-1 were similar among three groups ($p>0.05$)

Given low levels of MMPs (Figure 4B-E) and high level of bone metabolism mediators (Figure 5A-C) in NG compared with H and EG, the lesions of NG exhibits less soft tissue remodeling and greater bone metabolism.

Figure 4. The patterns in angiogenesis and soft tissue remodeling during experimental and naturally occurring gingivitis.

The expression level of (A) angiogenin (B) MMP-1 (C) MMP-3 (D) MMP- 8 (E) MMP-13 (F) MMP-7 (G) MMP-9 comparing healthy, experimental, and naturally occurring gingivitis group (n=26 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. (A) The expression of angiogenesis mediator was greater in naturally occurring gingivitis group than healthy and experimental gingivitis groups. (B-E) naturally occurring gingivitis group had lower expression of MMP-1, MMP-3, MMP-8 and greater level of MMP-13 release comparing healthy and experimental gingivitis groups. (F) Experimental gingivitis group had greater expression of MMP-7 than healthy and naturally occurring gingivitis groups. (G) Experimental gingivitis group had greater expression of MMP-9 than healthy group.

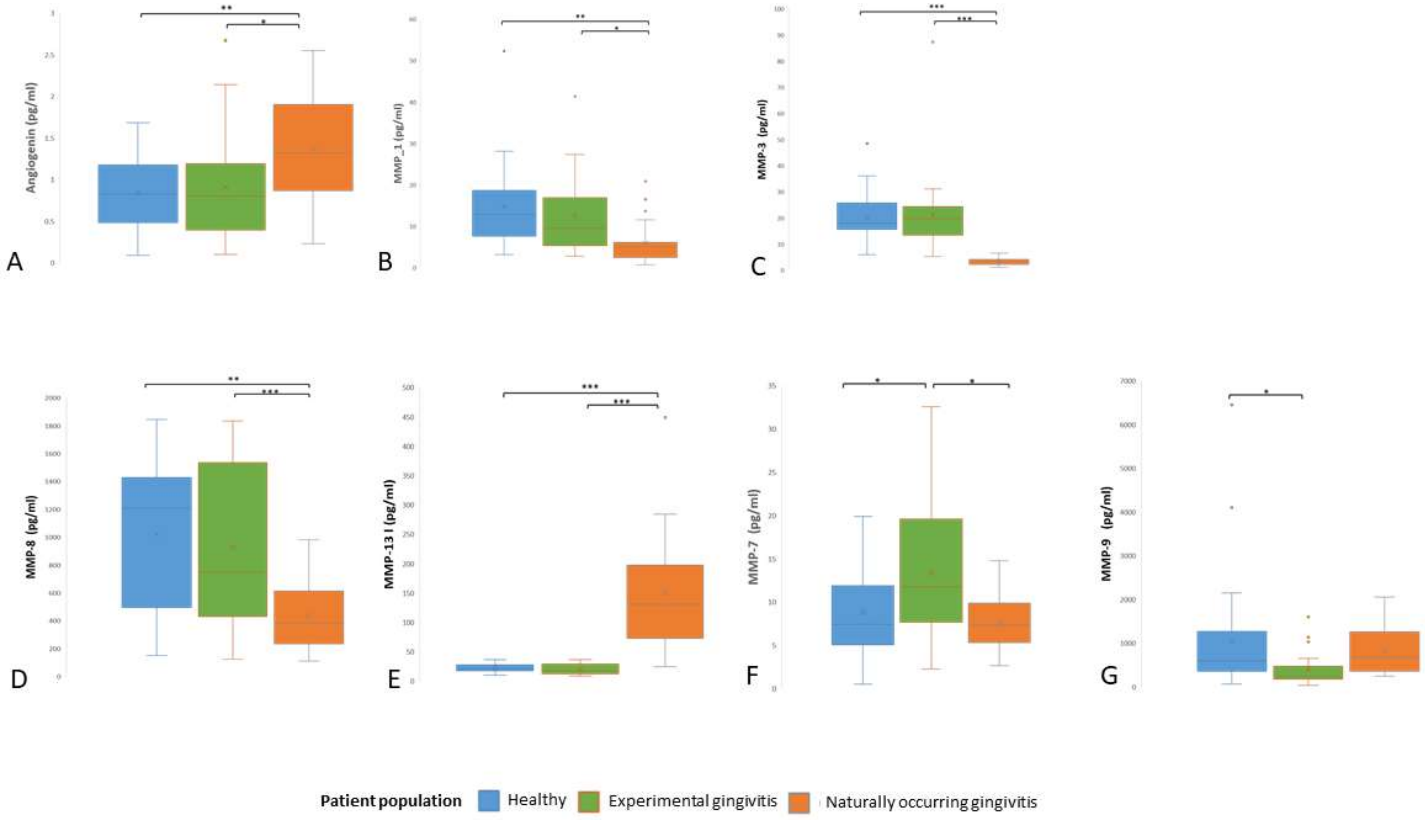
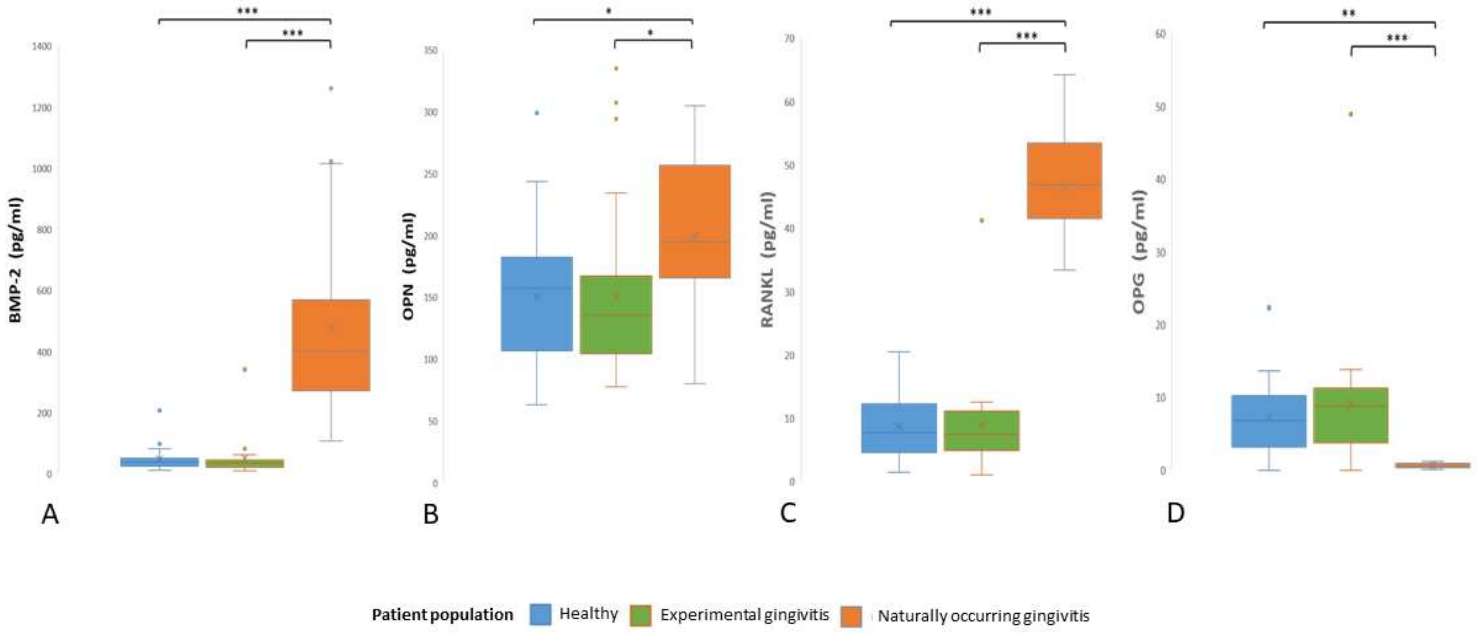


Figure 5. The patterns of bone metabolism during experimental and naturally occurring gingivitis.

The expression level of (A) BMP-2 (B) OPN (C) RANKL (D) OPG comparing healthy, experimental, and naturally occurring gingivitis group (n=26 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. (A-C) Naturally occurring gingivitis had significantly greater release of BMP-2, OPN, and RANKL compared with healthy and experimental gingivitis groups. (D)Naturally occurring gingivitis had significantly lower expression level of OPG compared with healthy and experimental gingivitis group.



3.4 Discussion

Our results indicate that EG and NG are not identical in immune regulation, angiogenesis, and tissue remodeling mediator patterns. To the authors' knowledge, this is the first study providing a comprehensive comparison of immune and tissue remodeling mediators between EG and NG.

During EG, the present findings in clinical indices reported that the gingival inflammation did not fully resolved after the resolution phase (21 days after prophylaxis) even though PI returned to the level of baseline. This phenomenon corresponds well with previous EG studies (Leite, Nascimento et al. 2022, Roberts, Yonel et al. 2022) at 14 days after prophylaxis in both clinical and immune regulatory mediators. Another EG study showed fully resolution of GI but remained slightly increases in bleeding index at the end of resolution phase (28 days after prophylaxis) compared with baseline(Offenbacher, Barros et al. 2010). Despite the length of resolution phase may contribute to the differences, it may also imply that gingivitis may not be fully reversible, as it was suggested from clinical and biochemical points of view, and may serve as a predisposing factor for further periodontal progression in susceptible individuals.

Consistent with our report, previous work has shown that EG displays increased plaque level, gingival inflammation, GCF volume. (Kinane, Adonogianaki et al. 1991, Chapple, Socransky et al. 1996, Wright, Chapple et al. 2003) A simultaneous increase in MPO alongside a reduction of IL-8 in EG has also been reported. (Meyer, Giannopoulou et al. 2017, Bamashmous, Kotsakis et al. 2021, Leite, Nascimento et al. 2022) Also, EG lesions had increased level of IL-1α, (Offenbacher, Barros et al. 2010) IL-1β, and alkaline phosphatase. (Chapple, Socransky et al. 1996, Wright, Chapple et al. 2003) In contrast, the level of TNF-α, IL-10, IL-17, GM-CSF, MIP-1β decreased during EG compared to H. (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou

et al. 2017, Leite, Nascimento et al. 2022) Inconsistent results among above mentioned studies were found regarding the released of IL-1ra, 2, 4, 6, INF-γ, and MMP-8. (Offenbacher, Barros et al. 2010, Salvi, Franco et al. 2010, Tsalikis 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022) Age distribution and EG clinical trial design may contribute to inconsistent findings. (Nonnenmacher, Helms et al. 2009, Salvi, Franco et al. 2010, Tsalikis 2010, Meyer, Giannopoulou et al. 2017, Leite, Nascimento et al. 2022) Also, inconsistencies between laboratory protocols make it difficult to compare across results of previous EG studies. Therefore, to minimize the potential discrepancies associated with patient population and experimental setting, our project used an age-matched population and worked closely with the previous investigators to assure the same GCF sample collection and laboratory protocols as well as identical multiplex panels across studies.

Previous literature (Nonnenmacher, Helms et al. 2009, Offenbacher, Barros et al. 2010, Bamashmous, Kotsakis et al. 2021) and our current findings indicate neutrophils are the predominant immune cells in the lesions of EG. (Baehni, Tsai et al. 1979, Meyer, Giannopoulou et al. 2017, Bamashmous, Kotsakis et al. 2021, Leite, Nascimento et al. 2022) Our data also reveal that NG displays more diversity in the types of immune cells, including neutrophils and macrophages, indicating that neutrophils are not the only predominating cells for immune regulation in NG. Immediately after plaque accumulation, neutrophils infiltrate the periodontium quickly and soon become the dominant immune cells in the earliest stage of inflammation, similar to other acute inflammatory conditions. (Ross and Odland 1968) As our current findings and previous reports, the acute phase of the lesion elevates gingival inflammation both in clinical and molecular levels, i.e., higher levels of GI and IL-1β, (Trombelli, Scapoli et al. 2010, Farina, Guarnelli et al. 2012) increased level of MPO (indicator of neutrophils) and the decreased

expression of IL-8 (the chemoattractant of neutrophils). (Meyer, Giannopoulou et al. 2017, Bamashmous, Kotsakis et al. 2021, Leite, Nascimento et al. 2022) The lesions of NG display a different pattern, representing the chronic inflammatory condition. Compared with EG, NG lesions displayed lower levels of markers commonly associated with gingival inflammation (lower GI and expression of IL-1β), fewer neutrophils (decreased level of MPO), and more involvement of macrophages (the elevated level of Fractalkine/ CX3CL1 and MIF). The current findings regarding the elevated expression of mediators associated with macrophage upregulation (MIF), and suppression of neutrophil mobilization (C3a) comparing NG versus H corresponded well with previous literature. (Henry, Ungchusri et al. 1987, Gürkan, Eren et al. 2016) The duration and speed of plaque accumulation may contribute to the difference of the patterns on inflammatory responses during EG and NG (Trombelli, Scapoli et al. 2010). Therefore, although EG is a useful model to study gingival inflammation, a further understanding of the regulatory events that disrupt oral homeostasis during NG as an established, chronic lesion of gingivitis is necessary.

Angiogenesis plays a critical role in wound healing. Angiogenin induces cell migration, proliferation of endothelial cells and supports cell survival. (Kishimoto, Liu et al. 2005, Li and Hu 2012) For the first time, our data showed this major regulator of angiogenesis increased in the lesions of NG, indicating the formation of blood vessels and wound healing were ongoing; whereas the lesions of EG and H showed less release of angiogenin in similar levels. The greater expression of angiogenin and low release of anti-angiogenic mediator, IL-12p70, is consistent with NG displaying greater angiogenesis than EG group. Even though the regulation of angiogenin in the periodontium is still not fully understood, the production of angiogenin in periodontal ligament cells and gingival fibroblasts involved in the wound repair from

inflammation, may benefit further periodontal regeneration. (Kim, Kim et al. 2015, Janjić, Bauer et al. 2019)

Tissue remodeling of the periodontium is a major interest for clinicians since it directly affects the disease severity and the prognosis of teeth. Immune responses triggered by plaque accumulation alter the balance of tissue homeostasis as evidenced by the patterns of MMPs expression vary from the acute and chronic lesions (Saarialho-Kere 1998, Soliman and Barreda 2022). In acute inflammatory lesions, neutrophils elicit significant MMPs expression and contribute to degradation of extracellular matrix proteins. A critical equilibrium is usually carefully maintained between the inflammatory and tissue homeostasis in the acute lesions to ensure minimal tissue damages caused by the acute inflammation.(Soliman and Barreda 2022) However, very little is known concerning collagen degradation during EG (Offenbacher, Barros et al. 2010, Meyer, Giannopoulou et al. 2017) and NG (Noack, Kipping et al. 2017, Keles Yucel, Afacan et al. 2020) rendering our knowledge of tissue turnover lacking. Our current findings displayed different patterns of tissue remodeling during EG and NG. For example, the EG lesions had higher expression of PMN-derived MMPs (MMP-1, MMP-3, MMP-8, and MMP-9); whereas the NG had elevated level of MMP-13. Indeed, MMP-13 is considered as an important MMP during bone development which degrades pre-existing extracellular matrix protein before bone mineralization. (Luchian, Goriuc et al. 2022) Clinically, MMP-13 is also associated with progressive bony destruction and untreated periodontitis. (Kiili, Cox et al. 2002) Along with the increase of MMP-13, NG also showed elevated level of mediators associated with bone metabolism (i.e., RANKL, OPN, and BMP-2), indicating the potential transition of these chronic lesions towards periodontitis.(Page and Schroeder 1976) These findings were consistent with previous results from others.(Sharma and Pradeep 2006, Rajapriya, Thomas et al. 2011,

Abdullameer and Abdulkareem 2023) Interestingly, the level of TIMP-1 were similar among three groups in the current study, which conforms with previous data.(Emingil, Tervahartiala et al. 2006, Maeso, Bravo et al. 2007, Nascimento, Baelum et al. 2019) This is the first time that a comprehensive analysis of tissue remodeling mediators was performed in both the lesions of EG and NG. Similar to the different patterns of tissue healing between acute and chronic lesions, tissue remodeling patterns vary between the lesions of EG and NG that may hold a key to tissue homeostasis or irreversible tissue destruction. It tends to be more self-limited in EG to minimize tissue (especially bone) destruction. On the other hand, the persistent dysregulation of gingival inflammation during NG, when beyond the points of self-repair, may increase the risks of further bone remodeling due to the constant disruption of tissue homeostasis.

Regardless of EG and NG, it is noteworthy that some mediators displayed a wide range of expression levels, e.g., MIF, CXCL5, and MMP-8. This is consistent with other data demonstrating individual variability in EG and NG studies.(Offenbacher, Barros et al. 2010, Keles Yucel, Afacan et al. 2020, Bamashmous, Kotsakis et al. 2021) The impact of these highly variable responder phenotypes on disease progression is not known. The contribution of how the individual variability may influence the disease progression in the lesions of either EG or NG will aid in the development of novel preventative and therapeutic approaches in periodontitis.

The current findings highlight the differences between the models of acute inflammation (EG) and chronic inflammation (NG). The former represents an typical early inflammatory responses in gingiva; while the later displays a dysregulated form of inflammation.(Murakami and Hirano 2012) The findings in NG group reflect the long-standing inflammation, in which multiple immune mechanisms are involved and lead to altered tissue homeostasis. Despite this limitation, EG is a useful model to understand the initial host response due to its ability to control

rate of plaque accumulation in healthy periodontium compared to NG, which is variable in its duration of chronic inflammation and hence its progress to periodontitis. Ultimately, further study of differences in individual responses in both NG and EG, while acknowledging each tool's limitation, is required to gain a better understanding of a patient's susceptibility to periodontitis.

To sum up, the current findings demonstrate that NG displays more immune regulation, greater angiogenesis, and bone remodeling than EG, which is consistent with the findings from acute and chronic inflammatory lesions. The mediators released in the lesions of NG also have greater variation, possibly reflecting the human individual variety.

Chapter 4. Immune regulation and tissue remodeling during gingivitis and periodontitis

4.1 Introduction

As the one of the most common oral diseases, uncontrolled plaque-induced gingivitis and periodontitis lead to loss of periodontium and, eventually tooth loss if left untreated. Clinically, gingivitis is considered reversible since it is limited to soft tissue inflammation; whereas periodontitis involves both soft tissue and hard tissue destruction and requires intervention to restore alveolar bone loss (Caton, Armitage et al. 2018). Histologically, gingivitis showed the accumulation of lymphoid cells initially followed by macrophages and plasma cells in the connective tissues (Page and Schroeder 1976). It was proposed that the dominance of macrophage and lymphocyte activation facilitated the transition from gingivitis to periodontitis. In advanced lesions, the inflammation extends into periodontal ligament and alveolar bone along with the formation of periodontal pockets (Page and Schroeder 1976, Page, Offenbacher et al. 1997).

Advancements in technology have allowed a deeper understanding of the immune response and its effect on tissue turnover during periodontal inflammation. Indeed, immune responses were found to regulate periodontal inflammation via multiple signaling pathways, resulting in the chronicity and extension of the inflammatory damage during the transition of disease progression (Kurgan and Kantarci 2018). Gingival crevicular fluid (GCF) has been is a source of biomarkers for periodontal diseases, and have proven to be an additional diagnostic method for disease progression(Barros, Williams et al. 2016).

Gingival inflammation is the prerequisite for the development of periodontitis (Kurgan and Kantarci 2018) although gingivitis lesions do not always progress to periodontitis (Ammons, Schectman et al. 1972, Goodson, Haffajee et al. 1984). Therefore, in this manuscript, select site-specific features of gingivitis, periodontitis, and transition sites have been characterized and compared. A cross-sectional study approach was employed to compare gingivitis sites in individuals with gingivitis only to gingivitis sites in individuals that also contain periodontitis. The comparison of gingivitis sites in individuals that do not transition to periodontitis (i.e. gingivitis only) versus the gingivitis sites in individuals with periodontitis provides the first comprehensive characterization of the immune regulation and tissue remodeling programs in the sites undergoing disease progression.

4.2 Materials and methods

This cross-sectional study enrolled two patient populations: naturally-occurring gingivitis and untreated stage III grade B periodontitis. Depending on the sites and periodontal status of the subject, the sites were categorized into 3 groups: (1) gingivitis sites from patients with gingivitis only (G group); (2) gingivitis sites from patients with stage III grade B periodontitis (GP group); (3) periodontitis sites from patients with stage III grade B periodontitis (PP group). The study was approved by the human subjects ethics board of the University of Washington (UW IRB# STUDY00012410) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013.

4.2.1 Patient inclusion and exclusion criteria

Patient inclusion criteria included (1) patients with age of 18-75 years old, (2) systemically healthy, (2) For gingivitis only population, patients should have generalized gingivitis following the definition of the 2018 AAP/EFP disease classification without any history of periodontitis (Chapple, Mealey et al. 2018). (3) For periodontitis population, patients should have untreated generalized severe periodontitis (stage III grade B) following the definition of the 2018 AAP/EFP disease classification (Caton, Armitage et al. 2018).

Exclusion criteria included: (1) use of antibiotics or nonsteroidal anti-inflammatory medications within 30 days, (2) patients with diabetes, (4) current smokers or former smokers quitting for less than 11 years (Tomar and Asma 2000), (5) refusal to sign the informed consent or unable to communicate in English, (6) patients received active periodontal therapy such as scaling and root planning, and periodontal surgery within 2 years.

4.2.2 Calibration

The calibration process among examiners was performed on five volunteers not enrolling in this study prior to the beginning of the study. The same quadrant was probed two times separately between 1-3 weeks apart. The inter and intra-examiner agreement were achieved to ≥ 80% before the beginning of the study.

4.2.3 Clinical examination and GCF collections

Calibrated researchers (YH and MS) recruited, examined, and collected biospecimens. Patients were screened after informed consent signed. Within the same visit, patient demographic information was recorded, including age, gender, smoking habit, and health status. Periodontal examination was further performed, recording the following clinical indices, including clinical attachment level (CAL), probing depth (PD), bleeding on probing (BOP), visible plaque index

(PI) (Silness and Loe 1964), and gingival index (GI) (Loe and Silness 1963). In gingivitis patients, GCF samples and clinical indices were collected at 6 surfaces of 6 selected teeth with gingivitis (G group). In periodontitis patients, biospecimens collection and clinical indices were performed at 6 surfaces of 3 selected teeth with either gingivitis (GP group) and periodontitis (PP group). All GCF samples were collected using the protocol of 30 seconds and stored at -80^{o}C until processing.

4.2.4 Analysis of Gingival crevicular fluid

All GCF samples underwent an assay of 39 mediators of inflammatory/immune responses and tissue remodeling using commercially available bead-based multiplex immunoassays (YH and MS) (Procartaplex 39-plex Panel, Thermo Fisher Scientific, Waltham, MA, USA). The GCF samples were extracted using 200 µl of 0.01M PBS to each tube and rotating for one hour at 4^{o}C. An aliquot of 10µl per sample was transferred for protein analysis (VMax microplate reader; GMI, Ramsey, MN, USA. Based on the protein concentration, the GCF samples were diluted with 0.01M PBS to normalize samples to 10µg/ml. Bead based multiplex analysis was then performed on duplicated, standardized GCF samples (i.e., samples diluted to 10µg/ml) using the bead-based multiplex immunoassays according to manufacturer's protocol. The data was obtained using a flow-cytometry-based array reader (Bio-Plex 200 reader, Bio-Rad Laboratories, Hercules, CA, USA), by acquiring the signal from the fluorescent dye within each bead for assay identification along with the fluorescent signal from the reporter for quantification. The data was analyzed with a software (Bio-Plex Manager Software V6, Bio-Rad Laboratories, Hercules, CA, USA) and the concentrations of different mediators were calculated based on the respective standard curve for each chemokine with 5-parameter logistic (5PL) equation. Mediator data are

reported in mean value with the unit of pg/ml. The intra-plate variability is 7.75% in average (6.33-9.64%) and the inter-plate variability is 5.02%.

The analytes included angiogenin, angiopoietin-1, angiostatin, BMP-2, C-peptide, C3a, Cortisol, CRP, ENA-78/CXCL5, Fractalkine/CX3CL1, GCP-2/CXCL6, GM-CSF, ICAM-1, Interleukin (IL)-1beta, IL-12p70, IL-17A/CTLA-8, IL-6, IL-8, insulin, LOX-1, MCP-1/CCL2, MIF, MIP-1 alpha/CCL3, matrix metalloproteinase (MMP)-1, -2, -3, -7, -8, -9, -12, -13, MPO, OPN, OPG, RANKL, RANTES, SDF-1alpha, TIMP-1 VEGF-A.

4.2.5 Statistical analysis

Statistical and power analysis were performed using a statistical analytic software (JMP® software version, Pro 17, JMP Statistical Discovery LLC., Cary, NC, USA). A statistical power (1-β) of 0.8 and a type I error rate of 0.05 according to the clinical differences were set (Tayman, Kurgan et al. 2019) and 20 patients per group were necessary.

Clinical and mediator data were summarized into single average scores for each group per patient and were reported using boxplots showing medians and interquartile ranges. The out of range data of each analyte was removed from statistical analysis. The numbers of detected analytes and the limit of detection were included in table 12. The differences in clinical indices and mediator expression among groups were determined at a 95% confidence level ($p \leq 0.05$) by a two-way ANOVA with a post-hoc Tukey's test. A p-value < 0.05 was set to indicate statistical significance.

4.3 Results

4.3.1 Demographic information

A total of 48 patients were recruited in this study, including 24 patients with gingivitis only and 24 patients with stage III grade B generalized periodontitis. The gingivitis group enrolled 15 females and 9 males with an average age of 54.33± 14.93 years old, whereas the periodontitis group enrolled 10 females and 14 males with an average age of 55.88± 13.73 years old. Neither gender nor age among groups showed a significant difference ($p>0.05$).

Table 13 and Figure 6 summarize the comparison of demographics and clinical indices among the 3 groups of inflammatory sites. Clinically, PP showed the greatest PD ($p \leq 0.001$ for both comparisons) and CAL ($p \leq 0.001$ for both comparisons) while G and GP groups shared similar level of CAL ($p>0.05$ G vs. GP, Figure 6A and 6B). G and GP shared similar level of gingival inflammation ($p>0.05$ G vs. GP, Figure 6C) although G had significantly lower PI than GP ($p \leq 0.001$ G vs. GP, Figure 6D). Of note, G had the lowest PI comparing with GP and PP groups ($p \leq 0.001$ at both comparison) while GP and PP groups did not have significant difference in PI ($p>0.05$, Figure 6D).

4.3.2 The expression of mediators associated with immune regulation

Table 14 and Figure 7 summarize the comparison of immune regulatory mediators among 3 groups of inflammatory sites. Compared with G groups, PP groups showed significantly greater expression of CXCL6 ($p \leq 0.05$), Fractalkine/CX3CL1 ($p \leq 0.01$), IL-12 ($p \leq 0.05$), CCL2 ($p \leq 0.05$), and CCL5 ($p \leq 0.05$) (Figure 7A-E). Within the same individuals, interestingly, PP groups also have significantly greater expression of CCL5 ($p \leq 0.05$, Figure 7E), GM-CSF ($p \leq 0.05$, Figure 7F), and IL-6 ($p \leq 0.05$, Figure 7G) than GP groups. It is also noteworthy that CCL3 level

was significantly greater in GP groups compared to G groups ($p \leq 0.05$, Figure 7H) even though PP failed to show any significant difference compared to gingivitis groups (G and GP) ($p>0.05$).

The levels of CRP, ENA-78/CXCL5, ICAM-1, IL-1β, IL-8, IL-17, LOX-1, MIF, and MPO were also tested but did not show significant differences among 3 groups ($p>0.05$, Table 14).

Table 12. Information of each analytes

	Number of Analytes	limit of detection
Angiogenin	66	0.01-600
Angiopoietin-1	30	7.08-145600
Angiostatin	70	0.19-5150
BMP-2	59	1.16-303000
c-peptide	64	1.13-295700
C3a	68	0.54-142800
CRP	66	0.11-28800
ENA-78/CXCL5	54	0.11-29100
Fractalkine/CX3CL1	60	0.02-5750
CXCL6	50	0.02-6200
GM_CSF	42	0.19-49900
ICAM_1	66	1.91-501200
IL-1B	68	0.03-7000
IL_12p70	67	0.12-32300
IL_17A	64	0.04-11100
IL_6	62	0.16-42000
IL_8	69	0.04-9250
LOX_1	71	0.02-6300
CCL2	57	0.08-20000
MIF	68	0-1100
CCL3	44	0.04-9600
MMP_1	64	0.11-28800
MMP_12	57	0.1-25100
MMP_13	61	0.12-32500
MMP_2	48	0.12-32700
MMP_3	56	0.03-7100
MMP_7	63	0.08-21200
MMP_8	62	0.39-101700
MMP_9	54	0.02-4400
MPO	68	1.31-344100
OPN	59	0.28-73300
OPG	51	0.01-2800
RANKL	40	0.12-30500
CCL5/RANTES	61	0.01-3500
SDF_1_alpha	44	0.24-63400
TIMP_1	61	044-116300
VEGF_A	59	0.08-20000

Table 13. Comparison of demographics and clinical parameters among 3 groups of inflammatory groups

	Gingivitis only (n=24)	Gingivitis sties in Periodontitis (n=24)	Periodontitis sties in Periodontitis (n=24)
Demographics/ mean (SD)			
Age (y/o)	54.33 (14.93)	55.88 (13.73)	55.88 (13.73)
Gender	15 females and 9 males	10 females and 14 males	10 females and 14 males
Clinical Parameters/ mean (SD)			
Probing depth (mm)	2.20 (0.23)	2.54 (0.30)	4.60 (0.43)
Clinical Attachment Level (mm)	2.44 (0.33)	2.78 (0.45)	5.53 (1.06)
Plaque index	0.54 (0.48)	1.18 (0.44)	1.41(0.39)
Gingival index	1.08 (0.31)	1.18 (0.39)	1.64 (0.47)

Table 14. Comparison of mediators among 3 groups of inflammatory groups

	Gingivitis only (n=24)	Gingivitis sties in Periodontitis (n=24)	Periodontitis sties in Periodontitis (n=24)
Protein concentration of standardized mediators (pg/ml)			
Angiogenin	1.26 (1.02)	5.30 (9.45)	2.79 (3.04)
Angiopoietin-1	49.02 (40.89)	53.35 (62.39)	122.70 (78.46)
Angiostatin	66.24 (90.65)	67.17 (77.30)	107.97 (131.49)
BMP-2	789.95 (1063.32)	741.76 (2005.77)	1720.70 (2954.41)
c-peptide	141.14 (77.95)	321.27 (287.26)	331.69 (292.77)
C3a	348.44 (507.68)	237.60 (336.63)	169.93 (166.17)
CRP	49.10 (39.64)	161.25 (631.52)	119.32 (272.92)
ENA-78/CXCL5	46.79 (63.99)	53.84 (31.50)	72.00 (52.23)
Fractalkine/CX3CL1	1.89 (1.55)	2.39 (1.39)	3.55 (2.23)
CXCL6	1.09 (0.84)	1.52 (1.35)	2.3 (1.64)
GM_CSF	59.20 (45.73)	42.23 (55.57)	138.77 (156.56)
ICAM_1	1234.97 (1276.87)	1907(1556.52)	1655.91 (1293.80)
IL-1B	20.77 (30.82)	24.31 (18.46)	38.23(31.62)
IL_12p70	14.92 (17.65)	18.18 (14.33)	28.83 (23.96)
IL_17A	22.19 (22.10)	22.92 (14.63)	36.02(24.61)
IL_6	43.72 (37.22)	34.83 (56.01)	85.18 (88.25)
IL_8	13.265 (12.96)	23.36 (23.08)	25.80 (22.81)
LOX_1	50.84 (55.87)	10168 (126.11)	117.26 (129.20)
CCL2	17.05 (16.43)	35.70 (20.23)	40.86 (27.21)
MIF	56.57 (56.02)	26.20 (27.79)	44.77(41.90)
CCL3	7.25 (7.73)	18.38 (10.63)	15.76 (10.21)
MMP_1	54.88 (48.82)	43.01 (33.59)	58.13 (12.69)
MMP_12	102.65 (86.58)	60.32 (36.26)	135.15 (112.30)
MMP_13	112.70 (102.87)	100.63 (94.73)	84.33 (62.23)
MMP_2	57.82 (59.49)	53.13 (31.68)	149.40 (112.52)
MMP_3	14.26 (11.82)	31.71 (31.23)	32.16 (24.79)
MMP_7	24.50 (15.71)	22.15 (11.46)	61.66 (44.53)
MMP_8	794.63 (921.98)	1688.20 (1683.89)	2185.55(2554.89)
MMP_9	1140.25 (839.80)	2250.63 (2082.98)	2278.95 (2075.83)
MPO	24919.76 (15794.76)	26060.88(15714.11)	31791.94 (17152.94)
OPN	226.68 (115.50)	247.37 (106.91)	325.51 (121.23)
OPG	0.69 (0.75)	4.73 (5.50)	5.94 (7.04)
RANKL	10.51 (6.89)	18.05 (8.34)	22.20 (12.00)
CCL5/RANTES	3.2 (2.64)	3.23 (2.11)	7.83 (6.11)
SDF_1_alpha	91.39 (112.33)	133.52 (152.21)	313.78(325.57)
TIMP_1	556.48 (468.05)	576.15 (430.79)	754.36 (490.65)
VEGF_A	31.84 (27.72)	29.11(17.00)	49.21 (43.25)
Total protein (ug/ml)	658.73 (109.91)	439.89 (430.82)	1470.50 (1210.95)

Figure 6. Clinical Indices among 3 groups of inflammatory sites.

The expression level of (A) Probing depth (B) Clinical attachment level (C) gingival index (D) plaque index comparing gingivitis sites in gingivitis (G), gingivitis sites in periodontitis patients (GP) and periodontitis sites in periodontitis patients (PP) (n=24 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. G sites showed the shallowest probing depth and PP sites showed the greatest probing depth among 3 groups (figure A). G and GP sites showed the similar level of clinical attachment and gingival inflammation (figure B-C). GP and PP sites shared similar level of plaque index, which is significantly greater than G sites (figure D).

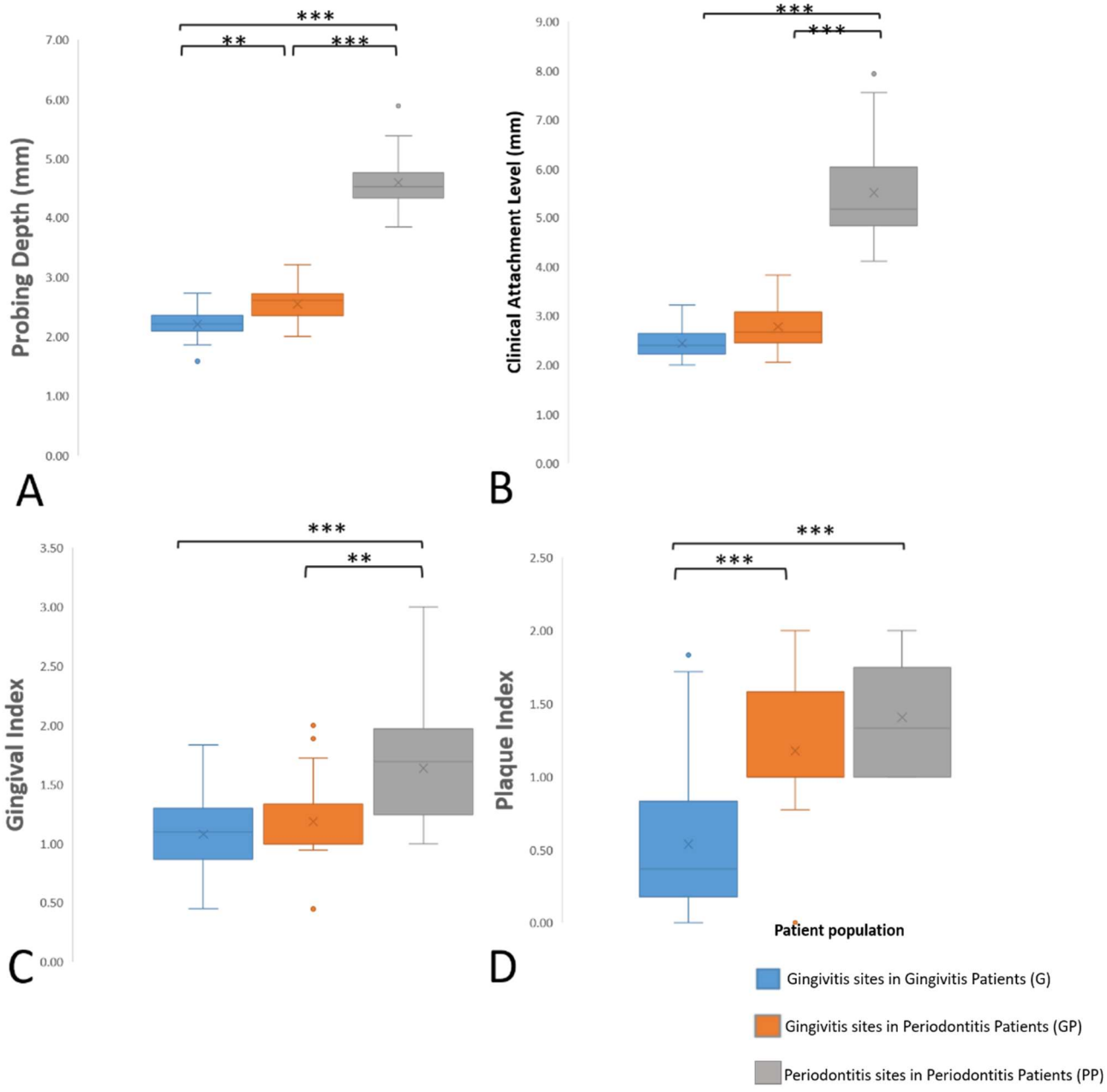
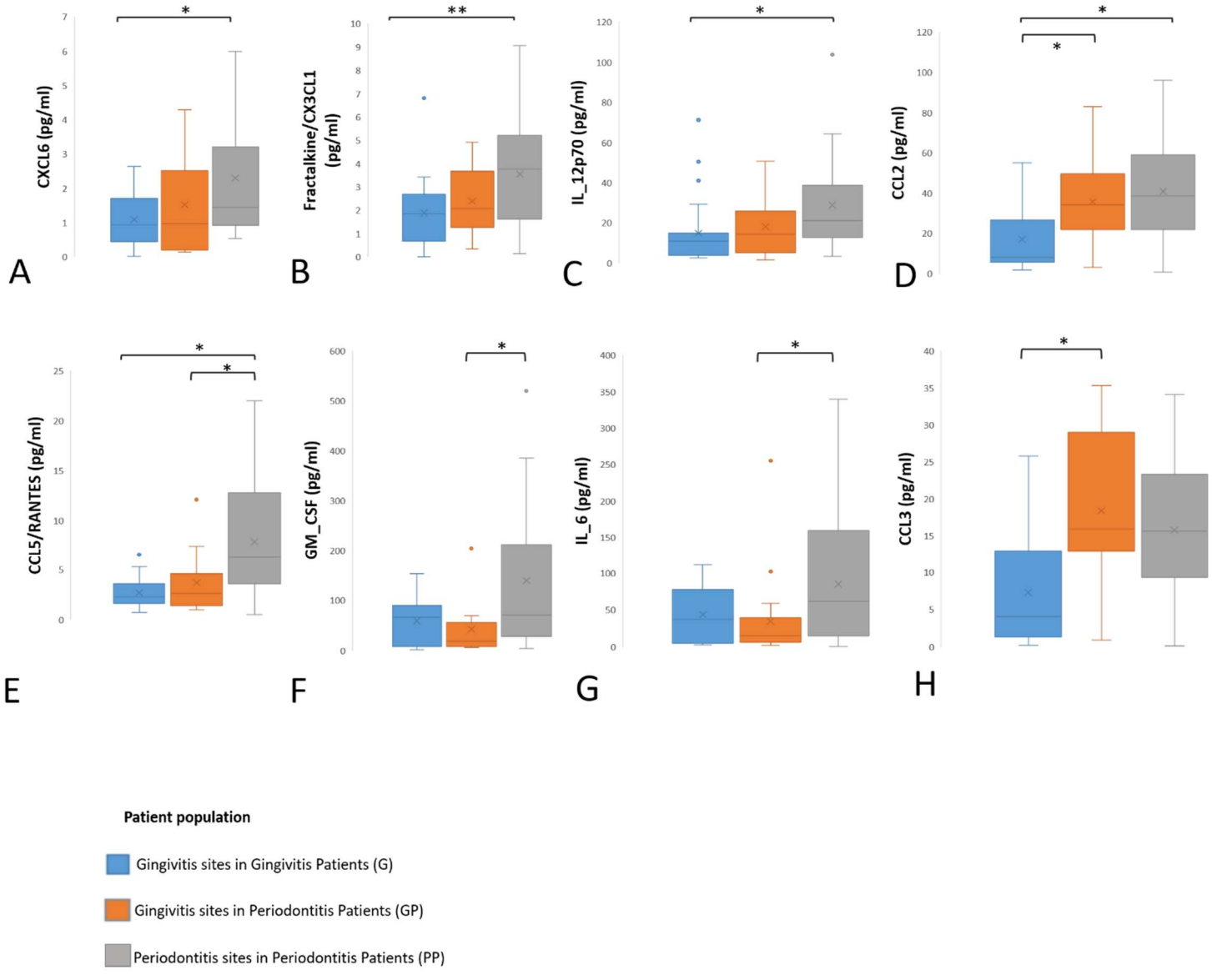


Figure 7. Levels of immune regulatory mediators among 3 groups of inflammatory sites.

The expression level of (A) CXCL6 (B) Fractalkine/CX3CL1 (C) IL-12 (D) CCL2 (E) CCL5/RANTES (F) GM-CSF (G) IL-6 (H) CCL3 comparing gingivitis sites in gingivitis (G), gingivitis sites in periodontitis patients (GP) and periodontitis sites in periodontitis patients (PP) (n=24 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. PP sites showed greater expression of CXCL6, Fractalkine/CX3CL1, IL-12, CCL2, and CCL5/RANTES among 3 groups (figure A-E). GP sites had less expression of CCL5/RANTES, GM-CSF and IL-6 than PP sites (figure E-G). G had lower level of CCL2 and CCL3 than GP sites (figure D and H).



4.3.3 The expression of mediators associated with angiogenesis and tissue turnover

We then examined the tissue responses during periodontal inflammation. Table 14 and Figure 8 summarizes the comparison of tissue remodeling and angiogenesis mediators among 3 groups of inflammatory sites. In general, PP groups had the greatest expression in these mediators. G and GP groups showed similar level of mediators related to angiogenesis and tissue turnover without significant statistical differences ($p>0.05$, Figure 8A-I).

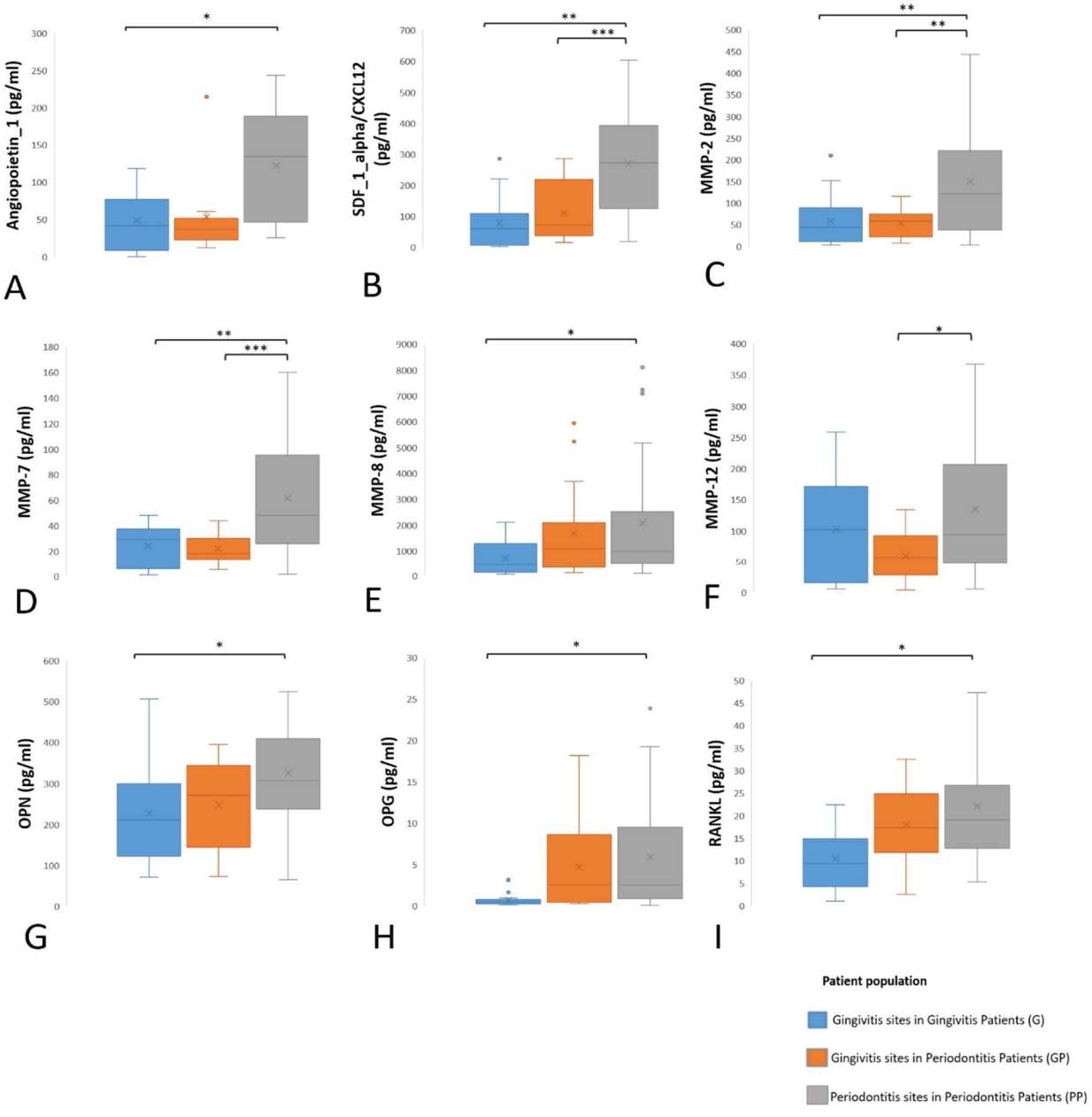
For those mediators associated with angiogenesis, PP groups showed greater expression of angiopoietin-1 ($p \leq 0.05$, Figure 8A) and SDF-1/CXCL12 ($p \leq 0.01$, Figure 8B) than G. Within individuals, PP also had greater level of SDF-1/CXCL12 than GP ($p \leq 0.001$, Figure 8B). The level of angiogenin, angiostatin and VEGF were also tested but did not show significant differences among groups ($p>0.05$).

Compared with G, PP groups also had the greatest level of mediators related to soft tissue remodeling, i.e., MMP-2 ($p \leq 0.01$, Figure 8C), MMP-7 ($p \leq 0.01$, Figure 8D) and MMP-8 ($p \leq 0.05$, Figure 8E). Within individuals, greater expression of MMP-2 ($p \leq 0.01$, Figure 8C), MMP-7 ($P \leq 0.001$, Figure 8D) and MMP-12 ($p \leq 0.05$, Figure 8F) were also found in PP compared with GP groups. The level of the abovementioned soft-tissue related mediators did not show a significant difference between G and GP groups ($p>0.05$). In summary, the level of MMP-2 (Figure 8C) and MMP-7 (Figure 8D) in PP groups were found the highest among 3 groups, whereas a statistically significance difference was only achieved between G and PP groups in the expression of MMP-8 ($p \leq 0.05$, Figure 8E). Due to the large standard deviation in the G and PP groups, although GP groups had the lowest expression of MMP-12 statistical differences were only found between GP and PP groups ($p \leq 0.05$, Figure 8F). The expression of MMP-1, 3, 9, 13 and TIMP-1 were also tested. Table 14 showed PP group had the greatest level of MMP-1,

MMP-3, and MMP-9 than G and GP groups. Of note, G group had the greatest expression of MMP-13 than GP or PP groups. However, these differences in MMP-1, 3, 9, and 13 did not show significant differences among 3 groups ($p>0.05$).

Figure 8. Expression of mediators associated with angiogenesis and tissue turnover among 3 groups of inflammatory sites.

The expression level of (A) angiopoietin-1 (B) SDF-1alpha/CXCL12 (C) MMP-2 (D) MMP-7 (E) MMP-8 (F) MMP-12 (G) OPN (H) OPG (I) RANKL comparing gingivitis sites in gingivitis (G), gingivitis sites in periodontitis patients (GP) and periodontitis sites in periodontitis patients (PP) (n=24 for each group). Boxes represent data and medians ± interquartile ranges (IQR); whiskers and outliers > 1.5 IQR below (above) the 25th (75th) percentile. Differences among each group are shown above the groups and their significance level indicated by asterisks. Significance levels: Significance levels: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. G had less expression of these mediators related with angiogenesis and tissue turnover than PP sites (Figure A-I). GP had lower level of SDF-1alpha/CXCL12, MMP-2, MMP-7, and MMP-12 than PP sites. (Figure B-D and F)



4.4 Discussion

Further understanding of disease progression offers the opportunity to provide more individualized tailored therapeutic strategies for periodontitis. In general, our findings demonstrated that gingivitis and periodontitis exhibited two distinct phenotypes both clinically and the corresponding host response. The GP group, i.e., the gingivitis sites in periodontitis population, expressed mediators found in both gingivitis and periodontitis. This information is consistent with the clinical observation that periodontitis sites can facilitate more advanced disease progression. Consistent with this, within the same individuals, PP sites showed more diverse immune regulation and more significant activity in angiogenesis and tissue turnover than GP sites. Despite the similar level of inflammation in clinical appearance, it is essential to point out the different levels of CCL2 and CCL3 between G and GP groups, indicating that the overall periodontal status of the individuals could influence the gingivitis sites at the molecular level.

Understanding gingivitis requires both a full-mouth clinical evaluation and analysis of inflammatory mediator expression. Clinically, G when compared to GP groups showed a similar level of gingival inflammation and CAL, but less plaque accumulation. The differences of PD between these two groups was within 0.3mm, which may not represent any clinical significance (Greenstein 2003). These data reveal that G and GP are similar in their clinical appearance (Figure 6), which is similar to their effect on angiogenesis and tissue remodeling (Figure 8). However, it is noteworthy that G had significantly less expression of monocyte chemoattractant protein-1 (CCL2) and macrophage inflammatory protein-1 alpha (CCL3) (Figure 7D and 7H) as well as PI (Figure 6D) than GP groups. As well-defined chemotactic chemokines, CCL2 and CCL3 exhibit polymorphonuclear neutrophils chemoattractant ability (Domachowske, Bonville et al. 2000) and are involved in the pathogenesis of various inflammatory diseases (Bhavsar,

Miller et al. 2015, Zhang, Wang et al. 2017). Indeed, both CCL2 and CCL3 mediate the recruitment of monocytes/macrophages and PMN's respectively in response to bacteria (Cekici, Kantarci et al. 2014). Therefore, the increased expression of CCL2 and CCL3 in the GP group is consistent with the increased inflammation associated with GP sites when compared to G sites. Furthermore, in the periodontium, CCL2 is correlated with NF-kB pathway (Zhang, Wang et al. 2017) and CCL3 may enhance osteoclastogenic activity via migration of RANKL+ cells (Repeke, Ferreira Jr et al. 2010). In contrast to difference in expression when G and GP are compared, CCL2 and CCL3 display similar levels when GP and PP groups are compared. These findings identify CCL2 and CCL3 as potential targets for therapeutic interruption due to their selective increased expression in gingivitis sites in individuals with periodontitis.

In contrast to the lack of information comparing gingivitis in individuals with and without periodontitis, the differences between gingivitis and periodontitis patient populations have been widely reported previously (Page and Schroeder 1976). Compared with stable gingivitis, periodontitis shows significantly greater tissue remodeling and diverse immune regulation, including neutrophils, plasma cells, monocytes and macrophage (Page, Offenbacher et al. 1997, Thorbert-Mros, Larsson et al. 2015). Clinically, periodontitis exhibits hallmark evidence of inflammation, bone destruction, along with deeper PD and CAL (Page, Offenbacher et al. 1997, Caton, Armitage et al. 2018). It has been shown that immune cells contribute to bone metabolism (Cekici, Kantarci et al. 2014, Brylka and Schinke 2019). For example, SDF-1/CXCL12 directly interacts with macrophages and up-regulates bone resorption via increasing MMP-9 expression and stimulating RANKL expression (Grassi, Cristino et al. 2004, Kim, Kim et al. 2014). CCL2 and CCL5 are then induced by RANKL during osteoclast differentiation (Kim, Day et al. 2005). The production of CCL2 further promotes osteoclastogenesis and inflammation via activation of

the PI3K cascade, p38 mitogen-activated protein kinase and JAK–signal transducer and activator of transcription pathways (Zhu, Liu et al. 2021). CCL5 contributes with CCL3 in the development of inflammatory bone loss, cooperating the migration of cells with RANKL (Repeke, Ferreira Jr et al. 2010). Interestingly, CCL5 also acts on osteoblasts and enhances their chemotaxis. Furthermore, , Fractalkine/CX3CL1 expressed by osteoblasts then further promote osteoclast-mediated bone loss via inducing chemotaxis and terminal differentiation of osteoclast progenitors (Brylka and Schinke 2019). Taken together, this orchestration of cell migration and mediator expression effectively contributes to bone remodeling found in periodontitis groups and are consistent with our findings. It explains well the greater expression of angiogepoitein-1, OPN, OPG, and RANKL in periodontitis groups along with the increased level of CCL2, CCL5, SDF-1/CXCL12, and Fractalkine/CX3CL1.

Another important finding from this study is the clarification of the contribution of bacterial load and bacterial composition to periodontitis. Both the GP and PP groups had a similar PI. Since our current setting compares GP and PP sites within the same individuals, differences in the host immune response such as inflammatory responder types (Offenbacher, Barros et al. 2010, Bamashmous, Kotsakis et al. 2021, Kerns, Bamashmous et al. 2023) are not contributing to the expression of the inflammatory mediator differences we observed. Rather, in the comparison of GP, our findings showed that PP had greater expression of majority of the investigated mediators and clinical indices, suggesting PP sites have much more periodontal damage when compared to GP sites despite these sites share similar PI. This data highlights the contribution of local bacterial compositions as opposed to bacterial load on sites with different degrees of disease severity within the same individuals.

Specifically, the major difference between GP and PP groups within the same individual are the expression of GM-CSF, IL-6, CCL5, SDF-1/CXCL12, MMP-2 and MMP-12. As major cytokines associated with inflammation, IL-6 and GM-CSF enhance a cascade of tissue destructive processes by the increase of inflammation and the stimulation of immune cells, such as neutrophils and monocytes (Koch 2005, Graves 2008, Cekici, Kantarci et al. 2014). Patients with periodontitis were found to have greater levels of IL-6 (Isola, Giudice et al. 2021), CCL5/RANTES (Gamonal, Acevedo et al. 2000), and GM-CSF (Balci, Cekici et al. 2021) compared with healthy controls. Indeed, a recent study suggested the inhibition of IL-6 receptor reduced inflammatory bone loss (Apolinario Vieira, Aparecida Rivas et al. 2021). Our current findings correspond well with these results.

With the limitations of a cross-sectional study, it remains unknown the fate of the GP sites regarding disease progression. However, these mediators (GM-CSF, IL-6, CCL5, SDF-1/CXCL12) may become potential biomarkers reflecting the disease progression. The limitation of the cross-sectional setting in this study makes it difficult to identify the interaction between mediators and the casualty of these mediators on periodontal destruction.

The current findings provide a comprehensive analysis of the inflammatory mediator expression profiles in gingivitis, periodontitis, and gingivitis sites in periodontitis individuals providing potential biomarkers for disease diagnosis and progression. Periodontitis exhibits more diversity in immune regulation and greater activities in tissue turnover, which also presents in those sites with shallow probing depth within the same individuals. Therefore, it should be kept in mind that the gingivitis sites in patients with periodontitis should be considered and treated as diseased sites as well since they are featured similar to periodontitis sites. Further investigation is

still required to evaluate the role of CCL2 and CCL3 on periodontium as well as the interaction among GM-CSF, IL-6, CCL5, SDF-1/CXCL12 and their association of disease progression.

In short, the current findings presented two distinct features of periodontal diseases that periodontitis exhibits more diversity in immune regulation and greater activities in tissue turnover, whereas less complexity in immune regulation and tissue remodeling was found in gingivitis. Gingivitis sites within the patients with periodontitis showed the features bridging gingivitis and periodontitis.

Chapter 5. Conclusion and Significance

5.1 Summary and conclusion

Periodontal diseases are common oral diseases in humans. Further understanding of the transition from health to different disease states with respect to immune regulation and tissue remodeling will facilitate capturing the signs of disease progression at early stage, providing novel avenues for early intervention approaches. Furthermore, comprehensive analyses of these mediators in gingival crevicular fluid (GCF) in the different states of periodontal inflammation are crucial for a better understanding of the immune and tissue homeostasis during gingivitis. The overall objective of this thesis is to investigate the molecular changes at gingivitis sites in immune regulation and tissue remodeling in individuals with different states of periodontal diseases.

The setting of experimental gingivitis (EG) is a common approach to investigate sites with gingivitis by inducing gingival inflammation over a short period. In chapter 2, we conducted a systematic review to summarize the changes of immune regulatory and tissue remodeling mediators during EG induction. Along with clinical plaque accumulation and inflammation, the findings revealed an increase in neutrophils and inflammatory mediators, such as MPO, IL-1α and IL-1β in GCF, confirming both clinical and molecular changes of these mediators during induced gingival inflammation. The expression of MIP-1β and MCP-1/CCL2 decreased during EG induction, indicating the reduction of monocytes/macrophages migration. The lack of significant changes in biomarkers associated with angiogenesis showed its limited involvement in vessel vascularity. However, the results also suggested there was great variability in expression of MIF, CCL20, ENA-

78/CXCL5, fractalkine/CX3CL1, PGE_2, IL-2, IL-8, TNF-α, IFN-γ, MMP-8, and MMP-9. The GCF levels of these mediators were modulated by several factors, including age, stress level, and the diabetes status of the volunteer populations. In this study, we characterized the profile of immune and tissue regulation during EG induction that consistent with a lesion showing acute inflammation and self-limiting characteristics. EG represents the characteristics of the initial lesion when gingival inflammation is just induced, which corresponded well with the previous studies that increased leukocytes migrate into the junctional epithelium and gingival sulcus for elevated inflammatory responses (Page and Schroeder 1976, Page and Kornman 1997). However, the variation in the results in tissue remodeling and the lack of significant changes in angiogenesis biomarkers during EG induction highlighted its self-limiting feature and lack of involvement of angiogenesis and tissue remodeling. These data reveal that EG may not be a good tool to investigate disease progression and the transition between gingivitis to periodontitis.

We further investigated the differences between EG and naturally- occurring gingivitis in immune regulation, angiogenesis, and tissue remodeling. In chapter 2, the systematic review reported the heterogeneity of EG results due to the lack of standardization/normalization protocols of samples. In chapter 3, therefore, we analyzed GCF samples collected from the same protocols in two different human studies (with matched genders and ages) using commercially available bead-based multiplex immunoassays in the same lab with the identical lab protocol. Our results also demonstrate that EG and NG are not similar in immune regulation, angiogenesis, and tissue remodeling mediator patterns. The findings showed greater level of IL-1β and MPO in EG compared with naturally occurring gingivitis, confirming findings from previous studies (Trombelli, Scapoli et al. 2010, Farina, Guarnelli et al. 2012). The fact that EG showed neutrophil-predominant lesions with less involvement of angiogenesis and bone remodeling is consistent with EG representing an acute inflammatory lesion whereas naturally- occurring gingivitis a chronic inflammatory lesion. Our results also imply that naturally occurring gingivitis may not be fully reversible right after a prophylaxis as a chronic lesion, as it is suggested from clinical and

biochemical points of view; and may serve as a predisposing factor for further periodontal progression in susceptible individuals.

To investigate the disease transition from gingivitis to periodontitis, in chapter 4, we compared inflammatory sites in patient populations with gingivitis only and with severe periodontitis (stage III grade B). In a cross-sectional setting, we recruited patients with gingivitis only and stage III grade B periodontitis. There were no significant differences in gender and age among these populations. GCF samples were collected along with clinical assessments on these patients. The results revealed that periodontitis exhibits more diversity in immune regulation and greater activities in tissue turnover, whereas gingivitis has less complexity in immune regulation and tissue remodeling. Gingivitis sites in gingivitis and periodontitis patients shared many similarities in their GCF compositions despite greater levels of CCL2 and CCL3 found in the latter group. Within individuals with periodontitis, the main differences between gingivitis and periodontitis sites are the expression of GM-CSF, IL-6, CCL5, SDF-1/CXCL12, MMP-2 and MMP-12. The fact that gingivitis sites in the periodontitis population show a GCF composition of mediators found in both gingivitis and periodontitis indicates its role in the disease transition from gingivitis to periodontitis. It also serves as a reminder to treat those sites with shallow pockets proactively in periodontitis patients since these sites already exhibit the subclinical features of diseases, similar to periodontitis sites.

In this thesis, the characteristics of gingivitis across various stages were delineated. Gingival inflammation initiates concomitantly with plaque accumulation, evident during experimental gingivitis induction. In naturally occurring gingivitis lesions, the inflammation proceeds into a more stable form similar to chronic inflammatory lesions. Once the tissue homeostasis is disrupted, gingivitis sites in patients with periodontitis display the features of dysregulated immunity and tissue degradation. Despite similar clinical presentations such as gingival index, bleeding upon probing, and shallow probing depth, gingivitis sites can exhibit distinct profiles of

GCF compositions, indicative of variations in immune and tissue regulatory mechanisms. The overall periodontal conditions at the sites of gingival inflammation significantly influence the maintenance of immune and tissue homeostasis, requiring further exploration.

5.2 Significance

Our results demonstrate that GCF compositions reflect different periodontal disease status, indicating GCF could detect the disease status and changes in a non-invasive approach.. Diagnosis of periodontal diseases currently relies on clinical and radiographic examinations. GCF biomarkers allow detection of subtle alterations before the clinical changes and provide a sensitive tool for early diagnosis and alert of disease progression.

To our knowledge, this thesis is the first study summarizing the features of EG and providing the most comprehensive comparisons between experimental and naturally occurring gingivitis in terms of immune regulation and tissue remodeling. Our results demonstrate that EG is a good tool to investigate the initial inflammatory responses in the periodontium; whereas naturally occurring gingivitis is a better setting to test therapeutic products for their efficacy.

Currently gingivitis is commonly perceived as completely reversible once dental biofilm is eliminated (Trombelli, Farina et al. 2018). However, emerging evidence increasingly suggests that gingivitis may not be entirely resolved following prophylaxis (Syndergaard, Al-Sabbagh et al. 2014, Roberts, Yonel et al. 2022). Our findings align with these insights, indicating that naturally occurring gingivitis may persist due to its chronic nature, characterized by complex immune dysregulation involving neutrophils, monocytes, and macrophages. Individualized immune regulation then plays a pivotal role in disease progression and potential resolution after

plaque removal. In addition to plaque control, the maintenance of immune and tissue homeostasis should be considered in the prevention of further disease progression. Therefore, personalized periodontal therapy tailored to individual variations in immune and tissue regulation is imperative for optimal treatment outcomes.

5.3 Future direction

This thesis builds a foundation for a more complete understanding about those mediators involved in the transition observed in periodontal diseases. Additional investigations based upon this work are described below:

1. **Interaction of these biomarkers**: it remains unclear how these biomarkers orchestrate to maintain immune and tissue homeostasis in the periodontium. With the advent of AI and machine leaning, it is feasible to learn the temporal and spatial relationships among these mediators and their roles in periodontal disease pathogenesis.
2. **The comparison between experimental gingivitis and naturally occurring gingivitis in disease resolution.** Our findings reveal the speed and duration of gingivitis induction may be associated with the composition of immune dysregulation and their fate to chronicity. More exploration during the phase of disease resolution benefits learning more about in the therapeutic targets for periodontal therapy.

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