Chronic periodontitis is associated with platelet factor 4 (PF4) secretion

by

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Abstract

Aim: Platelets contribute to chronic inflammation but their role in periodontitis is not well understood. The aim of this study was to compare platelet recruitment and activation in healthy and inflamed periodontium.

Material and Methods: Gingival crevicular fluid (GCF) samples were obtained from sites of healthy periodontium, gingivitis and periodontitis. Platelets were quantified in the GCF by staining and microscopy. GCF concentrations of platelet factor 4 (PF4) $[PF4]_{GCF}$ and glycoprotein IIbIIIa ($[GPIIbIIIa]_{GCF}$) were determined by ELISA. Blood samples were obtained from the 3 patient groups. Platelets were isolated from the whole blood and stimulated with lipopolysaccharides (LPS) from *P. gingivalis* to evaluate and compare the LPS-induced PF4 release.

Results: Compared to controls, platelet recruitment was increased at gingivitis and periodontitis sites, based on platelet counts and ($[GPIIbIIIa]_{GCF}$). $[PF4]_{GCF}$ was elevated in periodontal pockets but not at gingivitis or healthy sites. Circulating plasma levels of PF4 were higher in patients with generalized severe periodontitis (SP), compared to patients with gingivitis or healthy periodontium. Platelets isolated from SP patients contained and released more PF4 in response to *P. gingivalis* LPS than platelets from gingivitis or periodontally healthy patients.

Conclusions: Periodontitis is associated with increased platelet activation and PF4 release, both locally and systemically.

Preface

This dissertation is an original intellectual product of the author, Mathieu Brousseau-Nault. All research involving human subjects was approved by the Clinical Research Ethics Board (CREB) at the University of British Columbia ("Platelet function in human periodontal disease", certificate number H14-00100; and "Cytokines in human gingival crevicular fluid", certificate number H14-00102). Mathieu Brousseau-Nault and Hugh Kim collected the gingival crevicular fluid (GCF) samples. Mathieu Brousseau-Nault collected all blood samples. Mathieu Brousseau-Nault performed all of the laboratory analysis with the exception of confocal microscopy, which was performed by Hugh Kim.

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List of abbreviations

ANOVA	One-way analysis of variance
APC	Antigen-presenting cells
BOP	Bleeding on probing
CCL	Chemokine C-C motif ligand
CXCL	Chemokine C-X-C motif ligand
DGJ	Dento-gingival junction
ELAM-1	Endothelial cell leukocyte adhesion molecule-1
ELISA	Enzyme-linked immunosorbant assay
FITC	Fluorescin isothiocyanate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
GCF	Gingival crevicular fluid
GPIIbIIIa	Glycoprotein IIbIIIa
HMGB	High mobility group box
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
JE	Junctional epithelium
LPS	Lipopolysaccharide
MGI	Modified Gingival Index
MMP	Matrix metalloproteinase
OCS	Open canalicular system
PAF	Platelet activation factor
PBS	Phosphate buffered saline
PD	Probing depth
PDL	Periodontal ligament
PDGF	Platelet-derived growth factor
PI	Plaque index
PF4	Platelet factor 4
PMN	Polymorphonuclear neutrophils
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of the mean
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Tregs	Regulatory T-cells

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I would first like to thank my supervisor, Hugh Kim, who has inspired me to continue my work in this field. Your guidance was much appreciated.

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To my parents Gilles and Michèle, my godmother Nathalie and godfather Marc, for their unconditional love and support throughout all my studies and my career. You have inspired me to become a better person. Merci.

Dedication

To my sisters Camylle and Alice:

Throughout my life, I have always strove to fulfill my potential in order to become the best person I could be. With this decision, however, came the painful sacrifice of spending less time with you. May my accomplishment be a source of motivation for you, and I hope you can appreciate that each of you can realize whatever dream you cherish, as long as you are ready to put forth the necessary hard work and discipline.

Chapter One: Literature review

Periodontitis: clinical and histological features

Definition, prevalence and significance

Periodontitis, commonly referred to as "gum disease", is a chronic oral infection that is characterized by the degradation of the tooth-supporting tissues, collectively termed the periodontium **(Fig. 1)**. Based on data from the large-scale National Health and Nutrition Examination Survey, periodontitis affects up to half of the adult population of the United States (Eke et al., 2015). Left untreated, the continued destruction of tooth-supporting structures eventually leads to significant morbidity and tooth loss. In addition, considerable evidence indicates that periodontitis confers an increased susceptibility to serious systemic diseases such as diabetes (Taylor et al., 2013) and atherosclerosis (Lockhart et al., 2012; Bartova et al., 2014a) Consequently, there is considerable impetus to improve upon existing diagnostic strategies, risk assessments and therapeutics for this highly prevalent disease.

Anatomy of the healthy periodontium

Teeth are supported by ***bone***, a ***periodontal ligament*** and soft ***gingival*** tissues ***(gingiva)***; these tissues are collectively referred to as the ***periodontium*** **(Fig. 1)**. The aspect of the gingiva facing the oral cavity is the oral epithelium. The cells of the oral epithelium project downwards into the underlying connective tissue; these projections are called rete pegs. The gingiva attaches to the tooth at the ***dento-gingival junction***; this is a critical interface whose integrity is essential for periodontal health. The ***gingival sulcus*** is the crevice between the gingiva and the tooth surface. This space is lined with sulcular epithelium and contains a fluid called gingival crevicular fluid (GCF). The adhesion between gingiva and teeth is mediated by a ***junctional epithelium*** (Nanci

and Bosshardt, 2006). The gingival connective tissue is subjacent to the junctional epithelium and is firmly attached to the root surface by collagen fibers known as Sharpey's fibers. The fibroblast is the predominant cell in the gingival connective tissue; mast cells, macrophages and other inflammatory cells are also present (Lindhe et al., 2008; Golijanin et al., 2015).

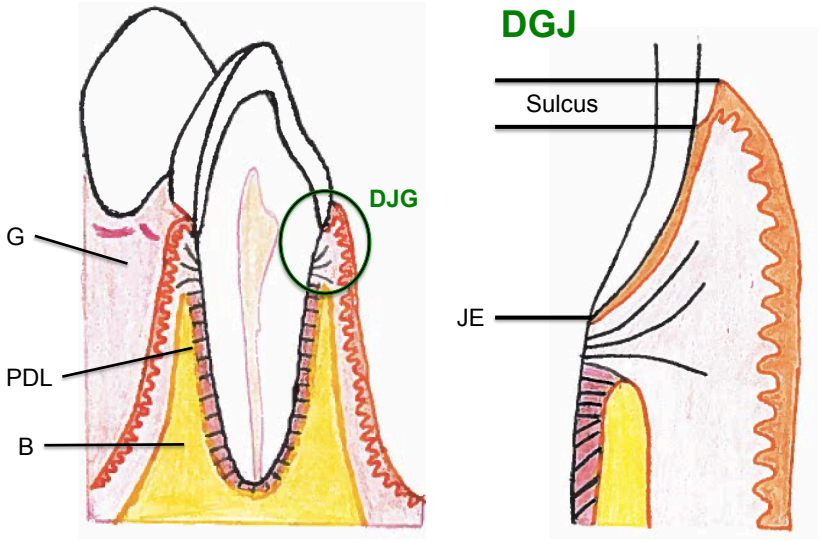

Figure 1. Schematic diagram depicting the structure of the periodontium. *Left panel:* Cross-section of a tooth illustrates the bone **(B)**, the gingiva **(G)** and the periodontal ligament **(PDL)**. The dento-gingival junction is circled in green. *Right panel:* Detailed schematic of the dento-gingival junction (**DGJ**, green circle). The crevice between the tooth and the gingiva is the sulcus; the base of the sulcus is termed the junctional epithelium **(JE)** that mediates gingival adhesion to the tooth.

Clinically healthy gingival tissues have a firm consistency, are coral pink in color and exhibit knife-edge margins **(Fig. 2)**. In addition, periodontal health is quantified by measurements of sulcus depth, or ***probing depth*** **(Fig. 3)**. Sulcus (probing) depths of 1-3 mm are consistent with periodontal health. Conversely, tissue degradation from periodontal disease results in pathologically deepened sulci that are termed ***periodontal pockets*** **(Fig. 3)**.

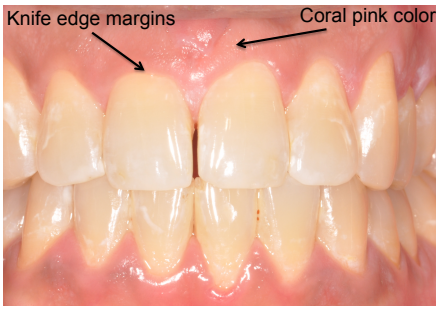

Figure 2. Photograph depicting healthy gingival tissues. Clinically, healthy gingiva is characterized as coral pink, with a firm texture and knife-edge margins (arrows).

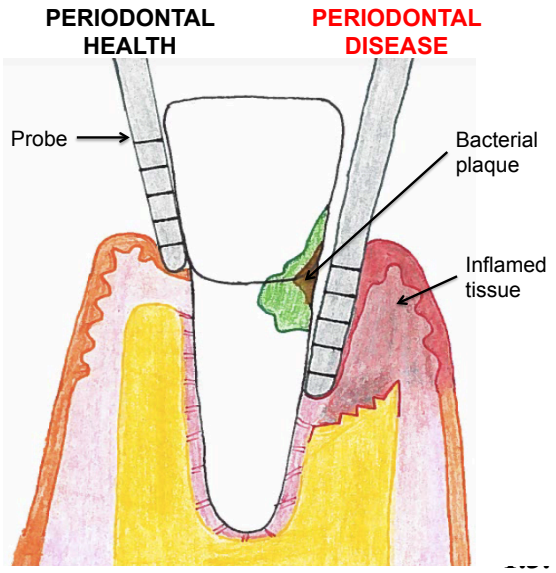

Figure 3. Schematic diagram illustrates periodontal probing as a quantifiable measure of periodontal health. *Left:* healthy gingival sulci are 1-3 mm deep. *Right:* in diseased periodontium, tissue destruction leads to pathologically deepened sulci, called periodontal pockets. Note the bacterial plaque at the dento-gingival junction. Probing depths are recorded at several points **(sites)** around each tooth.

Clinical and histological features of the diseased periodontium

In broad terms, most periodontal diseases are classified as either (1) gingivitis, that is confined to the soft gingival tissues; and (2) periodontitis, characterized by concomitant degradation of the periodontal ligament and alveolar bone (Armitage, 1999). Periodontal diseases are initiated by the accumulation of bacteria-laden dental plaque at the ***dento-gingival junction*** **(Fig. 3)**.

Gingivitis: clinical features

During gingivitis, the tissues exhibit the four (4) cardinal signs of inflammation, namely redness, bleeding, swelling and less commonly, pain (Page and Schroeder, 1976; Armitage, 2004b; Lindhe et al., 2008). A classical study of experimentally-induced gingivitis determined that clinically-observable gingivitis occurs in 10 to 21 days following abstention from oral hygiene measures (Loe et al., 1965).

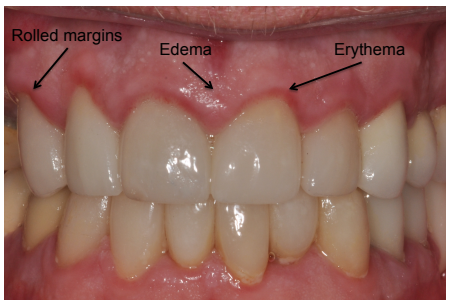

Figure 4. Clinical photograph depicting gingivitis. Gingival inflammation is characterized by erythema (redness), edema and rolled gingival margins (arrows). Clinically detectable gingivitis corresponds with the "early" or "established" gingival lesions described by Page and Schroeder (1976).

Gingivitis: histological features of the *initial lesion*

Page and Schroeder published a classical paper detailing the histopathological features of gingivitis and periodontitis (Page and Schroeder, 1976). The authors divided the gingivitis lesions into 3 distinct phases, describing them as the "initial", "early" and "established" lesions. The clinical and histological features of these lesions are illustrated in **Figure 5** and summarized in **Table 1**. First, the *initial* lesion emerges within 2-4 days of exposure to the dental plaque (Page and Schroeder, 1976) and is a subacute form of gingivitis that is not clinically detectable. Histologically, the initial lesion is characterized by local vasodilation, an increase in vascular permeability, and the influx of polymorphonuclear cells (PMNs) into the junctional epithelium and gingival sulcus. Within the gingival connective tissues, the perivascular collagen is degraded, creating additional space for the influx of fluid and inflammatory cells (Page and Schroeder, 1976).

Gingivitis: histological features of the *early lesion*

After 4-7 days of plaque accumulation, the initial lesion intensifies and becomes known as the *early* lesion (Page and Schroeder, 1976). Continued degradation of the gingival connective tissues results in a 60-70% loss of collagen, thus providing even more space for the inflammatory infiltrate (Page and Schroeder, 1976). In addition to PMNs, the cellular infiltrate of the early lesion contains macrophages and lymphocytes within the gingival connective tissue (Listgarten and Ellegaard, 1973; Payne et al., 1975; Seymour et al., 1983; Brecx et al., 1987). Histologically, there is marked proliferation of both basal cells of the junctional epithelium and the epithelial rete pegs (Schroeder, 1970; Schroeder and Munzel-Pedrazzoli, 1973). During the early lesion, the increased permeability and proliferation of the

vasculature result in clinically detectable gingival erythema and edema (Lindhe and Rylander, 1975).

Gingivitis: histological features of the *established lesion*

The *established* lesion follows from the early lesion and occurs within 2-3 weeks of plaque accumulation (Page and Schroeder, 1976). In contrast to the early lesion, the established lesion is distinguished by the predominance of antibody-producing plasma cells within the connective tissues (Page and Schroeder, 1976). Accordingly, there are large amounts of immunoglobulin present in the connective tissues and in the junctional epithelium (Page and Schroeder, 1976). The lesion is still confined to the gingival connective tissue although there is continued tissue degradation (Page and Schroeder, 1976). There is also ongoing proliferation, apical migration, and lateral extension of the junctional epithelium as rete pegs that extend deeper into the connective tissue (Lindhe et al., 2008). Clinically, the established lesion can manifest as red or cyanotic gingiva with rolled margins. The interdental gingiva (between adjacent teeth) can become bulbous, flattened, blunted or cratered. The established lesion can remain stable and stay confined to the gingival connective tissues for an indefinite period of time; alternatively, the established lesion can convert to an *advanced* lesion characterized by destruction of the underlying periodontal ligament and alveolar bone (Page and Schroeder, 1976).

Periodontitis: the *advanced* lesion

In contrast to the established gingivitis lesion that is limited to the gingival connective tissues, the *advanced* lesion involves degradation of the tooth-supporting alveolar bone and

periodontal ligament; the corresponding clinical condition is termed periodontitis. In addition to the alveolar bone loss that is discernable on dental radiographs, a critical feature of periodontitis is the downward migration of the junctional epithelium onto the root surface (Page and Schroeder, 1976; Armitage, 2004c), thus creating a periodontal pocket that is clinically measurable with a periodontal probe **(Fig. 3)**. As is observed in the established lesion of gingivitis, plasma cells dominate the advanced lesion of periodontitis (Berglundh and Donati, 2005). Eventually, in the more advanced stages of the disease, teeth become mobile due to erosion of bone support, potentially leading to tooth loss.

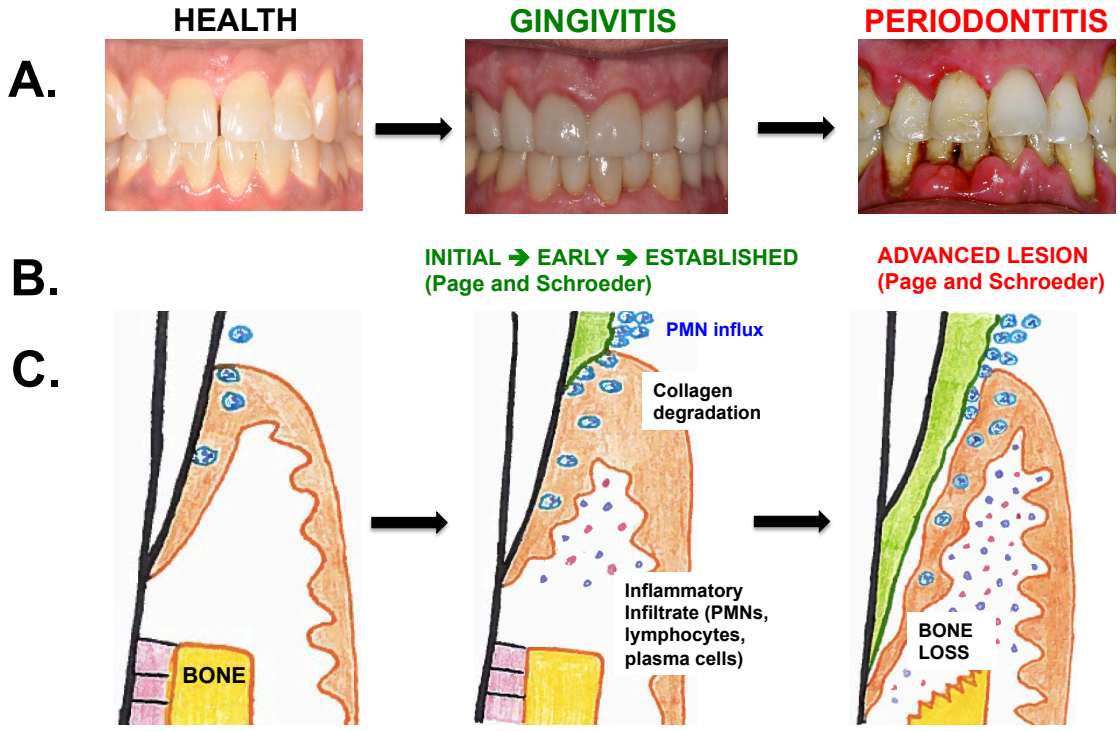

Figure 5. Diagram illustrating the correlation between the clinical presentation **(A)**, the Page and Schroeder classification **(B)** and the histological features **(C)** of periodontal health, gingivitis and periodontitis. The early and established lesions described by Page and Schroeder (1976) correspond with clinically detectable gingivitis in the absence of bone loss; the advanced lesion corresponds to periodontitis featuring bone loss that is visible on a dental radiograph.

Table 1. Summary of the major histological and clinical features of the initial, early, established and advanced lesions described by Page and Schroeder (1976).

	Gingivitis **Initial lesion**	**Gingivitis** **Early lesion**	**Gingivitis** **Established lesion**	**PERIODONTITIS** **Advanced lesion**
Time after plaque accumulation	2-4 days	4-7 days	14-21 days	
Major cells	PMNs	Lymphocytes	Plasma cells	Plasma cells
Major structural changes	Vascular dilation Perivascular collagen loss	Increased collagen loss (70% loss) Loss of coronal portion of JE	Extravascular IgG present JE converts to pocket epithelium	Loss of connective tissue, PDL and alveolar bone Irreversible clinical attachment loss
Clinical findings	Tissues appear clinically healthy	Erythema Bleeding on probing	Marked edema Can remain stable for months/years or convert to advance lesion	Increased probing depth (pocket) Bone loss observed on radiograph and clinically

Clinical progression of periodontal disease

In susceptible hosts, the clinical and histological changes that occur in response to plaque accumulation occur at different rates in different individuals (Loe et al., 1986; Michalowicz, 1994; Kornman et al., 1997). A classical study on the natural history of untreated periodontal disease in humans (Loe et al., 1986) found that 11% of the patient cohort exhibited stable gingivitis lesions that did not progress to periodontitis. Among those who did develop periodontitis, there was considerable variability in the rate of disease progression, with 81% of the patients exhibiting “moderate” progression and 8% of patients exhibiting “rapid” disease progression. This wide variation in the incidence of disease onset (and speed of disease progression) is likely attributable to individual differences in the molecular-level host response to bacterial pathogens (Bartold and Van Dyke, 2013; Silva et al., 2015). However, the precise

signaling mechanisms in the periodontium that regulate the onset and speed of disease progression remain incompletely understood.

Pathogenesis of periodontal diseases

Overview: bacterial plaque and host-mediated tissue destruction

The primary etiology of periodontal disease is the colonization of the dento-gingival junction by gram-negative, anaerobic pathogens present in dental plaque (Loe et al., 1965; Hasan and Palmer, 2014). More than 15 microorganisms have been identified as putative etiologic agents for periodontal disease (1996; Perez-Chaparro et al., 2014). A "red complex" of pathogens has been identified, whose presence in the periodontium is closely correlated with periodontal disease severity (Socransky et al., 1998). The three pathogens in this notorious "red complex", *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, exhibit virulence factors that exert noxious effects on the periodontium (Holt and Ebersole, 2005b). For instance, *P. gingivalis* secretes cysteine proteases known as gingipains that degrade connective tissues (Andrian et al., 2004; Carvalho-Filho et al., 2016). Notably, the bacterial cell walls of gram-negative pathogens contain lipopolysacharide (LPS), which activates the host inflammatory response and initiates periodontal tissue destruction by engaging toll-like receptors (TLRs) on the surface of immune cells in the periodontium, such as neutrophils, monocytes, macrophages and dendritic cells (Nair et al., 1983; Kayal, 2013; How et al., 2016).

Activation of the host response immune signaling cascade ultimately accounts for the bulk of the tissue destruction observed in periodontitis **(Figs. 6-8)**. For example, neutrophils (PMNs) remove bacteria by phagocytosis but also leach proteolytic enzymes that degrade the connective tissue. Moreover, the macrophages, lymphocytes and plasma cells that populate periodontal

lesions communicate through soluble, low molecular weight proteins known as cytokines (Graves, 2008; Deo and Bhongade, 2010). Cytokines serve to recruit additional inflammatory cells to the periodontium, and also signal the release of collagen-degrading enzymes such as matrix metalloproteinases (MMPs) by resident cells of the gingival connective tissues **(Fig. 7)**. Therefore, while the immune response is essential for the host's defense against bacterial infection, an overactive immune response and/or over-secretion of host-derived cytokines exacerbates periodontal tissue destruction.

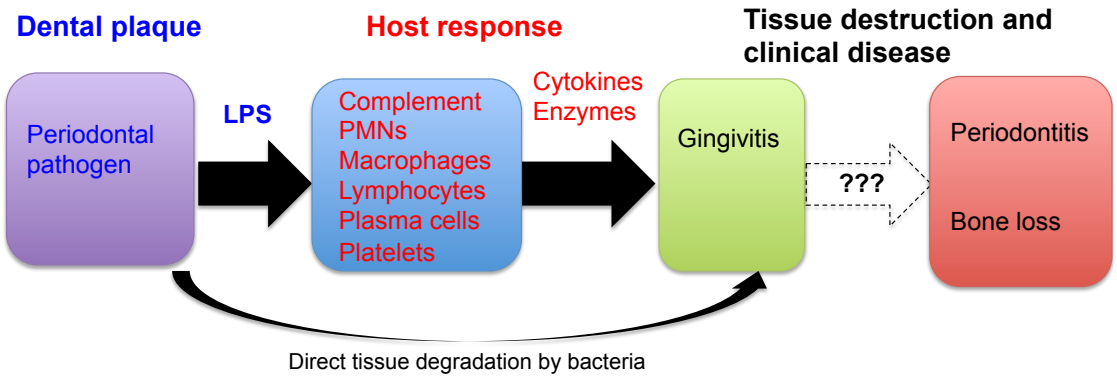

Figure 6. Flow diagram illustrating the relationships between bacterial infection (blue type), the host response (red type), and clinically detectable periodontal disease. The host response encompasses the inflammatory cells recruited to the periodontium and the cytokines/enzymes secreted in response to the bacterial infection. Although bacteria can degrade tissue directly (e.g. gingipains from *P. gingivalis*), the bulk of the tissue destruction is secondary to the host immune response.

Pro-inflammatory cytokine signaling in the periodontium

Initial lesion: Neutrophils (PMNs) are the dominant cell type of the initial lesion. At the outset of periodontal infection, the junctional epithelium **(Fig. 1)** secretes interleukin-8 (IL-8), a cytokine that recruits PMNs to the dento-gingival junction in a process known as chemotaxis (Tonetti et al., 1998). Bacteria also release N-formylmethionine-leucyl-phenylalanine (FMLP), a peptide that is chemotactic for PMNs (Scott and Krauss, 2012). In addition, the complement

system consists of a group of nine (9) proteins whose sequential activation leads to lysis of bacterial cells; one of the complement proteins (C5) is chemotactic for neutrophils (Van Dyke et al., 1983; Hajishengallis, 2010). Once the PMNs are recruited to the site of infection, vascular adhesion molecules such as ELAM-1 facilitate the PMN's exit from the circulation into the tissues (Moughal et al., 1992) through a process known as diapadesis.

While the bactericidal properties of PMNs are central to the innate immune response, prolonged PMN activity and degranulation results in the release of PMN-derived proteases that contribute to periodontal tissue destruction (Ohlsson et al., 1974; Scott and Krauss, 2012).

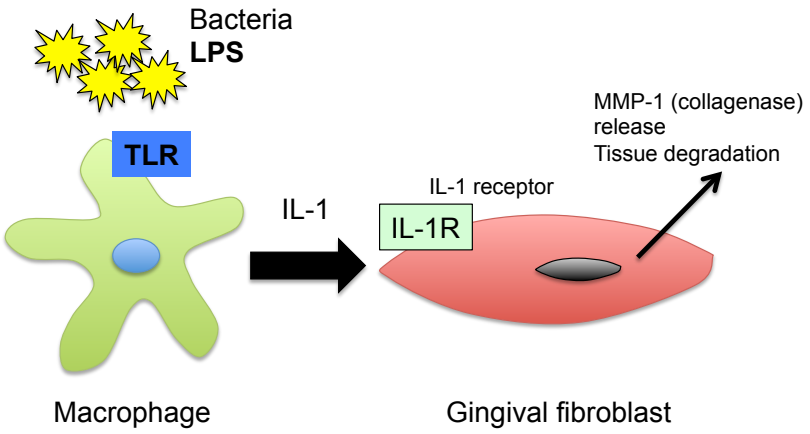

Figure 7. An example of pro-inflammatory cytokine signaling in the periodontium. LPS from gram-negative pathogens activate toll-like receptors (TLRs) on macrophages triggering release of interleukin-1 (IL-1) stimulates the release of tissue-degrading collagenase from resident gingival fibroblasts.

Early and established lesions: The early and established gingivitis lesions are populated by macrophages, lymphocytes and plasma cells. Like PMNs, the activities of macrophages and lymphocytes can propagate periodontal inflammation and tissue degradation. For example, macrophages destroy bacteria by phagocytosis but also secrete interleukin-1 (IL-1), a cytokine that signals the release of MMP-1, a collagen-degrading enzyme, from human gingival fibroblasts (Havemose-Poulsen and Holmstrup, 1997; Domeij et al., 2002) **(Fig. 7)**. Macrophages also release tumor necrosis factor-alpha (TNF-α), a cytokine which promotes the formation of bone-resorbing osteoclasts (Ukai et al., 2008; Hienz et al., 2015).

The interplay between macrophages, lymphocytes and plasma cells in the "established" gingival lesion can be summarized as follows **(Fig. 8)**. After ingesting pathogens, macrophages serve as antigen-presenting cells to helper T-lymphocytes (Sloan-Lancaster et al., 1993) that respond by secreting interleukin-4 (IL-4) and interleukin-5 (IL-5). These cytokines signal the differentiation of B-lymphocytes into antibody-producing plasma cells (Berglundh and Donati, 2005). Antibodies against periodontal pathogens activate the complement system thus facilitating bacterial destruction (Cekici et al., 2014), however, the chemotactic effects of complement serve to recruits additional PMNs whose continued degranulation contributes to tissue degradation.

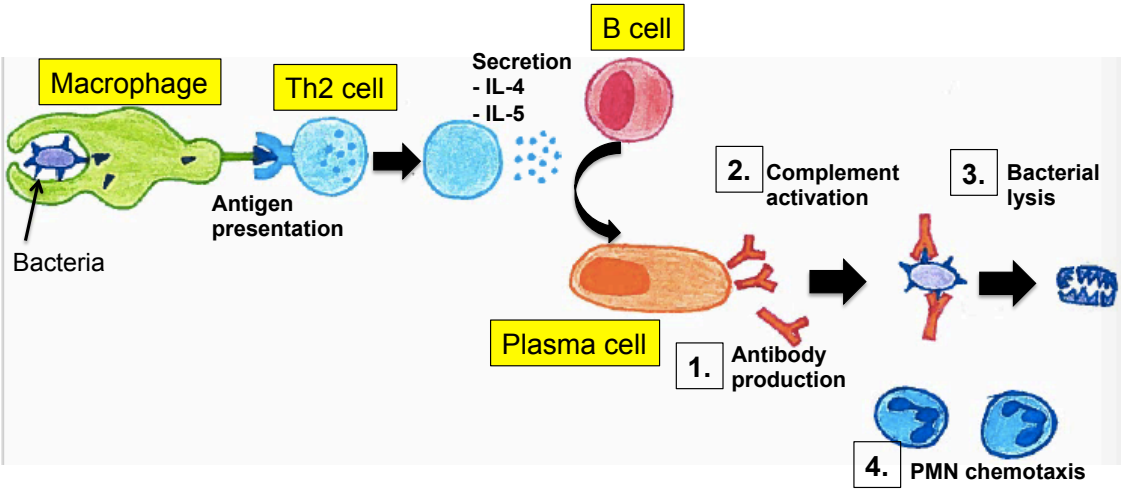

Figure 8. Cytokine signaling in the established lesion. Diagram illustrates crosstalk between macrophages, T-lymphocytes and B-lymphocytes (plasma cells). Macrophages phagocytose bacterial pathogens (e.g. *P. gingivalis*) and function as antigen-presenting cells to Th2 helper lymphocytes, who in turn secrete IL-4 and IL-5, thus signaling the differentiation of B-cells into IgG-producing plasma cells. Antibodies produced **(1)** serve to activate complement **(2)** which facilitates bacterial lysis **(3)** but also promotes PMN chemotaxis **(4)** whose degranulation promotes further tissue destruction in the periodontium.

SUMMARY: Gram-negative periodontal infections trigger a local influx of immune cells (PMNs, macrophages, T-lymphocytes, B-lymphocytes), and activation of the complement cascade. These responses can be both protective and destructive for the periodontium. The functions of these immune cells in the periodontium are controlled by complex (and incompletely understood) cytokine signaling networks that determine an individual's disease susceptibility and rate of disease progression.

Platelets as inflammatory cells

The roles played by PMNs, macrophages and lymphocytes (and their cytokines) in orchestrating and propagating periodontal inflammation have been extensively reported in the scientific literature (Yucel-Lindberg and Bage, 2013; Hajishengallis, 2014; Hajishengallis et al., 2014; Hajishengallis and Sahingur, 2014; Silva et al., 2015). In contrast, much less is known about how platelets contribute to periodontal disease pathogenesis. Platelets are small anucleate blood cells approximately 1-3 μm in diameter, and are released into the bloodstream by megakaryocytes in the bone marrow. In their quiescent state, platelets circulate as small discs for 8-9 days prior to being cleared by the liver. Platelets are by far the most numerous cells in the bloodstream, circulating at concentrations of 150,000-450,000 cells per microliter of blood.

Platelet structure, activation and secretion

Like other eukaryotic cells, platelets have a membrane-bound cytoplasm containing organelles including a Golgi apparatus and rough endoplasmic reticulum (Ts'ao, 1971; Lu et al., 2013). The plasma membrane contains multiple cell surface receptors **(Fig. 9)** that recognize extracellular signaling molecules, or ligands (Saboor et al., 2013). A unique feature of platelets is that they contain three types of secretory granules: the alpha-granules, the dense granules and the lysosomes (Gremmel et al., 2016). Upon activation by soluble agonists or vascular injury, resting platelets rapidly change shape, aggregate and secrete the contents of their granules **(Fig. 9)**. Notably, the plasma membrane is continuous with an extensive invaginated network of membranes, collectively known as the open canalicular system, which opens to the extracellular space, thus providing a conduit for the release of granular content (White and Krumwiede, 1987).

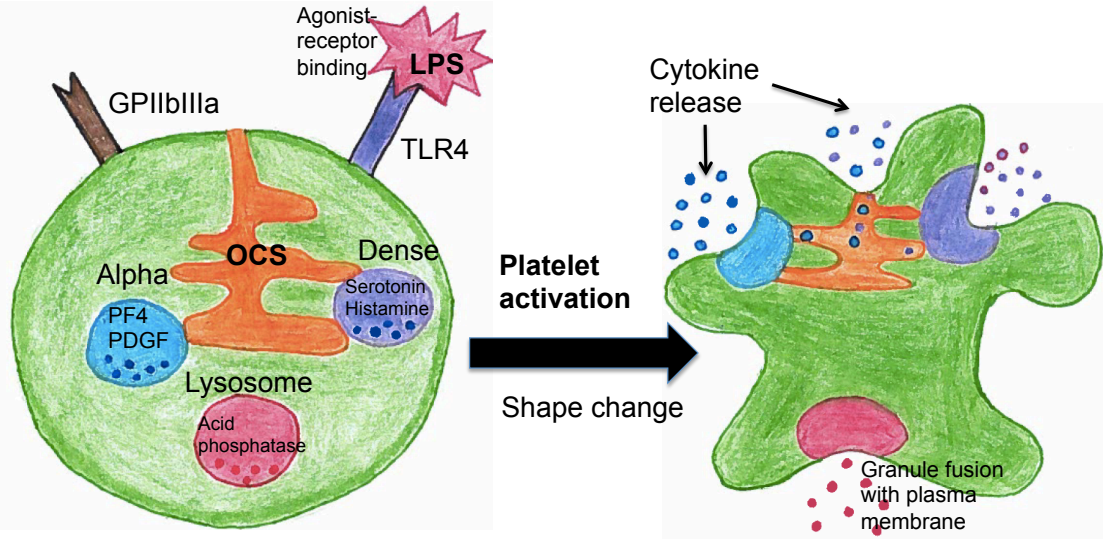

Figure 9. Schematic diagram of platelet activation. *Left:* resting platelets store biological mediators in 3 principal types of granules: α-granules, dense granules and lysosomes. The plasma membrane is contiguous with a complex of invaginated membranes known as the open canalicular system (OCS). Cell surface receptor TLR4 on platelets recognizes LPS from periodontal pathogens. *Right:* Upon binding of receptors (e.g. TLR4) with a soluble agonist (e.g. LPS), platelets become activated and change shape. Activation is accompanied by fusion of the granule membranes with the plasma membrane and OCS and extracellular relase of cytokines.

Platelets in chronic inflammatory diseases

Platelets are best known for their functions in hemostasis and thrombosis. Platelet granules store and release over 30 different cytokines, many of which have documented roles in inflammation **(Table 2)**. Accordingly, a growing body of evidence suggests that platelets are important contributors to chronic inflammation. For example, arthritic disease is milder in thrombocytopenic mice compared to controls (Semple et al., 1996). Humans affected by rheumatoid arthritis show elevated platelet activity *in vitro* (Mac Mullan et al., 2010) and also exhibit elevated plasma levels of soluble P-selectin (Sfikakis et al., 1999) and soluble CD40 ligand (Goules et al., 2006), two molecules released by activated platelets. Similarly, platelet activity is elevated in patients with inflammatory bowel disease (Danese et al., 2004; Yoshida

and Granger, 2009; Scaldaferri et al., 2011; Stadnicki, 2012). Collectively, these data support the association between platelet activation and chronic inflammation.

Platelets in periodontal disease: indirect evidence

Recently published data point to a general association between platelet activity and periodontitis. In an experimental periodontitis study in rats, systemic administration of anti-platelet drugs attenuated the severity of experimentally-induced bone loss while reducing circulating levels of the pro-inflammatory mediators interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Coimbra et al., 2011). In a separate animal study, the same authors found that systemic administration of the antiplatelet drug clopidogrel reduced neutrophil infiltration at sites of experimental periodontitis (Coimbra et al., 2014), suggesting that platelets have a net pro-inflammatory function. In humans, platelet counts are reportedly higher in patients with chronic periodontitis versus controls (Al-Rasheed, 2012a). Moreover, platelets from periodontitis patients are more responsive to oral bacteria than platelets from control subjects (Nicu et al., 2009a). The same group studied plasma levels of platelet-derived molecules in periodontitis patients (Papapanagiotou et al., 2009). They reported that periodontitis was associated with increased plasma levels of soluble P-selectin. Taken together, these data are consistent with the notion that periodontal inflammation is directly correlated with systemic platelet activity.

Platelet factor 4 (PF4)

The pro-inflammatory properties of platelets are perhaps most likely attributable to platelet factor 4 (PF4), an 8-kDa platelet-specific cytokine that is the major constituent stored and released from platelet alpha-granules. PF4 accounts for approximately 25% of the platelet's protein

content (Zucker and Katz, 1991). Significant evidence points to PF4 as a pro-inflammatory cytokine. For example, PF4 stimulates neutrophil degranulation (Bebawy et al., 1986) and is also chemotactic for monocytes and promotes their differentiation into macrophages (Scheuerer et al., 2000). Moreover, PF4 appears to nullify the anti-inflammatory properties of regulatory T-lymphocytes (Tregs) (Liu et al., 2005). In addition, PF4 is implicated in the pathogenesis of several chronic inflammatory diseases, including atherosclerosis (Aidoudi and Bikfalvi, 2010b), suggesting that PF4 is the cytokine that drives the pro-inflammatory effects of platelets. However, the role of PF4 has not been specifically evaluated in the context of periodontal disease.

Evaluating a direct role of platelets in the periodontal disease

Despite indirect evidence of the contribution of platelets to periodontal inflammation, there have been no direct studies of platelet recruitment, activation and secretion in the periodontium. Since platelets, like neutrophils, are chemotactically responsive to bacteria-derived FMLP (Czapiga et al., 2005), it is plausible that platelets are recruited to the inflamed periodontium. Conceivably, platelets recruited to the periodontium are readily activated by bacterial LPS since platelets express toll-like receptor 4 (TLR4), the receptor for bacterial LPS (Zhang et al., 2009a). These data support a biologically plausible mechanism by which platelets could contribute to periodontal inflammation. A logical approach to evaluating platelet recruitment and activity in the periodontium would be to study platelet-specific markers in the gingival crevicular fluid (GCF), a secretion obtained from the microenvironment of the dento-gingival junction.

Table 2. Partial list of platelet-derived cytokines, their cellular location, known physiological effects and (possible) role in periodontal inflammation.

Location in platelet	Cytokine	General role	Potential relevance to the periodontium	References
Alpha Granule	PF4	Activates monocytes	Promotes inflammation	(Engstad et al., 1995)
	RANTES	Attracts and activates macrophages and lymphocytes	Increase recruitment of inflammatory cells	(Gamonal et al., 2000b)
	IL-8	Chemoattractant cytokine for neutrophils	Increases neutrophil influx	(Bickel, 1993)
	PDGF	Improves soft tissue healing	Promotes regeneration	(Lynch et al., 1989)
	IGF	Reduces apoptosis in PDL fibroblasts	Promotes regeneration	(Han and Amar, 2003)
	TGF-β	Promotes matrix formation	Stimulates collagen synthesis	(Sodek and Overall, 1992)
	Thymosin-B4	Promotes wound healing	Diminishes inflammation	(Sosne et al., 2010)
	Epithelial growth factor	Promotes stimulation PGE2	Promotes bone resorption	(Carpenter and Cohen, 1979; Chang et al., 1996)
	Hepatocyte growth factor	Promotes epithelial invasion	Contributes to periodontal disease progression	(Gherardi and Stoker, 1991; Rudrakshi et al., 2011)
	CCL3	Stimulates osteoclast differentiation	Promotes bone resorption	(Watanabe et al., 2004)
	CXCL1	Chemotactic for neutrophils, supports PMNs adhesion to endothelial cells	Promotes periodontal disease progression	(Bender et al., 2008)
	CXCL5	Chemotactic for PMN	Increase neutrophil influx	(Leppilahti et al., 2014)
	Thrombocidins	Antibacterial peptides	Promotes pathogen destruction	(Clemetson, 2011; Semple et al., 2011)
Dense Granule	Serotonin	Stop osteoblast proliferation	Disrupts bone homeostasis	(Carvalho et al., 2010)
Lysosome	Collagenase	Collagen degradation	Destruction of connective tissue	(Mancini et al., 1999)
	Elastase	Involved in destruction of PDL	Promotes progression of periodontitis	(Ujiie et al., 2007)

Lysosome	Cathepsin	Proteolytic activities	Promotes tissue destruction	(Soell et al., 2002)
Not known	CCL7	Chemotactic to monocytes, activates T-lymphocytes, eosinophils and basophils	Promotes inflammation	(Ong et al., 2003; Wetzel et al., 2007)
	IL-1β	Promotes bone resorption, stimulates secretion of PGE2 and MMP	Promotes periodontal breakdown	(Gamonal et al., 2000a)
	HMGB1	Activate monocytes, releases pro-inflammatory mediators	Promotes periodontal tissue destruction	(Luo et al., 2011)
Plasma membrane	PAF	Promotes leukocytes degranulation	Promotes inflammation and tissue destruction	(Emingil et al., 2001a; Chen et al., 2010)
	CD40	Upregulates inflammatory response in numerous cells	Promotes inflammation	(Noelle, 1996; Chaturvedi et al., 2015)

Gingival crevicular fluid (GCF)

Overview

As noted earlier, the dento-gingival junction harbors a gingival crevicular fluid (GCF) that flows continuously through the sulcus **(Fig. 10)**. The GCF is a transsudate originating from the blood vessels in the gingival connective tissues and contains soluble proteins secreted by local host inflammatory cells (Delima and Van Dyke, 2003). Leukocytes, predominantly neutrophils, are the most abundant inflammatory cells in the GCF (Egelberg, 1963; Attstrom, 1970; Skapski and Lehner, 1976; Wilton et al., 1976; Kowolik and Raeburn, 1980; Sandholm, 1984; Saito et al., 1987; Kennett et al., 1997). The GCF serves to dilute bacterial toxins (Goodson, 2003) and also contains antibacterial proteins such as complement proteins, immunoglobulins, lactoferrin and alpha-1 antitrypsin (Kido et al., 2012; Huynh et al., 2015). Moreover, the outward flow of GCF

helps to physically expel bacteria from the sulcus (Frank and Cimasoni, 1972; Charon et al., 1982; Delima and Van Dyke, 2003).

The flow rate and composition of GCF reflect the health of the immediate periodontal microenvironment. For example, the GCF flow rate is ~30-fold higher in periodontal pockets compared to healthy sulci, suggesting that GCF flow increases as a host defense mechanism in response to a greater bacterial load (Goodson, 2003). The molecular constituents of the GCF have been extensively studied as potential markers of periodontal health or disease (McCulloch, 1994; Barros et al., 2016). Previous studies have correlated periodontal disease severity with GCF levels of collagenase (Golub et al., 1976), aspartate aminotransferase, a marker for cell death (Sheth and Verma, 2011) and beta-glucoronidase, a marker for neutrophil degranulation (Sanara et al., 2015). However, further research is required to achieve the still-elusive goal of identifying biomarkers with diagnostic or prognostic value in periodontitis (Armitage, 2004a), for example, a GCF-based marker expressed specifically in patients at risk for disease progression.

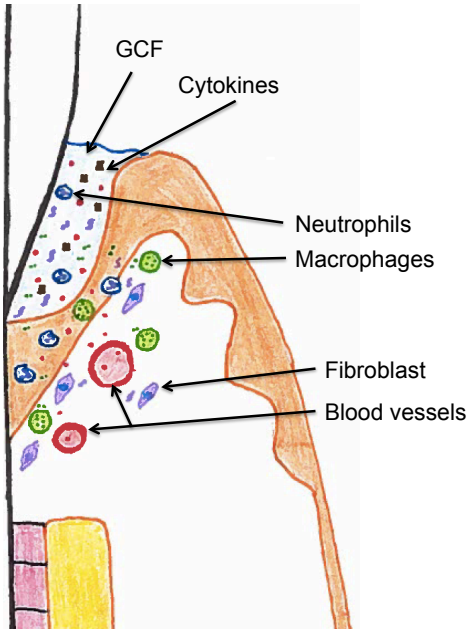

Figure 10. Schematic diagram illustrates the origin of gingival crevicular fluid (GCF) and its constituents. Cytokines released by immune cells (e.g. neutrophils and macrophages) recruited to the dento-gingival junction are detectable in the GCF.

GCF collection methods

The analysis of gingival crevicular fluid (GCF) is a well-established research tool that is reported in over 3000 published articles dating back to 1960. Importantly, GCF is readily collected from clinically healthy gingiva as well as periodontal pockets (Alfano, 1974). Three (3) different methods for GCF collection are described in the literature: (1) gingival washing; (2) collection of GCF on to absorbent filter paper strips; and (3) the use of micropipettes (Griffiths, 2003). The three methods are summarized in **Table 3**.

Table 3. Comparison of three methods for gingival crevicular fluid (GCF) collection.

	Gingival washing	**Paper strips**	**Micropipettes**
Method	The sulcus is washed with an isotonic solution	A paper strip is placed at or inside the entrance of sulcus	A micropipette is placed at or inside sulcus entrance; GCF migrates up the tube by capillary action
Advantage(s)	Allows maximal harvesting of cells	Ease of sample collection	Ease of sample collection Pure, undiluted GCF
Disadvantage(s)	Elaborate and time-consuming GCF fluid is diluted Site-based analysis is impossible	Proteins must be eluted from paper strip Protein binding to strip Apparatus (Periotron) required to calculate GCF volume (cost)	Centrifugation is necessary for sample recovery

Intracrevicular washing method

To collect GCF by washing, the sulcus or pocket is perfused with an isotonic solution, such as Hank's balanced salt solution. Oppenheim described a method for gingival washing that

employs a custom-made hard acrylic appliance to isolate the gingival tissues from the rest of the mouth (Oppenheim, 1970). A peristaltic pump is then used to rinse the crevicular area from one side to the other for 15 minutes. This method allows for maximal harvesting of non-adherent cells, in addition to soluble GCF components. However, the complexity of this procedure is a major disadvantage. In addition, the washing method collects GCF across multiple sites simultaneously, thus precluding accurate analysis of the GCF composition at individual sites.

Absorbent filter paper strips

A considerably faster and simpler method for GCF collection involves the use of absorbent filter paper strips inserted to the base of the pocket or until "minimal resistance is felt" (Brill, 1962). A modification of this method involves placing the paper strip just at the entrance of the crevice to minimize irritation of the sulcus (Loe and Holm-Pedersen, 1965). While GCF collection by paper strips is technically straightforward, the fluid and associated analytes must then be eluted off of the paper strip in a separate step. It should also be noted that the GCF proteins may become permanently bound to the paper strips (Johnson et al., 1999). Moreover, calculating the concentration of specific molecules in a given sample requires an accurate determination of the sample volume. This is achievable with the Periotron®, an electronic device that calculates the GCF sample volume based on measurements of the electrical capacitance of the GCF-embedded paper strip (Ciantar and Caruana, 1998). While the Periotron® allows for accurate determinations of GCF volume (Golub et al., 1984; Almas et al., 2003; Mokeem et al., 2014), the cost of this additional armamentarium may present a disadvantage for some investigators.

Capillary tubing or micropipettes

A third method for collecting GCF involves the use of glass micropipettes or capillary tubes. The micropipette is placed at the entrance of the gingival crevice, allowing the GCF to migrate up into the tube by capillary action **(Fig. 11)**. In contrast to the other 2 methods described, the use of micropipettes yields an undiluted GCF sample that does not require elution from any surface. The use of tubes of known internal diameter and length allow for accurate determinations of GCF volume (Mann, 1963; Kaslick et al., 1968) by simply measuring the distance migrated by the GCF **(Fig. 11)** (Sueda et al., 1969). Since the length and total volume capacity of the tube are known, one can then accurately calculate the sample volume (Griffiths, 2003). The GCF is recovered from the micropipette by centrifugation into a clean microfuge ("Eppendorf") tube.

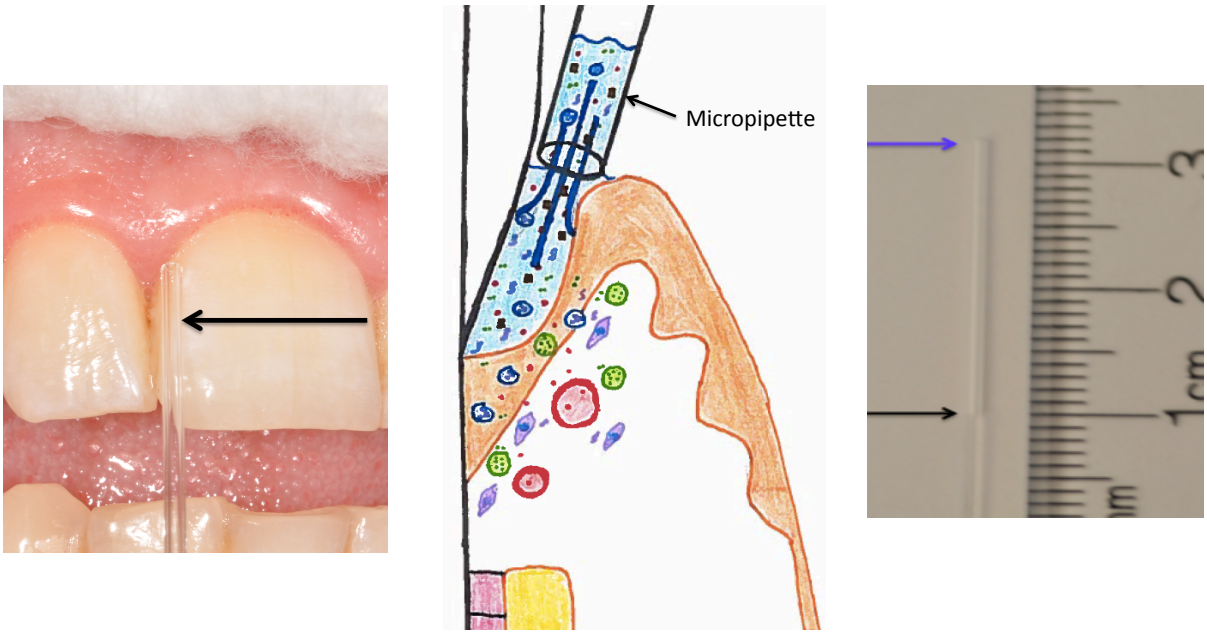

Figure 11. *Left:* photograph depicting GCF collection by the micropipette method. *Center:* illustration depicts the migration of GCF up the pipette (arrow) via capillary action. *Right:* the volume of GCF collected is calculated based on the length of fluid migration into the tube of known diameter.

Caveats of GCF sample collection

The three major sources of contamination while collecting GCF are blood, saliva and dental plaque (Griffiths, 2003). Samples visibly contaminated with blood **(Fig. 12)** are normally discarded. Saliva contamination can be prevented by isolating the sites to be sampled with cotton rolls, and gently air-drying the area **(Fig. 12)**. As an additional quality control measure against saliva contamination, GCF samples can be analyzed for the presence of the salivary enzyme alpha-amylase (Griffiths et al., 1992). Finally, fluids present in dental plaque can also contaminate GCF samples (Stoller et al., 1990; Griffiths et al., 1992; D'Aoust and Landry, 1994) Supragingival dental plaque should therefore be gently removed with a curette prior to sampling **(Fig. 12)**.

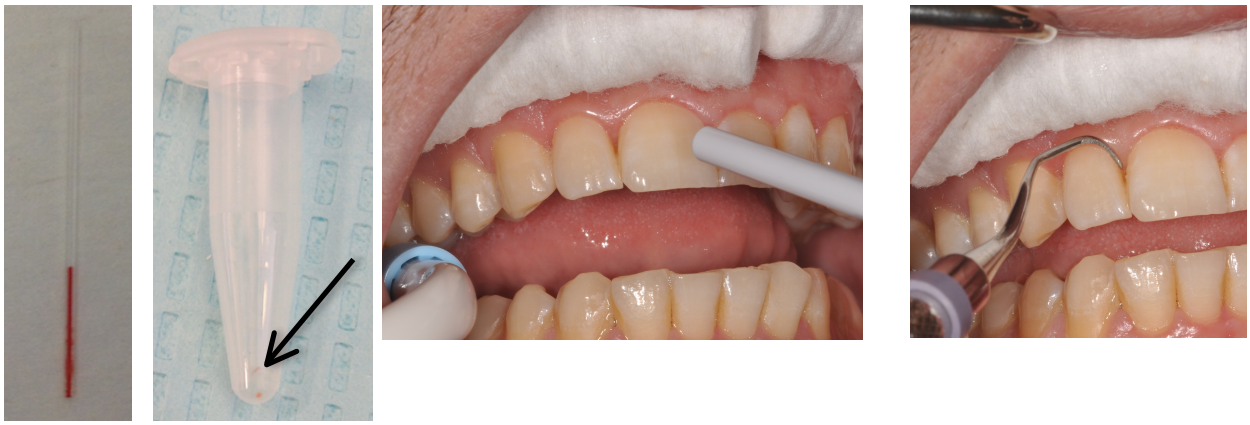

Figure 12. *Left:* Blood contamination is typically obvious at the time of collection; samples should also be double-checked prior to laboratory analysis (arrow). *Center:* To prevent saliva contamination, sites to be sampled are isolated with cotton rolls and suction. *Right:* Supragingival plaque is gently removed with a curette.

Since GCF collection typically yields small volumes from any single site, sampling times are sometimes extended to amplify the yield of GCF (Griffiths, 2003). However, the prolonged presence of a sampling device in the gingival sulcus can introduce artifacts into the GCF (Curtis

et al., 1988). For example, the abnormal presence of immunoglobulins (IgM) was detected in GCF samples that were harvested over long periods of time (Griffiths et al., 1997). The authors concluded that the prolonged sampling induces an inflammatory response, resulting in the influx of IgM into the GCF. Therefore, GCF sampling times should be minimized to avoid introducing artifacts into the GCF sample. Fortunately, the availability of highly sensitive laboratory assays can largely compensate for limitations imposed by small sample volumes.

Laboratory methods for GCF analysis

The protein constituents of the GCF can be quantified by Western blotting, also known as immunoblotting (Overall et al., 1991; Ingman et al., 1996; Golub et al., 1997; Kiili et al., 2002; Soell et al., 2002; Euzebio Alves et al., 2013). Western blotting is a universally-employed molecular biology technique used to detect proteins within a biological mixture, such as a cell lysate or GCF. Proteins in the GCF sample are separated based on size by electrophoresis and transferred (blotted) on to a membrane that is then probed with an antibody against the target protein (Mahmood and Yang, 2012). Although inexpensive, the relative lack of sensitivity of this method is a significant disadvantage when detecting proteins in low-volume GCF samples.

A much more sensitive method to quantify GCF proteins is the enzyme-linked immunosorbent assay, or ELISA (Lee et al., 1995; Ingman et al., 1996; Engebretson et al., 1999; Gamonal et al., 2000a; Mogi et al., 2004; Bostanci et al., 2007; Gan and Patel, 2013). In this method, GCF samples are incubated on surfaces pre-coated with an antibody against the target protein (Gan and Patel, 2013). The bound antigen-antibody complexes emit a colorimetric signal whose intensity is directly proportional to the concentration of the antigen in the GCF sample.

The high sensitivity of this assay allows the precise quantification of GCF proteins, even those present in low amounts (Gan and Patel, 2013).

Statement of the problem, aim and hypothesis

STATEMENT OF THE PROBLEM: The role of platelets in the pathogenesis of periodontal disease is undefined, despite considerable evidence that platelets contribute to chronic inflammation.

Aim: Evaluate the association between platelets and periodontal inflammation by comparing platelet recruitment and activation in healthy and inflamed periodontium.

Hypothesis and rationale:

I hypothesized that local *and* systemic concentrations of platelet factor 4 (PF4) would be elevated during periodontal inflammation. The previously documented pro-inflammatory functions of PF4, combined with its specificity to platelets, made PF4 an ideal choice as a marker of platelet activation. This study is the first to directly evaluate the role of a platelet-specific cytokine in the context of human periodontitis, from both a local and systemic perspective.

Summary of key findings:

I studied gingival crevicular fluid (GCF) and platelet samples obtained from patients with gingivitis, periodontitis as well as healthy controls. I report that both gingivitis and periodontitis are characterized by increased platelet recruitment to the periodontium. Moreover, PF4 is markedly more abundant in the GCF of periodontitis sites, but not in the GCF of gingivitis or healthy sites. Further, platelets isolated from periodontitis patients store and secrete more PF4 in response to stimulation with LPS from the periodontal pathogen *Porphyromonas gingivalis*.

Chapter Two: Chronic periodontitis is associated with platelet factor 4 (PF4) secretion

Materials and methods

Recruitment of human subjects for collection of gingival crevicular fluid (GCF)

All subjects were recruited from the clinics at the Faculty of Dentistry, University of British Columbia and from local private dental practices (North Vancouver, BC and Surrey, BC, Canada). Approval for GCF sample collection was obtained from the University of British Columbia Clinical Research Ethics Board (CREB). Written informed consent was obtained from each subject before enrolment in the study. To be included, participants needed to be in good general health. Exclusion criteria included smoking, systemic diseases, pregnancy, the use of nonsteroidal inflammatory drugs, the use of systemic antibiotics in the past 6 months and periodontal therapy in the past 6 months. Human donors of gingival crevicular fluid (GCF) were recruited separately from blood donors.

Clinical parameters for collection of gingival crevicular fluid (GCF)

Clinical data was obtained from potential sampling sites according to three parameters: (1) the degree of gingival inflammation, based on the visual Modified Gingival Index (MGI) scoring system (Lobene et al., 1989); (2) the presence or absence of bone loss, as determined from dental radiographs; and (3) probing depth (PD), the distance between the gingival margin and the base of the periodontal pocket or sulcus. Based on these data, sites for GCF harvesting were classified as follows:

1. Periodontally healthy: sites with no sign of gingival inflammation (MGI score=0), no radiographic evidence of alveolar bone loss and PD≤3 mm.

2. Gingivitis: sites with visible gingival inflammation (MGI≥1) but no radiographic evidence of alveolar bone loss and PD<5 mm.
3. Periodontitis: sites with periodontal pocketing (PD≥5 mm) and alveolar bone loss.

Collection of gingival crevicular fluid (GCF)

For GCF collection, the initial site selection was based on visual clinical assessment and information obtained from existing patient records **(Fig. 13)**. To avoid trauma to the periodontal tissues, we refrained from probing the sites prior to GCF harvesting. The previously documented pocket depths were verified by probing the sites after GCF collection. All sampling sites were isolated with cotton rolls to prevent saliva contamination. Supra-gingival plaque was gently removed and the area was dried with a gentle air stream. GCF was collected via capillary action using a microcapillary tube (Drummond Scientific, Broomall, PA). The microcapillary tube was inserted less than 1 mm into the gingival crevice to minimize tissue irritation. Samples with visible blood or plaque contamination were discarded. Immediately following the GCF collection, the probing depths of the corresponding sites were re-measured to ensure appropriate sample classification. The volume of GCF collected was calculated by measuring the length of the microcapillary tube occupied by GCF. The GCF was removed from the microcapillary tube by centrifugation and diluted at a 1:50 ratio with phosphate buffered saline (PBS) supplemented with a protease inhibitor cocktail. Samples were stored at -20^{o}C until analysis. The samples were visually re-inspected for contamination just prior to laboratory analysis. The absence of blood contamination by visual assessment was validated using an enzyme-linked immunosorbent assay (ELISA) for hemoglobin content (Abcam, Cambridge, MA, USA).

GCF analysis

To quantify local platelet recruitment in the periodontium, cells in the diluted GCF samples were fixed with 2% paraformaldehyde and stained for 30 minutes with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD42a (Beckman-Coulter, Mississauga, ON). The GCF suspension was centrifuged onto a cytospin preparation and analyzed with a Zeiss spinning disk confocal microscope. Confocal images were processed using SlideBook software (Intelligent Imaging Innovations, Denver, CO). For each sample, platelets were counted in at least 15 fields of view. To determine the concentrations of platelet glycoprotein IIbIIIa (GPIIbIIIa) and platelet factor 4 (PF4) in the GCF samples, enzyme-linked immunosorbent assay (ELISA) kits were used in accordance with manufacturer's instructions (Abcam, Cambridge, MA, USA and R&D Systems, Minneapolis, MN, USA).

Recruitment of blood donors and clinical parameters for blood collection

Peripheral venous blood was obtained from fifteen (15) systemically healthy dental patients with approval obtained from the University of British Columbia Clinical Research Ethics Board (CREB) and informed consent in accordance with the Declaration of Helsinki. The subjects were classified as follows:

1. Healthy periodontium (n=7): patients showed no sign of gingival inflammation (MGI score=0) and no radiographic evidence of alveolar bone loss.
2. Gingivitis (n=4): patients with visible gingival inflammation (MGI≥1) but no radiographic evidence of alveolar bone loss and no PD>4 mm.
3. Generalized severe periodontitis (n=4): patients with clear radiographic evidence of alveolar bone loss and periodontal pocketing (PD≥5 mm) at a minimum of 30% of sites.

The exclusion criteria that were applied to GCF donors were also applied to potential blood donors. The detailed periodontal characteristics of the blood donors are summarized in **Table 4**.

Human platelet preparation

Platelets were isolated by sequential centrifugation of whole blood and the resultant platelet-rich plasma. The platelet pellet was washed twice with a washing buffer (140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6.0) supplemented with prostaglandin-1 (Sigma). Washed platelets were resuspended in resuspension buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, pH 7.4) containing apyrase (Sigma) and allowed to rest for 30 minutes at 37^{o}C prior to use.

Measurement of LPS-induced release of PF4

For quantification of PF4 release, resting platelets were activated over a time course ranging from 30 seconds to 30 minutes with lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (Invivoven, San Diego, CA). The reactions were stopped by centrifugation at 4^{o}C, and the ensuing pellet (cell fraction) and supernatants (releasate fraction) were separated. The corresponding pellets were solubilized in lysis buffer. The concentrations of PF4 in the cell and releasate fractions were determined by ELISA as described above.

Statistical analysis

A one-way analysis of variance (ANOVA) and Bonferroni post-hoc multiple comparison tests were used to assess the effect of periodontal status (healthy, gingivitis, periodontitis) on the various tested parameters. Statistical significance was set at $p<0.05$.

Results

To evaluate the local recruitment of platelets to the periodontium, we analyzed gingival crevicular fluid (GCF) samples, which is a well-established approach to analyzing cells and host- and pathogen-derived products released in the periodontium (Delima and Van Dyke, 2003; Armitage, 2004a; Buduneli and Kinane, 2011). To exclude the possibility of blood contamination, any visibly blood-contaminated GCF samples were discarded. As an additional quality control measure, we validated our visual assessment by quantifying hemoglobin levels (ELISA, Abcam) in GCF samples with and without visible blood contamination. Visibly contaminated GCF samples had a mean hemoglobin concentration of 133.9 ± 2.88 ng/mL (mean ± SD, n=6) but there was no detectable hemoglobin in the samples that passed the visual assessment test. Similarly, we used a colorimetric amylase detection kit (Abcam) to objectively exclude the possibility of saliva contamination in the GCF. Amylase levels were 26.5 ± 2.88 nmol/well (mean ± SD, n=6) in saliva-spiked GCF samples but was not detectable in samples collected with cotton roll isolation.

Confocal microscopy of cytospin preparations revealed the presence of platelets in the gingival crevicular fluid (GCF) obtained from healthy control sites **(Fig. 14A)**, sites with gingivitis **(Fig. 14B)** as well as periodontal pockets **(Fig. 14C)**. Microscopic counts of platelet numbers showed that platelets were ~3 times more abundant in gingivitis sites relative to healthy control sites ($p<0.0001$), and were further elevated in periodontal pockets **(Fig. 14D)**. As a complementary approach to platelet quantification, we used ELISA to quantify the platelet-specific glycoprotein

IIbIIIa (GPIIbIIIa) receptor on the platelet surface (Watson et al., 2005). There was a ~3-fold elevation ($p<0.0001$) in the concentration of GPIIbIIIa in sites with gingivitis (n=34) compared to healthy control sites (n=29). As was observed in the cytospin preparations, the highest levels GPIIbIIIa were found in periodontal pockets (n=22) **(Fig. 14E)**. The concordant results from these 2 assays indicate that platelets are recruited to sites of periodontal inflammation.

To assess the activation status of platelets recruited in the periodontium, we quantified the concentration of platelet factor 4 (PF4) in GCF samples obtained at sites with healthy periodontium, gingivitis and periodontitis. There was no difference ($p>0.05$) in the concentration of PF4 between healthy control sites and gingivitis sites **(Fig. 15A)**. However, periodontal pockets harbored ~20 times more PF4 than GCF from the other 2 groups ($p<0.0001$) **(Fig. 15A)**. We then stratified these data according to different ranges of PF4 concentration $[PF4]_{GCF}$ **(Fig. 15B)**. Notably, none of the periodontally healthy (n=25) and gingivitis sites (n=20) had a $[PF4]_{GCF}$ in excess of 0.5 ng/mL. In contrast, the $[PF4]_{GCF}$ exceeded 0.5 ng/mL in 59% of periodontal pockets **(Fig. 15B)**. These data indicate that platelets are preferentially activated during periodontitis but not gingivitis, despite being recruited in comparable numbers in both disease states.

Since PF4 levels were locally elevated at individual periodontal pockets, we then set out to determine whether platelets would be functionally different in patients with generalized severe chronic periodontitis (SP), compared to controls. The circulating concentration of PF4 was ~2 times higher ($p<0.05$) in patients with generalized SP, compared to patients in the periodontally healthy and gingivitis groups **(Fig. 16A)**. In addition, resting platelets isolated from the whole

blood of SP patients contained more PF4 than platelets from the other 2 patient groups although this was not statistically significant ($p>0.05$) **(Fig. 16B)**.

Published evidence indicates that platelets are activated by periodontal pathogens (Li et al., 2008; Nylander et al., 2008; Assinger et al., 2011; Yu et al., 2011). To determine whether platelets from SP patients released more PF4 in the context of periodontitis, we stimulated the isolated platelets *in vitro* with lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, a pathogen central to the etiology of periodontitis (Holt and Ebersole, 2005a). Secretion of PF4 was observed in all 3 patient groups (healthy, gingivitis, SP) following LPS stimulation **(Fig. 17A)**. Notably, the unstimulated release of PF4 was highest in platelets from the SP group, suggesting that severe periodontitis is associated with an ongoing, agonist-independent release of PF4. In addition, while the PF4 release from platelets in the healthy and gingivitis groups plateaued after 1 minute of LPS stimulation, PF4 release in the SP group continued to rise over the 30-minute post-stimulation period **(Fig. 17A)**. Finally, when the secretion data are expressed in terms of percentage of total PF4 content **(Fig. 17B)**, the platelets from the severe periodontitis group release a greater proportion of their total PF4 content in response to *P. gingivalis* LPS. Taken together, these data show that platelets isolated from patients with generalized severe periodontitis contain more PF4 and are more responsive to LPS stimulation than platelets isolated from periodontally healthy or gingivitis subjects.

Table 4. Periodontal characteristics of blood donors

	Healthy (n=7)	Gingivitis (n=4)	Periodontitis (n=4)
Age (mean ± SD)	43.4 ± 7.8	47.8 ± 11.7	51.2 ± 13.5
Mean PD (mm)	2.5 ± 0.8	2.3 ± 0.9	5.0 ± 2.1
PD range (mm)	1-4	1-4	1-15
PD≤4 mm (% sites)	100	100	48
5mm<PD<7 mm (% sites)	0	0	41
PD≥7 mm (% sites)	0	0	11
BOP (% ± SD)	1.6 ± 1.5	28.9 ± 6.1	69.0 ± 24.6
PI (% ± SD)	0.4 ± 1.1	16.2 ± 1.5	86.5 ± 16.3

Legend:

SD: Standard deviation
PD: Probing depth
BOP: Bleeding on probing
PI: Plaque index

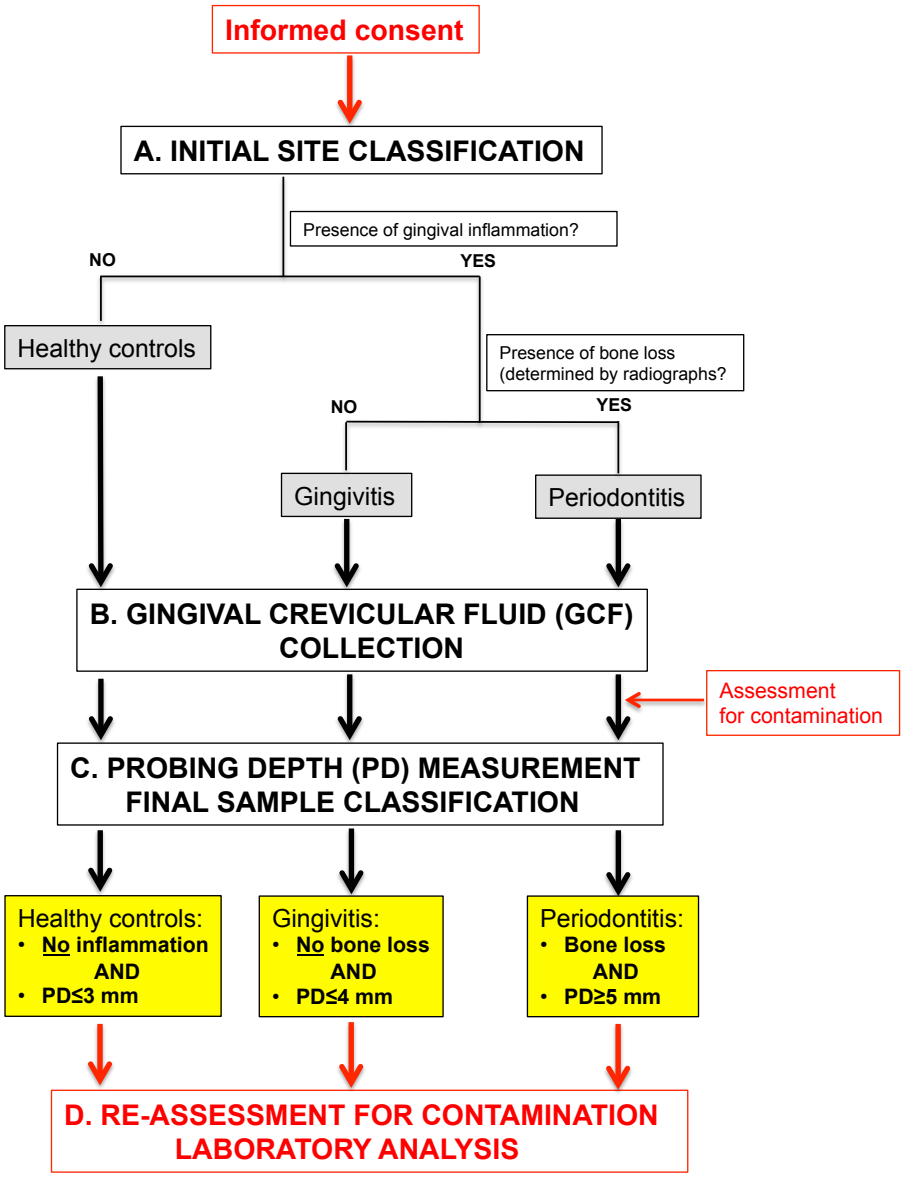

Figure 13. Algorithm for GCF sample collection and classification. **A**. After obtaining informed consent, GCF donor sites were initially triaged based on the presence or absence of gingival inflammation; sites with no visible gingival inflammation were assigned to the healthy control group. Sites with gingival inflammation but no bone loss were assigned to the gingivitis group. Sites with gingivitis and bone loss were assigned to the periodontitis group. **B.** To avoid tissue trauma, periodontal probing was avoided until after GCF harvesting. Following sample collection, the GCF was visually inspected for contamination. **C.** Probing depth was then measured to ensure classification of the GCF samples into the appropriate groups. **D.** Following a second independent visual inspection, samples were subjected to laboratory analysis.

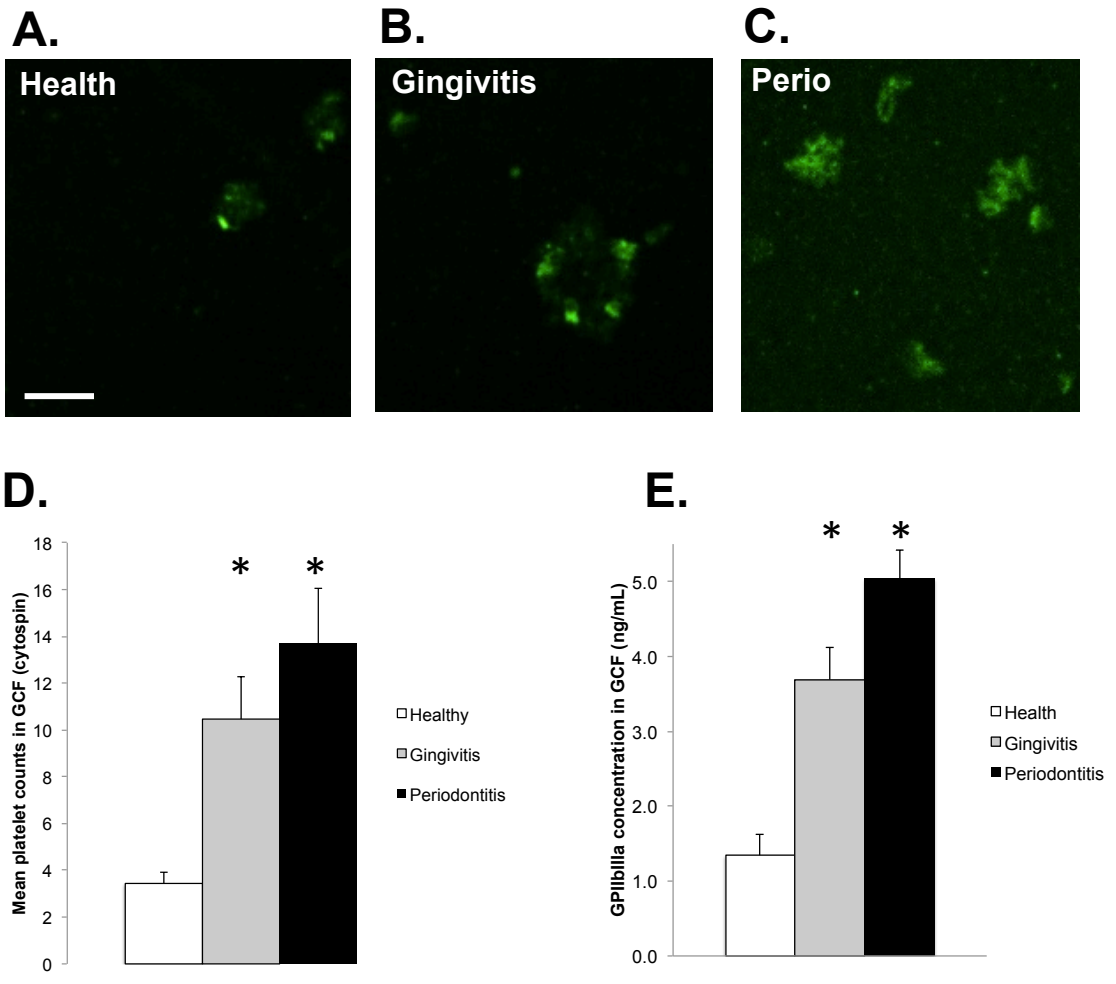

Figure 14. Platelets are recruited to sites of periodontal inflammation. A-C. Confocal micrographs of cytospin preparations illustrate the presence of platelets in the gingival crevicular fluid (GCF) obtained from healthy periodontium **(A)**, a site with gingivitis **(B)** and a periodontal pocket **(C)**. Cells were stained with a FITC-conjugated antibody against the platelet-specific marker CD42a. Bar=10 μm. **D.** Histogram compares the relative recruitment of platelets in the GCF of periodontally healthy sites (white bar, n=3), sites with gingivitis (grey bar, n=3) and periodontitis sites (black bar, n=3). For each cytospin preparation, the number of platelets was counted in 15 fields field of view. Data are expressed as mean ± SEM. *, $p<0.0001$, based on Bonferroni multiple comparison tests. **E.** Histogram illustrates the concentration of glycoprotein IIbIIIa (GPIIbIIIa) in the GCF of periodontally healthy sites (white bar, n=29), sites with gingivitis (grey bar, n=34) and periodontitis sites (black bar, n=22). GPIIbIIIa levels were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean ± SEM. *, $p<0.0001$, based on Bonferroni multiple comparison tests.

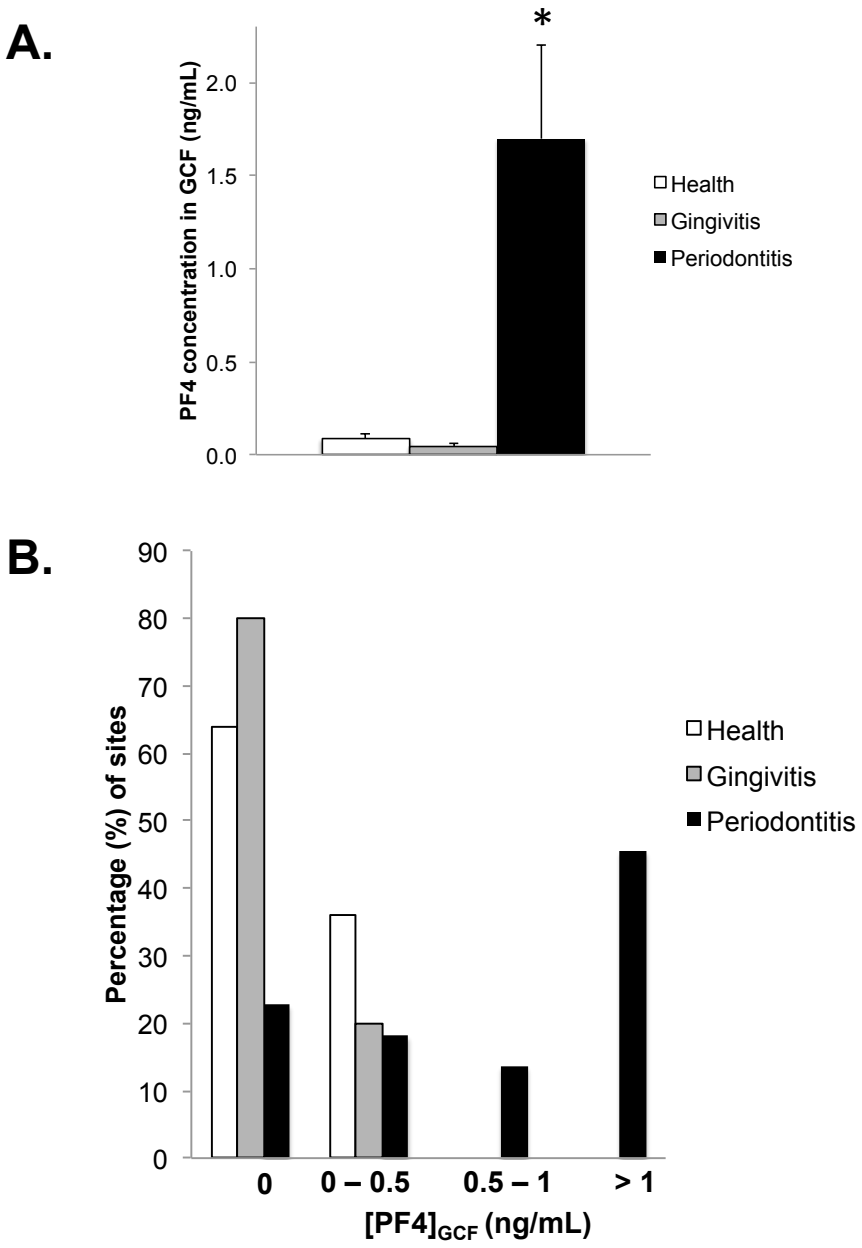

Figure 15. Gingival crevicular fluid (GCF) concentrations of platelet factor 4 (PF4) are elevated in periodontal pockets. A. Histogram illustrates the mean platelet factor 4 (PF4) concentration in the GCF harvested from periodontally healthy sites (white bar, n=25), sites with gingivitis (grey bar, n=20) and periodontitis sites (black bar, n=22). PF4 concentration was determined by ELISA. Data are expressed as mean ± SEM. *, $p<0.0001$, based on Bonferroni multiple comparison tests. **B.** Histogram illustrates the distribution of test sites (healthy, gingivitis, periodontitis) within the various ranges of PF4 concentration ($[PF4]_{GCF}$).

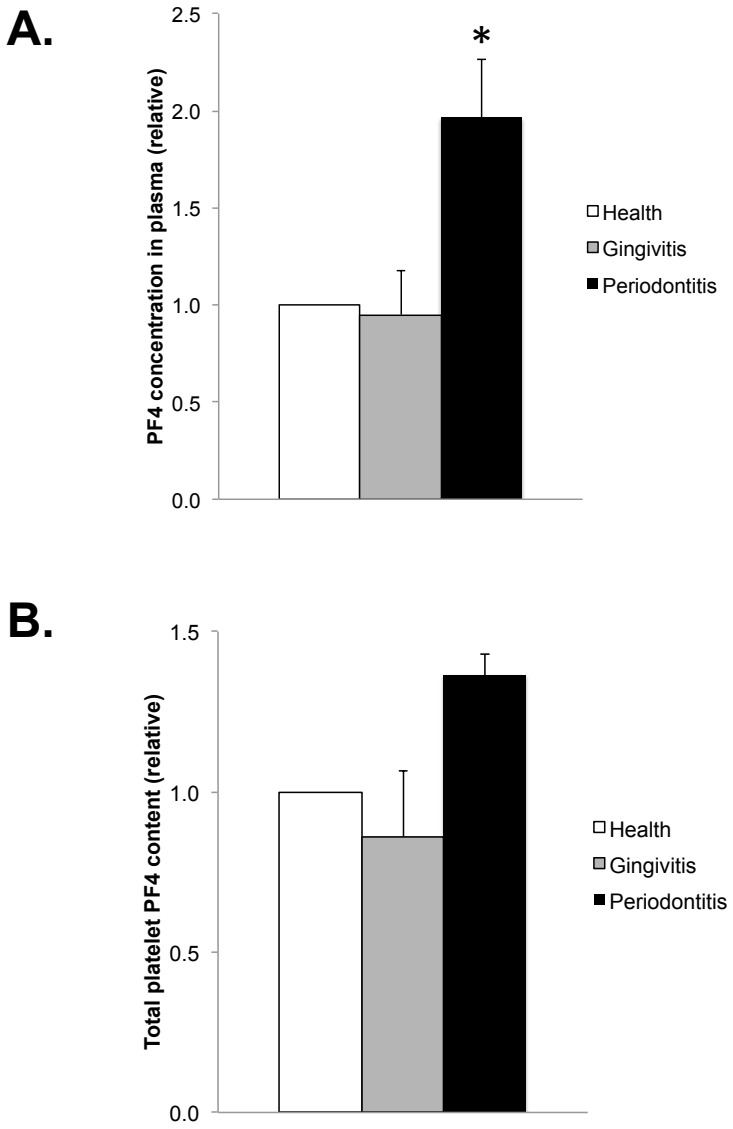

Figure 16. Circulating PF4 and platelet loading of PF4 is elevated in patients with generalized severe chronic periodontitis. A. Histogram illustrates the relative circulating levels of PF4 in the platelet-poor plasma of patients with healthy periodontium (white bar, n=7), patients with gingivitis (grey bar, n=4) and patients with generalized severe chronic periodontitis (black bar, n=4). Data are expressed as mean ± SEM, with patients from healthy periodontium set at 1. **B.** Histogram illustrates the total PF4 content in platelets obtained from patients with healthy periodontium (white bar, n=7), patients with gingivitis (grey bar, n=4) and patients with generalized severe chronic periodontitis (black bar, n=4). Data are expressed as mean ± SEM, with platelets from periodontally healthy subjects set at 1.

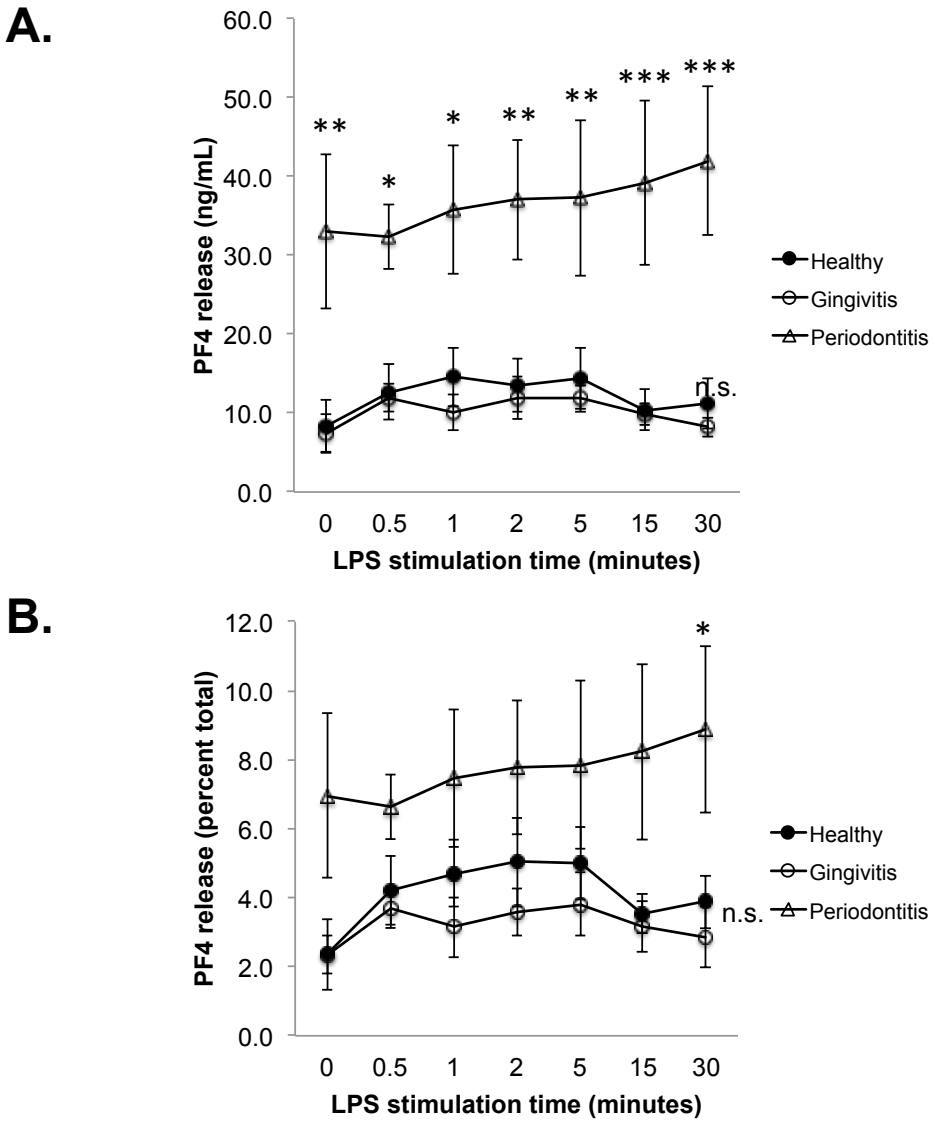

Figure 17. LPS-induced PF4 release is amplified from platelets isolated from patients with generalized severe chronic periodontitis. A. Line graph illustrates the LPS-induced release of PF4 from equal numbers of platelets isolated from the blood of patients with healthy periodontium (closed circles, n=7), patients with gingivitis (open circles, n=4) and patients with generalized severe chronic periodontitis (open triangles, n=4). Data are expressed as mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., not significant, based on Bonferroni multiple comparison tests. **B.** Line graph illustrates the LPS-induced release of PF4, expressed as percent of total PF4 content, from equal numbers of platelets isolated from the blood of patients with healthy periodontium (closed circles, n=7), patients with gingivitis (open circles, n=4) and patients with generalized severe chronic periodontitis (open triangles, n=4). Data are expressed as mean ± SEM.

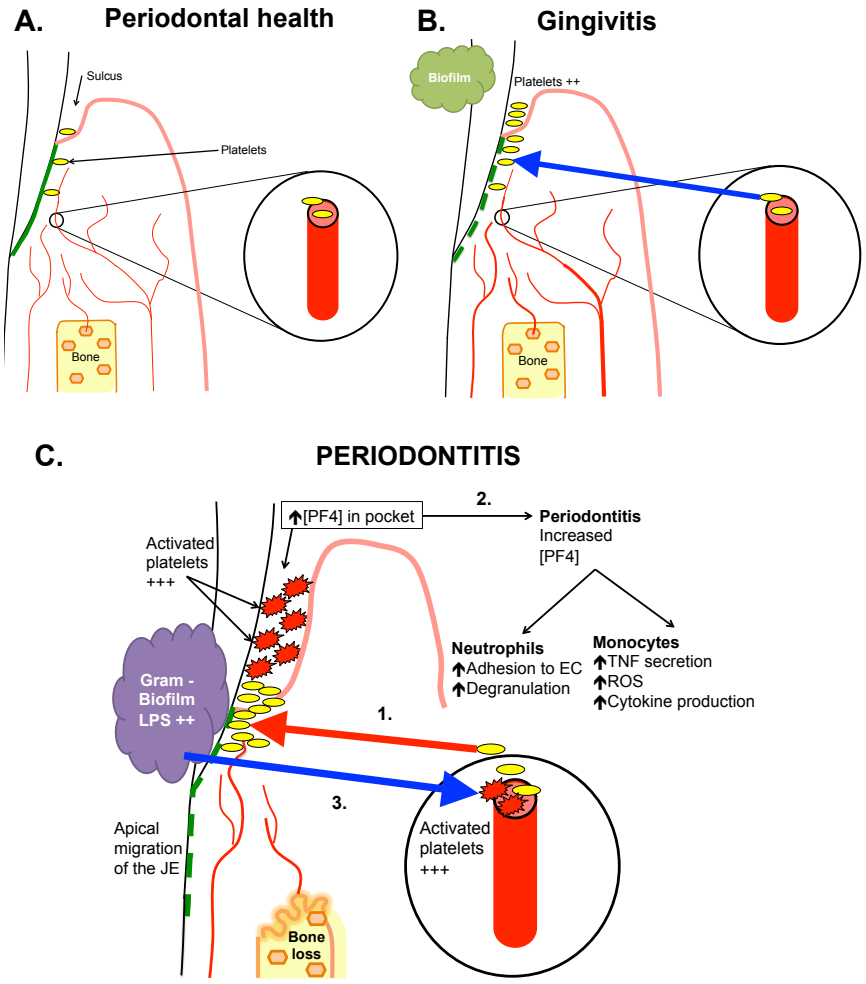

Figure 18. Proposed model outlining platelet recruitment and PF4 release in the context of periodontitis. **A.** Diagram depicts the cross section of the dento-gingival junction (tooth/sulcus in a healthy periodontium and the relative absence of LPS challenge. Inset (bottom-right) depicts platelets (yellow) exiting the systemic circulation. **B.** During gingivitis induced by bacterial plaque (green), increased numbers of platelets are recruited to the dento-gingival junction (red arrow) but remain generally quiescent and do not release significant quantities of PF4. **C.** During periodontitis, the gram-negative, LPS-laden biofilm (purple) provides continued stimulation of the recruited platelets **(1)**, thus inducing the release of PF4 **(2)**, which potentiates neutrophil and monocyte activity thus promoting periodontal tissue degradation. Bone degradation and apical migration of the junctional epithelium (JE) occur. Inset: The elevated plasma concentration of PF4 in the systemic circulation likely accentuates activation of local leukocytes. **(3)** Severe periodontal infection likely provides a source of periodontal pathogens and LPS in the systemic circulation, thus creating a feedback mechanism **(blue arrow)** that could maintain elevated levels of circulating PF4.

Discussion

Periodontitis is a highly prevalent and destructive inflammatory disease, and the molecular determinants of its pathogenesis remain undefined. Current evidence supports the existence of a general association between platelet count and activation and periodontitis (Nicu et al., 2009b; Papapanagiotou et al., 2009; Al-Rasheed, 2012b; Kumar et al., 2014). However, the present study is the first to investigate a specific role for platelet factor 4 (PF4), which is the major constituent of platelet granules (Lambert et al., 2007). Here we demonstrated that local and systemic elevations of PF4 are correlated with periodontitis in humans. This conclusion is based on our finding that periodontal pockets contained ~20 times more PF4 than gingival sulci associated with healthy periodontium or gingivitis. Moreover, platelets isolated from patients with generalized severe periodontitis stored and secreted more PF4 than platelets from periodontally healthy or gingivitis subjects. In addition, basal circulating concentrations of PF4 were doubled in patients with severe periodontitis relative to controls. This is the first study analyze PF4 concentration in the context of periodontal health and disease.

Platelets are recruited to sites of gingival inflammation

Our analysis of gingival crevicular fluid (GCF) with 2 complementary approaches indicates that platelets are recruited to the dento-gingival junction during gingival inflammation. Since blood contamination was excluded as a source of platelets in the GCF, we conclude that platelets exit the circulation at sites of gingival inflammation. This is consistent with early reports that platelets respond to chemotactic signals (Lowenhaupt, 1978; Lowenhaupt, 1982; Lowenhaupt et al., 1982) and that platelets enter inflamed tissues by diapadesis and extravasation (Beasley et al.,

1989; Feng et al., 1998). The underlying mechanism for platelet recruitment to the periodontium is unclear. One possibility is that the clinical onset of gingivitis is accompanied by the expression of an unidentified bacteria- or host-derived platelet chemokine. Alternatively, since periodontal inflammation is characterized by marked neutrophil influx (Hajishengallis et al., 2014), it is plausible that platelets are recruited in tandem with neutrophils given the extensive and well-documented crosstalk between the two cell types (Li et al., 2000; Ghasemzadeh and Hosseini, 2013; Page and Pitchford, 2013).

PF4 is preferentially secreted during periodontitis

A striking finding from our study was the ~20-fold elevation of PF4 concentration in periodontitis sites relative to gingivitis sites, despite there being roughly equivalent platelet recruitment in both conditions. This finding indicates that platelet activation is specifically accentuated in advanced periodontal lesions. This may be explained by the relative abundance of gram-negative pathogens (Holt and Ebersole, 2005a) in advanced periodontitis lesions relative to early gingivitis lesions. Notably, platelets express toll-like receptor 4 (TLR4), which recognizes the lipopolysaccharide (LPS) component of gram-negative periodontal pathogens such as *Porphyromonas gingivalis* (Andonegui et al., 2005; Cognasse et al., 2008; Zhang et al., 2009b). In addition, direct platelet activation by periodontal pathogens, including *P. gingivalis*, is well documented (Li et al., 2008; Nylander et al., 2008; Assinger et al., 2011; Yu et al., 2011). The local abundance of PF4 may also be explained by the presence of other known platelet agonists in periodontitis lesions, such as thromboxane (Dewhirst et al., 1983; Bons-Sicard et al., 1998) and platelet-activating factor (McManus and Pinckard, 2000; Emingil et al., 2001b) that could potentiate platelet activation along with LPS.

There are several plausible explanations to support a role for PF4 in the progression of periodontitis. For example, PF4 promotes neutrophil degranulation (Bebawy et al., 1986; Petersen et al., 1996; Vandercappellen et al., 2011), which contributes to tissue proteolysis in periodontitis lesions (Klebanoff, 2005). Moreover, PF4 promotes monocyte survival and secretion of tumor necrosis factor-alpha (TNF-α) (Scheuerer et al., 2000; Srivastava et al., 2010), a pro-inflammatory cytokine that in turn stimulates the production of collagen-degrading matrix metalloproteinases (MMPs) by gingival fibroblasts (Domeij et al., 2002). In addition, PF4 induces the formation of reactive oxygen species (ROS) in macrophages (Pervushina et al., 2004) that also contribute to degradation of the periodontal tissues (Waddington et al., 2000; Dahiya et al., 2013). In this context, our findings are consistent with the notion that PF4 propagates the pro-inflammatory and tissue-degrading activities of neutrophils and monocytes in the periodontium **(Fig. 6)**.

Periodontitis is correlated with systemic levels of circulating PF4

We found platelet PF4 content and circulating PF4 levels to be elevated in patients with generalized severe periodontitis, consistent with previous reports recording increased platelet activation in periodontitis patients (Nicu et al., 2009b; Papapanagiotou et al., 2009). Notably, in our study, platelets from the periodontitis group secreted a higher percentage of their total PF4 content in response to LPS, suggesting that their platelets are also abnormally reactive to LPS stimulation. In addition, oral bacteria enter the bloodstream following innocuous procedures such as toothbrushing (Lockhart et al., 2008). It is therefore plausible that the platelets of untreated severe periodontitis patients are subjected to continuous LPS stimulation, thus creating a

feedback loop that would maintain elevated PF4 levels in the systemic circulation while further exacerbating inflammation and tissue degradation in the periodontium **(Fig. 18)**.

PF4 is also implicated in the pathogenesis of atherosclerosis, an inflammatory vascular disease characterized by the formation of plaques along the arterial wall. The putative relationship between periodontitis and atherosclerosis has been extensively studied (Bartova et al., 2014b; Teeuw et al., 2014). Notably, PF4 promotes the adhesion of monocytes to the endothelium and their eventual differentiation into lipid-laden macrophages that form the core of the atherosclerotic plaque (Gawaz et al., 2005; Aidoudi and Bikfalvi, 2010a; Gleissner, 2012). In this context, our discovery of increased PF4 levels in severe periodontitis may provide additional evidence linking the pathogenesis of the two diseases.

A limitation of our study was the small number of subjects available for the blood- and platelet-based analyses. This was largely due to our strict inclusion criteria that limited the periodontitis group to patients with generalized severe disease in the absence of any other contributing factors such as smoking. However, this shortcoming was mitigated by the large differences observed between the test groups as well as by the concordance of the platelet-based data with the results of the GCF studies that were conducted over much larger sample sizes. Another shortcoming is that our cross-sectional data do not permit the evaluation of PF4 as a causal factor in the initiation or progression of periodontitis.

Our finding that PF4 was largely absent from the GCF of healthy and gingivitis sites invites speculation that PF4 may contribute to the transition between gingivitis to periodontitis.

Conceivably, PF4 could represent a biomarker for periodontal sites at risk for disease progression. Future prospective, longitudinal studies could also query the significance of circulating PF4 levels as a novel tool for assessing individual susceptibility to periodontitis and/or atherosclerosis.

Chapter Three: Conclusions and Future Directions

Conclusions:

Our study identifies a novel role for platelets in the context of periodontal disease pathogenesis. While previously published data have suggested a general correlation between systemic platelet reactivity and periodontal status (Nicu et al., 2009a; Papapanagiotou et al., 2009), our study is the first to employ molecular probes for platelet-specific molecules (CD42, GPIIbIIIa, PF4) to demonstrate *in situ* platelet recruitment and activation in the inflamed periodontium.

Directions for future research:

1. Our finding of increased platelet factor 4 (PF4) levels in the gingival crevicular fluid in periodontitis, but not gingivitis, lesions raises the interesting possibility that PF4 may promote the shift from gingivitis to periodontitis. While this cannot be ascertained from our cross-sectional study, future prospective studies could measure both local (GCF) and systemic (plasma) concentrations of PF4 over time to determine whether higher concentrations of PF4 confer an increased risk for subsequent periodontal breakdown.
2. The hypothesis that platelet activation induces periodontitis could be tested with an experimental periodontitis model, for example, using thrombocytopenic mice and controls.
3. In patients with generalized severe periodontal disease, it would be of interest to determine whether successful periodontal therapy causes a systemic reduction in circulating PF4.
4. A limitation of our study was the evaluation of only one molecule (PF4) in the gingival crevicular fluid. Future studies could employ multiplex ELISA assay arrays capable of analyzing the concentration of multiple analytes simultaneously (Shimada et al., 2013). This

approach, while costly, could provide insight into the concentration of platelet-derived cytokines relative to those cytokines derived from other cell types.

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