Oxidative Stress – a Key Player in Periodontal Disease

by

Guy M. Aboodi

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy – Dentistry (Periodontics)

Faculty of Dentistry
University of Toronto

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University of Toronto
2015

Abstract

Background: The biological oxidative state reflects the net balance between reactive oxygen species (ROS) and antioxidants (AO). An increase in ROS levels will result in oxidative stress, which has been linked to aging as well as pathologic conditions, including inflammatory, degenerative, and multi-organ diseases.

Periodontal disease is an inflammatory disease regulated in part by oxidative states in periodontal tissues. In its most common form, chronic periodontitis (CP) is an inflammatory disease initiated by subgingival biofilms, which induce a local immune response, host-mediated tissue destruction and unresolved inflammation. The well-documented heterogeneity of such immune responses among patients with CP is, in part, attributed to neutrophil functional alterations including generation of oxidative stress.

Goals: To investigate the factors affecting oxidative stress in oral neutrophils and their role in periodontal tissue destruction. I hypothesize that oral neutrophils in chronic periodontitis (CP) have a pro-oxidative phenotype. This phenotype presents as hyperactive oral neutrophils, which contribute to oxidative stress and tissue destruction during CP.

Results: Increase in cytoprotective proteins, including AO, was demonstrated in an experimental gingivitis model, suggesting that the regulation of physiologic oxidative state has a role in preventing clinical attachment loss (CAL). Increased oral neutrophil ROS production was associated with severe
periodontal tissue damage (CAL) in CP patients whose disease was resistant to treatment (refractory disease). Gene expression microarray completed in both oral and peripheral blood neutrophils revealed that oral neutrophils in CP patients present with a deficient oxidative stress response, which is mediated by the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. The role of the Nrf2 pathway in neutrophil function and periodontal tissue destruction was then further investigated in a murine periodontal disease model. Nrf2 null mice have a hyperactive neutrophil phenotype when activated. Furthermore, Nrf2 null mice have more severe bone loss and increased ROS-mediated damage compared to control mice in response to ligature-induced periodontitis.

Conclusions: Nrf2 down-regulation in oral neutrophils is associated with more severe periodontal tissue destruction. Reduced production of AO by neutrophils in the context of increased recruitment in the periodontium aggravates tissue destruction. It can therefore be concluded that reduced AO production by oral neutrophils in un-resolved periodontal inflammation may contribute to increased severity of CP through local oxidative stress.
Acknowledgments

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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>8-OHdG</td>
<td>8-hydroxy-deoxyguanosine</td>
</tr>
<tr>
<td>ABC</td>
<td>Alveolar bone crest</td>
</tr>
<tr>
<td>ABL</td>
<td>Alveolar bone loss</td>
</tr>
<tr>
<td>AgP</td>
<td>Aggressive periodontitis</td>
</tr>
<tr>
<td>AO</td>
<td>Antioxidants</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
</tr>
<tr>
<td>CAL</td>
<td>Clinical attachment loss</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementery DNA</td>
</tr>
<tr>
<td>CEJ</td>
<td>Cementoenamel junction</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>CP</td>
<td>Chronic periodontitis</td>
</tr>
<tr>
<td>CT</td>
<td>Gingival connective tissue</td>
</tr>
<tr>
<td>DBP</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>DHR</td>
<td>Dihydrorhodamine 123</td>
</tr>
<tr>
<td>EG</td>
<td>Experimental gingivitis</td>
</tr>
<tr>
<td>Epi</td>
<td>Sulcular epithelium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>fMLP</td>
<td>Formylmethionine leucyl-phenylalanine</td>
</tr>
<tr>
<td>fPR1</td>
<td>fMLP receptor 1</td>
</tr>
<tr>
<td>GCF</td>
<td>Gingival crevicular fluid</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidise</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>LC-ESI-MS/MS</td>
<td>Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry</td>
</tr>
</tbody>
</table>
LTF - Lactotransferrin
MDA - Malondialdehyde
MPO - Myeloperoxidase
MS - Mass spectrometry
NADPH Oxidase - Nicotinamide adenine dinucleotide phosphate-oxidase
NO - Nitric oxide
Nrf2 - Nuclear factor erythroid 2-related factor 2
O$_2^-$ - Superoxide
OH$^-$ - Hydroxide molecule
oROS - Oral neutrophil reactive oxygen species
PBS - Phosphate buffer saline
PD - Probing depth
PMA - Phorbol 12-myristate 13-acetate
qRT-PCR - Quantitative Reverse Transcription-Polymerase Chain Reaction
RCF - Relative centrifugal force
ROI - Regions of interest
ROS - Reactive oxygen species
SDD - Subantimicrobial dose doxycycline
SOD - Superoxide dismutase
TAOC - Total antioxidant capacity
TBST - Tris-Buffered Saline and Tween
TREM - Triggering receptor expressed on myeloid cells 1
WT – Wild type
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Publications from the thesis:


3. **G.M. Aboodi**, C. Sima, F.S. Lakschevitz, C. Sun, M.B. Goldberg, M. Glogauer. Nrf2 down-regulation is associated with chronic periodontitis and results in periodontal tissue destruction in animal model. *(Manuscript under review)*

Other Publications:


Chapter 1

Introduction

The biological oxidative state reflects the balance between reactive oxygen species (ROS) and antioxidants (AO). ROS have been shown to play a fundamental role in many physiologic processes, including innate immunity and signal transduction. However, alongside their central role in these processes, ROS have the potential to damage DNA, proteins, and lipids through oxidation reactions. The detoxification of ROS is carried out mainly by AO activity. AO neutralize excess ROS and maintain physiologic ROS levels. The balance between ROS and AO is the determining factor in the overall biological effect of the oxidative state. Periodontal disease is an excellent example of an inflammatory disease regulated in part by the oxidative state. This clinical research project will focus on the contribution of ROS and the AO defence system to the oxidative stress state observed during periodontal disease and the resulting periodontal tissue destruction.

1.1 Significance of Project

Previous observations of increased ROS production by hyperactive neutrophils in patients with chronic periodontitis (CP) suggest a role for ROS in the pathogenesis of inflammatory periodontal diseases. However there has been no definitive evidence implicating ROS mediated damage or the oxidative state in periodontal tissue destruction in CP. Therefore, my aim for this project was to identify a disease-specific oxidative oral neutrophil phenotype that results in increased periodontal tissue destruction.
During gingivitis, patients do not present with clinical attachment loss (CAL). Gingivitis has been shown to be a stable condition over time in some patients, with no periodontal tissue destruction following the inflammatory process. Characterizing the whole saliva proteome during gingivitis would allow us to identify any cytoprotective proteins, which contribute to a reduced oxidative state in these patients. Whole saliva proteome analysis was completed in an experimental gingivitis model.

Oral neutrophil responsiveness to stimulation to produce ROS was studied in a group of patients who were diagnosed with refractory CP. Refractory CP is characterized by persistent CAL after treatment in patients who present without known risk factors for disease progression (proper plaque control, systemically healthy, non-smokers). Clearly, the host response in refractory CP patients plays a key role in the disease pathogenesis. Oral neutrophil ROS production was assessed utilizing a flow cytometry approach. Based on oral neutrophil ROS production in response to stimulation, I report on 2 different patient groups – High and Low responders. Analysis of disease severity, as measured by CAL, suggests an association between high oral neutrophil responsiveness to produce ROS and increased disease severity.

Further characterization of oral neutrophil oxidative state in untreated patients with severe CP was performed in comparison with blood neutrophils and related to that of healthy individuals. Analysis of oral versus blood neutrophils within each of the study groups revealed that unlike healthy controls, CP patients present a significant downregulation in the nuclear factor erythroid 2-related factor 2 (Nrf2) mediated oxidative stress response in their oral neutrophils. The role of the Nrf2 pathway in neutrophil function in periodontitis was then investigated using a murine model of periodontitis in Nrf2/- mice.
1.2 Objectives

In summary, the specific objectives of this clinical research project are as follows:

i. To identify a disease-specific pro-oxidative oral neutrophil phenotype.

ii. To identify the correlation between hyper-responsive oral neutrophils (as defined by ROS production) and periodontal disease severity.

iii. To demonstrate that ROS-mediated tissue damage correlates with severity of periodontal tissue destruction.

1.3 Hypotheses

i. Salivary cytoprotective proteins which regulate oxidative state, are increased during the inflammatory phase of gingivitis.

ii. Oral neutrophils obtained from refractory CP patients are hyper-responsive to stimulation.

iii. Oral neutrophils obtained from patients with severe CP have a disease-specific pro-oxidative phenotype characterized by decreased AO production.

iv. This increased neutrophil-mediated oxidative stress contributes to periodontal tissue destruction in periodontitis.
Chapter 2

Background

2.1 Periodontal diseases

Periodontal diseases are a diverse group of inherited or acquired conditions that affect the tooth-supporting tissues in more than half of world population. Different pathogenic mechanisms including inflammatory, traumatic, genetic, and neoplastic contribute to the onset and progression of periodontal diseases (1). The main etiologic factor for these conditions is the bacterial biofilm. The most common forms of periodontal diseases are plaque-induced gingivitis and chronic periodontitis (CP). Although subgingival bacteria are necessary for onset of disease, they are not sufficient. Multiple host and environmental factors contribute to disease onset and progression, including smoking, age, gender, immune status, nutritional deficiencies, psychological stress, substance abuse, various medications, genetic disorders of the immune system and connective tissue, and systemic conditions (2,3).

Periodontal diseases classification is based on the American Academy of Periodontology 1999 classification system (4). The main diagnostic criteria is clinical attachment loss (CAL) – the combined measure of the periodontal probing depth (PD) and recession in each site (4). Measurements are done with a periodontal probe. PD measurements reflect the measured distance from the bottom of the periodontal pocket to the free gingival margin; recession measurements reflect the measured distance from the free gingival margin to the cementoenamel junction (CEJ) (2). A full periodontal examination includes measurements of pockets depth and recession at 6 sites around each tooth, and recording each of the following periodontal features: gingival bleeding on probing (BOP), supragingival bacterial plaque and
calculus, the presence of exudates, tooth mobility, and mucogingival deformities (5). The probing depths measured in healthy periodontium are 1-3 mm, while sites where periodontitis is suspected are typically measure deeper than 3 mm.

**Box 1: Periodontal diseases classification**

| **Plaque-induced Gingivitis**: An inflammation of the gingiva induced by bacteria located at the gingival margin. |
| **Chronic Periodontitis**: Inflammatory disease affecting the supporting tissues of the teeth, characterized by progressive loss of tooth attachment to periodontal tissues. It is associated with periodontal pocket formation and/or gingival recession. Both localized and generalized disease forms can occur. |
| **Aggressive Periodontitis**: A rare form of inflammatory periodontal disease, characterized with by rapid disease progression in patients who present with no common etiologies and risk factors for periodontal disease. Both localized and generalized disease forms can occur. |
| **Refractory Periodontitis**: A group of destructive inflammatory periodontal diseases affecting patients who do not respond to conventional periodontal therapy, and present with continued attachment loss over time, despite well-controlled known risk factors for disease progression. |

**Plaque-induced gingivitis** is defined as an inflammation of the gingiva induced by bacteria located at the gingival margin. The causative relationship between bacterial plaque (biofilm) and gingival inflammation was demonstrated in an experimental gingivitis study (6). Characteristic gingivitis clinical signs include erythema, edema, loss of gingival stippling and bleeding on probing (BOP) (7). Interestingly, the host response to similar plaque levels varies significantly among patients (8). Histologic changes in the tissue include proliferation of junctional epithelium, vasculitis of blood vessels adjacent to the junctional epithelium, collagen degradation, cytopathologic alteration of fibroblasts, and inflammatory infiltrate (9). Gingivitis is reversible upon removal of the etiologic biofilm (6).

Similar to gingivitis, CP is also an inflammatory disease caused by the bacterial biofilm. In fact, gingivitis is an established risk factor for CP (10). CP commonly develops in the 4th
decade of life, with highest prevalence in senior individuals. (11). CP is characterized by extension of gingival inflammation to the alveolar bone, connective tissue degradation and net loss of tooth attachment to periodontium. The disease is further defined by its extent (localized if <30% of sites are affected, and generalized if >30% of sites are affected), and by its severity, as measured by CAL (mild: 1-2mm CAL; moderate 3-4mm CAL; severe >5mm CAL) (4). As opposed to gingivitis, which is not associated with specific pathognomonic bacteria, several bacterial species are considered periodontogenic. The members of the red complex - *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* were consistently demonstrated to be elevated in CP patients compared to periodontally-healthy subjects, and in diseased sites compared to healthy sites of diseased subjects (12). Conventional periodontal therapy for CP includes scaling and root planing that focus on the removal of etiologic biofilms and calculus. In most cases this form of therapy allows for resolution of inflammation to occur and for periodontal tissues to heal (2). Surgical re-establishment of maintainable periodontal tissues generally follows scaling and root planing in severe cases of CP. If left untreated, CP results in pain, discomfort, tooth mobility, and eventually – tooth loss (1), and may represent a risk factor for multiple systemic conditions with underlying low grade inflammation such as metabolic syndrome, diabetes and cardiovascular disease (13-16).

Clinically, there is great diversity in patient responses to the bacterial biofilm. CP patients may differ in the rate of disease progression, severity of periodontal tissue destruction, and treatment outcomes (2). Furthermore, clinical studies have demonstrated that in some individuals gingivitis never progresses to CP, regardless of periodontal care (17). Although certain pathogenic bacteria in subgingival biofilms produce specific virulence factors that could cause direct damage to periodontal tissues, current evidence suggest that it is the host factors that drive periodontal tissue degradation at sites with CP. These factors include over-expression...
of inflammatory cytokines, proteolytic enzymes, and increased oxidative stress (18,19). The rate limiting steps in onset and progression of clinical attachment loss are incompletely understood. Increasing evidence that emerged in recent years indicates that failure to resolve biofilm-induced periodontal inflammation results in chronicity and pro-osteolytic environments (20-22).

Aggressive periodontitis (AgP) is comprised of a group of rare, often severe, rapidly progressing forms of periodontitis affecting up to 5% of population depending primarily on race/ethnicity (23). AgP has a distinctive familial pattern, and patients generally have non-contributory medical history (24). Unlike CP, AgP is more likely to be diagnosed in younger individuals (<30 years of age), although age per-se is no longer considered a diagnostic criteria (4). Similar to CP, AgP occurs in localized and generalized forms. The localized form most commonly affects permanent first molars and incisors, while the generalized form is characterized by generalized interproximal attachment loss affecting at least 3 permanent teeth other than the first molars and incisors (25). Common clinical features may include bacterial biofilm levels that are inconsistent with the severity of periodontal tissue destruction, elevated proportions of the periodontal pathogen Aggregatibacter actinomycetemcomitans (AA), and neutrophil function abnormalities (25). Antibiotic treatment in conjunction with scaling and root planing is recommended for AgP, as specific bacterial etiology has been demonstrated (25). Altered neutrophil function is a common finding among AgP patients, suggesting a key role of altered host responses in periodontitis severity and rate of progression. This role is further supported by the observation that disease progression may be self-arrested (25). AgP is treated in a similar manner to CP with more emphasis on the host than on the biofilm. The use of immune-modulatory therapeutics and antimicrobials are used more often than for CP with varying degrees of success.
In some instances patients with AgP or CP are refractory to conventional periodontal therapy and may be diagnosed with refractory AgP or refractory CP, respectively (4,26). Refractory patients have a recurring disease progression pattern and continuous loss of clinical attachment after treatment, which does not correlate with plaque levels, microbiology assessments, and treatment compliance (27-29). Therefore, patients with either CP or AgP are diagnosed with refractory disease only after active periodontal treatment, periodontal maintenance and follow-up (4,26). Refractory patients were demonstrated to lose a significantly higher number of teeth during periodontal maintenance when compared to non-refractory patients (29,30). Management of disease in refractory patients consists of long-term subantimicrobial dose doxycycline (SDD) in conjunction with routine periodontal maintenance. SDD was demonstrated to inhibit matrix metalloproteinase activity, thus preventing connective tissue destruction (31). A nine-month longitudinal study demonstrated significantly greater mean CAL and mean PD reductions in patients receiving daily SDD when compared to placebo treatment (32).
2.2 Reactive oxygen species

Box 2: Oxidative state components

*Reactive oxygen species (ROS)*: A collective term for reactive molecules containing oxygen. It includes free radicals (e.g. superoxide O$_2^-$, hydroxyl OH$^-$), or non-radical molecules such as hydrogen peroxide (H$_2$O$_2$).

*Antioxidants (AO)*: A diverse group of molecules that prevents oxidative damage by regulating ROS levels.

*Oxidative stress*: Characterized by increased ROS activity and/or decreased AO activity, resulting in a net increase in ROS activity.

2.2.1 Chemistry

Free radicals are highly reactive molecules, containing one or more unpaired electrons. ROS are a heterogeneous group of oxygen-containing free radicals (33,34). Superoxide (O$_2^-$) is considered the primary ROS as a result of observed biological functions in health and disease. It is the product of different metabolic processes or irradiation upon tissues, and mediates critical functions in non-self alteration as mechanism of defense and in self alteration when in excess. Further, O$_2^-$ serves as substrate for generation of secondary ROS, including hydrogen peroxide (H$_2$O$_2$) and hydroxide molecule (OH$^-$) (34). O$_2^-$ is formed by the addition of one electron to molecular oxygen. The major cellular sources of O$_2^-$ are the mitochondrial respiratory chain and the activated nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) (35,36). The oxidative burst process mediated primarily by the NADPH oxidase was identified in both phagocytic and non-phagocytic cells, but is most commonly observed in activated neutrophils, the main cellular component of the innate immune system (37).

In phagocytes, O$_2^-$ is the precursor to all ROS, through its dismutation to hydrogen peroxide (35). Further, it is a mediator of reactive nitrogen species synthesis, through reaction
with nitric oxide (NO) to generate peroxynitrite anion. This reaction is facilitated by inducible nitric oxide synthase, and takes place mainly in macrophages (19). Similarly, in the presence of \( \text{O}_2^- \) the myeloperoxidase-mediated peroxidation of chloride ions generates hypochlorous acid, a potent antimicrobial agent. \( \text{O}_2^- \), peroxynitrite and hypochlorous acid are key weapons the phagocytes uses against pathogens (Figure 2.1).

Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) is generated following \( \text{O}_2^- \) dismutation, either through spontaneous degradation, or through the more common enzymatic process carried out by super oxide dismutase (SOD) (See section 2.3) (38). As a relatively stable molecule (estimated half life: \( 10^{-5} \) seconds (39)), \( \text{H}_2\text{O}_2 \) is involved in many physiologic, pro-survival processes, mainly through the oxidation of specific molecules. This results in inhibition of enzymes and negative regulation of the involved pathways. As is the case with all ROS, high \( \text{H}_2\text{O}_2 \) concentrations may result in irreversible protein and tissue damage (38).

ROS are generated in multiple compartments within the cell (Figure 2.2). The mitochondria are the organelle with the largest oxygen consumption (estimated at up to 90% of cellular oxygen), and it is therefore the main generator of \( \text{O}_2^- \) in physiological conditions (40). ROS generation in the mitochondria is a bi-product of the oxidative phosphorylation process. An estimated 0.2-2% of oxygen consumption during oxidative phosphorylation processes contribute to \( \text{O}_2^- \) generation (34,40). ROS generation in the mitochondria is tightly regulated, and it does not significantly contribute to the cellular oxidative state (40,41).

A second cellular ROS production site is the peroxisome. Peroxisomes, cell organelles involved in cellular lipid metabolism, generate mainly \( \text{H}_2\text{O}_2 \) (produced by flavin oxidases which are found in high abundance in the peroxisome), but other ROS producing enzymes were also identified (42). High catalase (a hydrogen peroxide reducing antioxidant – see section 2.3) concentration and other AO in the peroxisome maintain oxidative state homeostasis (42).
Through high concentrations of both ROS producing and degrading enzymes, peroxisomes can significantly affect the cellular oxidative state. However, it has been demonstrated that despite its high catalase content, the peroxisome does not contribute to the reduction of external H$_2$O$_2$ (43). Its contribution to oxidative state homeostasis is suggested to be through maintenance of cellular membrane integrity by metabolizing lipophilic molecules, which may otherwise alter membrane structure when in excess (43). Yet under certain pathologic conditions, peroxisomes may release H$_2$O$_2$ in the cytoplasm and contribute to cellular oxidative stress (39). Therefore, the roles of peroxisomes in cellular oxidative state regulation are not fully understood.

The endoplasmic reticulum (ER) is a network of membranous tubules within the cytoplasm, continuous with the nuclear membrane. ER specializes in protein folding and assembly of multisubunits proteins. This process is oxidative state-dependent, with the ER lumen being an oxidizing environment (44). It is estimated that about 25% of cellular ROS is produced in the ER. The main contributor to ER ROS formation is the ER oxidoreductin 1 (Ero1), an ER membrane-associated protein, which generates O$_2^-$ during protein folding. Another proposed mechanism is ROS production during proteasome-mediated degradation of misfolded or unfolded proteins (44). In fact, an association between ER stress and oxidative stress has been linked to various chronic conditions. However, what remains unclear to what extent the ER ROS production directly affects the cellular oxidative state (45).

Unlike the previously discussed organelles, which generate ROS as a bi-product, the NADPH oxidase enzyme complex functions primarily as a O$_2^-$ generating system. NADPH oxidase is found in great abundance in neutrophils though it is not limited to them. Its critical role in innate host defense is clearly demonstrated in chronic granulomatous disease (CGD), a genetic disorder caused by mutations in one of the enzyme’s subunits. NADPH oxidase function in CGD neutrophils is inhibited, resulting in lack of O$_2^-$ production. CGD patients present with recurrent
severe infections that may lead to life-threatening sepsis (37). The functional enzyme is a multiunit protein: a membrane bound complex - flavocytochrome b558, comprised of 2 proteins (gp91, p22), and a cytosolic complex, comprised of 3 proteins (p67, p47, p40). Small GTP-binding protein Rac2 has also been shown to have an essential role in NADPH oxidase activation in human neutrophils. Upon stimulation, the cytosolic complex and the Rac2 GTPase migrate to the cell membrane where it binds to the flavocytochrome b558 complex for the assembly and activation of the enzyme (37). Activation of NADPH oxidase results in rapid $O_2^-$ production. $O_2^-$ is then rapidly converted to $H_2O_2$, a reaction that is mediated by SOD. Both $O_2^-$ and $H_2O_2$ are key components of the oxygen-dependent bacterial killing process, suggesting that NADPH oxidase activation is essential for neutrophil antibacterial activity (46). This transient process is termed “respiratory burst process” and is characterized by increased $O_2$ consumption and NADPH oxidase activation (Figure 2.1) (47).
Figure 2.1 – Superoxide generation by NADPH oxidase in neutrophil phagosome. Neutrophil activation by bacteria or bacterial products will result in superoxide generation and subsequent ROS production through the assembly and activation of the NADPH oxidase enzyme. Upon stimulation, the cytosolic complex and the Rac2 GTPase migrate to the cell/phagosome membrane where they bind to the flavocytochrome b558 complex. Abbreviations: NADPH - nicotinamide adenine dinucleotide phosphate; Rac - subclass of Rho family of GTPases; \( \text{O}_2^- \) - superoxide; \( \text{H}_2\text{O}_2 \) - hydrogen peroxide; \( \text{OH}^- \) - hydroxide molecule; iNOS - inducible nitric oxide (NO) synthase; ONOO\(^-\) - peroxynitrate; MPO - myeloperoxidase; HOCl - hypochlorous acid; Figure adapted with permission from creator Dr. C. Sima.
During NADPH oxidase activation, large quantities of O$_2^-$ are produced in a short amount of time – 3-6 nmol/min per 10$^6$ cells for an average of 2 minutes when exposed to formylmethionine leucyl-phenylalanine (fMLP) (48). This efficient process may result in excess O$_2^-$ production, which can lead to increased oxidative stress and irreversible lipid, protein and nucleic acid degradation. Several control mechanism are suggested to regulate the enzyme’s deactivation (47): (i) Myeloperoxidase (MPO), an abundant neutrophil enzyme, produces hypochlorous acid through its reaction with H$_2$O$_2$. MPO and its by-products have been suggested to be involved in NADPH deactivation. (ii) Cytoskeletal interactions during NADPH oxidase activation were also demonstrated to play a role in the enzyme’s deactivation, although this mechanism is not fully understood. (iii) Dephosphorylation as well as hyperphosphorylation of the NADPH oxidase subunits. (iv) Small GTP-binding protein Rac2 is a key component of the activated enzyme. GTP hydrolysis during the enzyme’s activation was suggested to mediate its deactivation. (v) Spontaneous enzyme degradation was demonstrated in cell-free systems, yet the physiologic relevance of this process has not yet been confirmed. Other possible mechanisms were suggested as well. It is possible that different deactivation mechanisms apply in different conditions (47).
Figure 2.2 – Cellular ROS Production. ROS are generated by several intracellular organelles. NADPH oxidase, through the oxidative burst process, is the predominant $\text{O}_2^-$ producing mechanism, contributing to increased ROS levels and oxidative stress-mediated damage of cells.
Figure 2.3 – ROS pathway in phagocytes. Intracellular $O_2^-$ is the precursor of all other ROS. $O_2^-$ can generate ONOO$^-$ through its reaction with Nitric Oxide (49), or it can be dismutated to $H_2O_2$ by SOD (34). $H_2O_2$ may produce hypochlorous acid through its reaction with myeloperoxidase, or, in the presence of transition metals, give rise to the highly reactive HO$^·$ radical. This reaction is known as the Fenton reaction, and is a rate limiting step in the formation of highly reactive ROS (50). Originally described with iron ($Fe^{2+}$) molecules, Fenton-like reactions can be carried out in the presence of other metal molecules, such as copper ($Cu^{+}$) (50). Regardless of the participating metal, the Fenton reaction results in the production of HO$^·$ radicals, which have the potential to cause direct DNA damage, as well as producing lipid radicals which will result in lipid peroxidation (34). SOD1 - Superoxide dismutase 1; HClO - hypochlorous acid; MPO – Myeloperoxidase; PRDx – Peroxiredoxin; GPx - Glutathione peroxidise
The destructive potential of ROS produced or released excessively intra and/or extra-cellularly, has been demonstrated in many pathologic conditions. Importantly, the ROS formation/release pathway is tightly regulated by AO activity (see section 2.3). Prior to the discussion of ROS-mediated pathogenic mechanisms, one should recognize the significance of ROS in physiologic processes.

### 2.2.2 Physiologic role

ROS participate in many intracellular processes, which are regulated by redox signaling. Redox signaling relates to a regulatory process where the signal is delivered through redox reactions. Under physiologic conditions the oxidative state of a cell is kept within a narrow range through regulators such as glutathione (GSH) and thioredoxin. The ratios of their reduced to oxidized states are maintained by GSH reductase and thioredoxin reductase, which act as oxidative buffers (34). Temporary changes in ROS/AO balance towards a more oxidative state will result in activation of specific cellular defense mechanisms. At the same time, the increase in oxidative stress activates several AO pathways, which aim to induce an AO protective response (34). A brief discussion of a number of ROS-regulated processes is presented below:

*Cell signaling* (signal transduction) is believed to be tightly regulated in oxidative stress conditions. In order to maintain signal transduction pathways, cells generate low concentrations of ROS through the oxidative burst process (51). Cytokines and growth factors stimulate signal transduction pathways through the tyrosine kinases receptors or through non-receptor protein kinases. ROS generated following cell signaling activation are believed to function as true second messengers, and participate in the activation of mitogen-activated protein kinases (MAPKs) signalling pathways and other ROS-mediated pathways that are involved in several processes including cell including proliferation, differentiation, and apoptosis (34,35).
**Intrinsic apoptosis pathway** is commonly activated following ROS-mediated intracellular damage. In general, a shift towards oxidative stress in the cell leads to apoptosis or necrosis (34). ROS can mediate apoptosis by regulating the expression of various apoptosis regulatory proteins. Bcl-2, a mitochondrial membrane-bound protein, is considered one of the major intrinsic apoptosis regulators (52). Several studies found that both O$_2^-$ and H$_2$O$_2$ are involved in Bcl-2 degradation, which enhances apoptosis (52,53).

**Neutrophil chemotaxis** was also demonstrated to be a ROS-dependent process (54). NADPH oxidase activity is required for efficient neutrophil chemotaxis. Reduced ROS levels was shown to affect directional migration towards chemoattractants (54). This was further validated in neutrophils isolated from CGD patients, which had severe chemotaxis defects, affecting both directionality and speed of migration (54). Previous work in our lab demonstrated NADPH oxidase regulation of directional migration using murine models of inflammation in mice knocked out for Rac GTPases, critical for NADPH oxidase complex assembly (55).

### 2.2.3 Mechanisms of cellular and tissue damage

Oxidative stress due to direct (excess production) or indirect (deficient neutralization) increased ROS levels, may lead to ROS-mediated damage through oxidation of cellular constituents including nucleic acids, lipids and proteins.

**DNA damage** is facilitated mainly by the hydroxyl radical (HO$^-$), damaging both the purine and pyrimidine bases (34). Other mechanisms include degradation of bases, single- or double-stranded DNA breaks, mutations, deletions or translocations, and cross-linking with proteins (35). ROS-mediated DNA damage was shown to be involved in many ROS-related pathologies, including carcinogenesis, aging, and neurodegenerative, cardiovascular, and autoimmune diseases (35). The interaction of HO$^-$ with nucleic acid bases leads to the formation
of 8-hydroxy-deoxyguanosine (8-OHdG) (41). 8-OHdG is recognized as a common marker for ROS-mediated damage (35,41), and gingival crevicular fluid (GCF) levels of 8-OHdG were suggested as biomarker for ROS-mediated DNA damage in the gingival tissues, and an indicator for periodontal disease severity (56). Formation of 8-OHdG in transcription factor binding sites can modify binding of transcription factors and thus change the expression of related genes (35).

**Lipid peroxidation** involves mainly polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. Following peroxidation, proteins are more susceptible to proteolytic degradation (34). ROS-mediated lipid peroxidation processes may disrupt the cell membrane lipid bilayer arrangement. This results in inactivation of membrane-bound receptors and enzymes, and increased membrane permeability (35). Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are common lipid peroxidation products, with HNE being the major toxic product of this process (34,35). The concentration of carbonyl groups is commonly used as an indicator for ROS-mediated lipid oxidation (34).

**Protein peroxidation** occurs through various mechanisms during oxidative stress conditions: fragmentation of the peptide chain, cross-linking, alteration of electrical charge of proteins, and oxidation of specific amino acids; all of which are ROS-mediated mechanisms that may lead to increased susceptibility to proteolysis by specific proteases (35). Cysteine and methionine residues are particularly more susceptible to oxidation (35).

The cumulative effect of the cellular ROS mediated damage contributes to tissue degradation and oxidative stress has been suggested as direct cause for tissue breakdown (Figure 2.4) (19). A brief review of the pathogenic role of oxidative stress in inflammatory diseases and in bone metabolism will be further discussed bellow.
Neutrophils are the main cellular component of the innate immune system, and as such, play a key role in inflammatory responses to non-self. Therefore, the inflammatory response is characterized by an increased influx of neutrophils at sites of stimulation. Neutrophils, a major source of ROS, contribute to increased oxidative stress during inflammatory conditions through two main mechanisms (57): First, NADPH oxidase activation results in generation of O₂⁻ and secondary ROS used in neutrophil antibacterial activity. Second, ROS mediate signal transduction that activates pro-inflammatory pathways. It has also long been recognized that increased ROS during the inflammatory process can lead to injury of the surrounding tissues (58). Indeed, neutrophil-derived ROS, including O₂⁻, H₂O₂, and HOCl were all associated with tissue damage seen in multiple acute and chronic inflammatory conditions (57).
Figure 2.4 – Changes in oxidative state and its biological effect. Adapted from (19).
In addition to their impact on tissue integrity at sites of inflammation, recent studies have pointed to a role of ROS in inflammation regulation. It was shown that ROS can act as second messengers during inflammasome activation (59). Inflammasomes are multi-protein oligomer platforms which play critical roles in initiating and sustaining inflammation (60). Their activation is characteristic to many conditions with an underlying inflammatory component (59).

ROS and oxidative stress involvement in bone metabolism and related pathology was previously described in the literature. In brief, ROS contribute to uncoupling of bone formation by osteoblasts and resorption by osteoclasts directly or indirectly through upregulation of receptor activator of nuclear factor-κB ligand (RANKL). This is believed to occur through modulation of kinases and transcription factor activities in both osteoclasts and osteoblasts (61). Rheumatoid arthritis, the most studied osteolytic inflammatory disease, is characterized by increased cellular ROS production in synovial cells at diseased sites (62). Similar to rheumatoid arthritis, CP is an inflammatory disease characterized by inflammation-mediated bone loss. ROS involvement in periodontal disease will be discussed in the next section.

2.2.4 Reactive oxygen species in periodontitis

As discussed earlier in this chapter, CP is an inflammatory disease affecting tooth-supporting structures (2). Due to increased bacterial load in CP compared to health, neutrophils are constantly recruited and activated in periodontium. When neutrophil-derived factors are produced in excess in periodontal tissues, they contribute to tissue breakdown. The pathogenic shift of subgingival biofilms seen in CP is controlled, in part, by this constant influx of neutrophils in the gingival crevice (63). Therefore neutrophils play a key role in the pathogenesis of periodontitis in the gingival crevice and epithelium, where they are the dominant inflammatory cell (64). Neutrophil contribution to oxidative stress through oxidative burst has been suggested
as a key mechanism in periodontal diseases pathogenesis and periodontal tissue damage (64,65). Therefore, the investigation of oxidative stress during CP has focused mainly on neutrophil function. However, what remains unclear is the relative importance of neutrophil ROS-mediated oxidative damage through excess ROS production per cell in the tissue, due to increased numbers of neutrophils in the tissue producing normal levels of ROS and the inability to neutralize ROS through AO activity locally.

Most of the studies investigating neutrophil function in CP were conducted on peripheral blood neutrophils (19). Several of these studies demonstrate consistent, significant increase in intracellular ROS production by peripheral blood neutrophils in patients diagnosed with CP compared to healthy controls. This suggests a hyperactive peripheral blood neutrophil phenotype in CP patients (66-69). One study reported increased extracellular ROS production by peripheral blood neutrophils in response to FcγR stimulation despite similar baseline ROS levels (19) Interestingly, it was also demonstrated that neutrophil hyperactivity (as measured by peripheral blood ROS levels) is not altered by periodontal treatment (66,68,70). This suggests that a hyperactive neutrophil phenotype may be an inherited trait rather than an acquired one, and a pre-existing determinant for CP. This is further supported by the observation that peripheral blood neutrophils from CP are primed to a pro-inflammatory phenotype, which may lead to hyper-responsiveness upon stimulation (71,72)

Yet the investigation of peripheral blood neutrophils may not provide a full understanding of the neutrophil’s roles in CP pathogenesis. The observation of different neutrophil phenotypes in tissues compared to peripheral blood has been reported by numerous studies (73,74). However, oral neutrophil function in CP and the possible role it plays in periodontal tissue destruction has not been extensively investigated. This site-specific neutrophil phenotype may be critical to the impact these cells have on diseased periodontal
tissues. Furthermore, previous work in our lab demonstrated site-specific gene expression in oral neutrophils obtained from healthy subjects, when compared to peripheral blood neutrophils (75). Nonetheless, gene expression of oral neutrophils obtained from CP patients demonstrated significant changes compared to healthy controls in favour of a pro-survival neutrophil phenotype (76).

In contrast to the limited studies on oral neutrophil function in CP, ROS-mediated damage during CP has been studied by various techniques, utilizing several ROS-mediated damage markers. Lipid peroxidation was studied through MDA and HNE levels during CP: Increased MDA and HNE levels in GCF and saliva samples of CP patients when compared to periodontally healthy controls were previously reported (77-80). Protein oxidation was measured in whole saliva samples of patients with CP of differing severity levels (81). Protein carbonyl concentration was measured as a marker for oxidative injury. Severe CP patients presented with increased concentrations of protein carbonyls in whole saliva samples compared to patients with moderate and mild CP (81). Quantification of 8-OHdG is used as measure of ROS-mediated damage to DNA. Whole saliva samples obtained from periodontitis patients present significantly higher levels of 8-OHdG compared to healthy control subjects (79,82). Similar findings were reported in GCF samples (56) and in gingival blood samples (83). Non-surgical periodontal treatment resulted in a significant decrease in GCF levels of 8-OHdG and correlated with healing and stability 3 months post-treatment, suggesting that cells damaged through DNA oxidation in inflamed periodontal tissue were the source of high GCF 8-OHdG (56). Whether local oxidative damage is due to neutrophil dysfunction in maintaining redox balance remains unknown.
2.3 Antioxidant species

The potential of ROS to generate tissue damage implies either high cellular production and release or low levels of scavengers (AO) to maintain pathologic intra- and extracellular ROS levels. The AO defense system plays an important role in balancing physiological oxidative state (35,84). Generally, AO are divided into two main categories: enzymatic and non-enzymatic (See table 2.1). AO can have rescuing functions through scavenging capacities manifested mainly in the extracellular environment or preventative functions manifested primarily through reduction of intracellular ROS. The latter have greater effect on cell function (19).

Table 2.1. Enzymatic and non-enzymatic antioxidants

<table>
<thead>
<tr>
<th>Enzymatic Antioxidants</th>
<th>Non-Enzymatic Antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Superoxide dismutase (SOD)</td>
<td>• Glutathione (GSH)</td>
</tr>
<tr>
<td>• Catalase (CAT)</td>
<td>• Ascorbic acid (Vitamin C)</td>
</tr>
<tr>
<td>• Peroxiredoxin</td>
<td>• α-tocopherol (Vitamin E)</td>
</tr>
<tr>
<td>• Thioredoxin</td>
<td>• Carotenoids (β-Carotene)</td>
</tr>
<tr>
<td>• Glutathione peroxidase (GPx)</td>
<td>• Flavonoids</td>
</tr>
<tr>
<td>• Glutathione transferase</td>
<td>• Uric acid</td>
</tr>
</tbody>
</table>

Within the non-enzymatic AO, glutathione (GSH) has the most significant impact on cellular oxidative state, and is the most common soluble AO in the cytoplasm, nuclei and mitochondria. GSH is a tripeptide and its oxidised form is glutathione disulphide (34). GSH regulates cellular oxidative stress and ROS-mediated signal transductions through several mechanisms. First, glutathione peroxidase (GPx) enzyme reduces H₂O₂ to H₂O. (Figure 2.2) (85). Second, GSH can regenerate AO vitamins C and E back to their active form, by reducing
semidehydroascorbate to ascorbate and tocopheroxyl to tocopherol (19). Through these same mechanisms GSH also regulates apoptosis and inflammatory pathways (19,35). Increased expression of GPx was demonstrated to be protective against ROS-mediated apoptotic stimuli. Furthermore, oxidant-dependant tissue injury was shown to increase in GPx null mice (85).

Among the enzymatic AO, superoxide dismutase (SOD), catalase (CAT) and GPx were most studied. The dismutation of $O_2^-$ to $H_2O_2$ is carried out by SOD, making SOD serve as the front-line defense against ROS. SOD has three isoforms: SOD1 (Cu/ZnSOD. Half-life of 6–10 minutes) – the major intracellular SOD; SOD2 (MnSOD. Half-life of 5–6 hours) – mitochondrial SOD; and SOD3 (ecSOD) – extracellular SOD (86). SOD1 is localized mainly in the cytosol, but was also demonstrated in the intermembrane space of mitochondria, nuclei, lysosomes, and peroxisomes. Its activity depends on the presence of copper (Cu$^+$) and Zinc (Zn$^+$) (86). Its high concentration in the cytosol makes it the main regulator of $O_2^-$ dismutation in the intracellular compartment. SOD1 was demonstrated to be upregulated in activated cells, in order to generate $H_2O_2$ and activate ROS-dependent cellular signals (86). Localized at the mitochondrial matrix, manganese (Mn)-containing SOD2 is produced in the cytoplasm and migrates into the mitochondria. It reduces $O_2^-$ generated by the mitochondrial respiratory chain. Its essential preventative role is demonstrated in early post-natal death of mice who had complete SOD2 depletion (86). SOD3, a secretory extracellular Cu/Zn-containing SOD is localized mainly in the extracellular matrix and on cell surfaces. It is mainly expressed in specific tissues such as blood vessels, the lung, kidney, uterus, and heart, and is upregulated in inflammatory cells during tissue injury (86). Similar to SOD2, SOD3 depletion in mice resulted in lethal rate of 85% one week following depletion (86).

Catalase (CAT) is the main $H_2O_2$-reducing enzyme catalyzing a reaction that produces $H_20$ and $O_2$ (87,88). Its tetrameric structure is very stable, allowing the enzyme to function in
wide pH and temperature ranges and have high resistance to enzymatic proteolysis (87). These properties suggest a critical function of CAT under stress conditions to reduce oxidative stress. Reduced CAT levels have been associated with several ROS-mediated pathologies such as atherosclerosis, development of diabetes, and schizophrenia (87). Its role in CP pathogenesis, as well as other enzymatic AO, will be discussed below (Section 2.3.1).

Several factors determine the efficiency of AO activity: location of action (intra/extracellular/membrane bound) and the nature of ROS challenged. Within these criteria, AO reducing upstream ROS (such as SOD, CAT, and GPx) will have a more pronounced effect on the entire ROS pathway (35). SOD, CAT and GPx, as well as some other enzymatic AO, are regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2), a critical transcription factor that regulates several important cytoprotective and oxidative stress response mediators (89,90). Nrf2 pathway is considered essential for antioxidant defence (91). Under physiologic conditions, Nrf2 is inactive through its direct interaction with the Nrf2 inhibitor Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. Once exposed to oxidative stress conditions, Nrf2 is released from Keap1 and migrates into the nucleus. Nrf2 is then free to bind to different transcriptional activators which then bind to the antioxidant-response element (ARE) to activate the transcription of a wide array of cytoprotective proteins (89). Nrf2 activity provides protection during oxidative stress conditions, and increases cell survival (90). Nrf2 activation was demonstrated in various pathologic conditions, including pulmonary, gastrointestinal and renal inflammation, atherosclerosis, and rheumatoid arthritis (92). Conversely, its down regulation was previously shown to contribute to oxidative stress and inflammation in various pathologic conditions (92).
2.3.1 Antioxidants in periodontitis

Most studies investigating the protective role of AO in periodontitis focus on total antioxidant capacity (TAOC) in serum, whole saliva and gingival crevicular fluid (GCF) samples. TAOC records extracellular AO, and whole saliva TAOC is mainly affected by uric acid concentration, the most abundant extracellular AO in whole saliva (93,94). Several studies demonstrated significantly lower TAOC in whole saliva (81,95) and serum samples (96) from CP patients compared to periodontally healthy controls. Others reported no differences in whole saliva and serum TAOC levels between CP patients and periodontally healthy controls (97,98), while some reported opposing results (99). GCF TAOC was demonstrated as an accurate indicator of the periodontal health (100). GCF TAOC levels were found to be significantly lower in periodontitis patients when compared to healthy controls (97,101).

Analysis of intracellular AO levels and activities in periodontal diseases was also investigated in previous studies. Reduced glutathione (GSH) levels were reported in whole saliva samples of CP patients when compared to periodontally healthy controls (77). Glutathione peroxidase (GPx) levels and activity in CP was also reported in several studies. Significant increased in GPx activity was reported in interproximal periodontal tissue biopsies from sites with CP compared to healthy sites (102). Most found reduced GPx salivary levels (98) and activity (79) in CP compared to health. Some reported similar GPx activity in whole saliva samples of CP patients and periodontally healthy controls (77). On the contrary, GCF levels of GPx were demonstrated to increase in CP patients when compared to healthy controls (103).

SOD activity was previously assessed in whole saliva and serum samples of CP patients as well. Reduced SOD activity in whole saliva samples of CP patients was reported (79,99,104). Reduced SOD activity in serum was also reported (104). Opposing results were also reported in
whole saliva (80,98), GCF and serum samples (80), as well as in gingival biopsies of patients with severe CP (105). The latter also reported no significant differences in SOD activity levels in GCF samples of CP patients and periodontally healthy controls (105). Following periodontal treatment (either surgical or non-surgical) SOD activity levels increased in both whole saliva (98,104) and serum (104).

These contradicting findings may be in part due to different experimental protocols and in part due to the fact that AO expression in the oral environment of CP patients at a specific point in time may reflect disease activity or remission. Existing evidence with regard to CP pathogenesis suggests that CP progression follows a pattern of apparently random episodes of activity when tissue breakdown occurs and remission when some healing/repair takes place.

Regardless of conflicting results in oral AO activity in CP the protective role of SOD and GPx activity is suggested by the negative correlations found between salivary levels of ROS-mediated tissue damage markers (MDA and 8-OHdG) and salivary SOD and GPx activities (79). The protective role of CAT in maintaining periodontal health was demonstrated in acatalasemia (Takahara's disease), a hereditary CAT deficiency. Acatalasemic patients are systemically healthy, but present with an increased incidence of periodontal infection (106). Similarly, acatalasemic mice developed normally, but are more prone to oxidant tissue injury (107). However, total CAT activity measured in interproximal tissue biopsies was found similar in CP patients and periodontally healthy controls (102).

Clearly, existing data does not provide conclusive evidence of the specific role TAOC or local periodontal AO in the progression of periodontal disease. Testing the hypothesis that reduced oral neutrophil AO production significantly contributes to disease activity and progression in CP will further our understanding of neutrophil biology in pathogenesis of inflammatory periodontal diseases.
Chapter 3

Increased abundance of salivary cytoprotective proteins during experimental gingivitis

G.M. Aboodi, C. Sima, E. Moffa, Y. Xiao, W.L. Siqueira, M. Glogauer. Increased abundance of salivary cytoprotective proteins during experimental gingivitis (Manuscript under review)

3.1 Abstract

Objective: The aim of this study was to identify changes in the salivary proteome during experimental gingivitis (EG).

Background: Host-related factors are considered to be responsible for heterogeneity in responses to bacterial biofilms. The protective mechanisms that maintain periodontal homeostasis in gingivitis and prevent periodontal tissue destruction are poorly understood.

Methods: The EG study was carried in 5 periodontally healthy individuals. EG subjects were asked to refrain from all oral hygiene procedures for 21 days and then resume normal OH. They were followed for another 14 days. Days 0, 21 and 35 were considered baseline and end points for the inflammatory and resolution phases of gingival inflammation respectively. Oral neutrophils and whole saliva samples were collected on days 0, 7, 14, 21, 28 and 35. Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry was completed for identification and quantification of protein content in pooled whole saliva samples.

Results: Recruitment of neutrophils in the oral cavity increased significantly by day 21. There was a significant increase in cytoprotective proteins through day 21 and a concordant decrease through day 35. These included proteins involved in inflammation regulation, antibacterial
activity, antioxidant and protease inhibitor proteins. Oral neutrophil numbers in gingival inflammation and resolution correlated moderately with salivary β-globin, thioredoxin and albumin and strongly with collagen alpha-1(XXVII) chain and G-protein coupled receptor 98.

Conclusions: We demonstrate an increase and subsequent decrease in cytoprotective proteins during the inflammatory and healing phases of gingivitis. The observed increase in cytoprotective proteins during gingivitis may prevent periodontal tissue destruction and clinical attachment loss. Changes in salivary cytoprotective proteins were associated with a similar trend in oral neutrophil recruitment and clinical parameters. Further investigation of the proteins identified in this study may suggest biomarkers for periodontal disease progression, as well as developing novel therapeutic approaches.
3.2 Introduction

Periodontal diseases are a diverse group of conditions that affect the tooth-supporting tissues (108). Differentiation is made between gingival diseases which affect the gingival tissue alone, and diseases that affect the periodontium, including the gingiva, periodontal ligament, cementum and alveolar bone (4). In its common form, periodontal disease is a destructive inflammatory disease caused by the subgingival bacterial biofilm, which initiates an acute inflammatory response. Clinically, there is significant heterogeneity in patient responses to the subgingival biofilms primarily due to host factors (108).

The mechanisms involved in periodontal disease progression from its reversible phase (gingivitis) to CP are poorly understood (108). Page & Schroder defined the different histologic stages of the periodontal lesion (9). The initial, early and established lesions present similar clinical findings to those of gingivitis, and correspond to the clinical progression of gingivitis (6). On the other hand, the timeline for the development of the advanced lesion was termed “unknown”. Page & Schroeder concluded that the established lesion may remain stable and might not progress to an advanced lesion at all (9). This was clinically demonstrated in the Sri Lankan tea labourers studies of the natural history of periodontal diseases (17). Within the study population of 161 non-treated subjects, 11% demonstrated no progression of periodontal disease beyond gingivitis while 81% demonstrated moderate progression and 8% demonstrated rapid progression in a 15-year interval.

Defining the temporal relationships between offensive and protective host factors in progression of gingivitis is essential for understanding rate limiting steps in chronic inflammation of periodontal tissues. The role of specific proteins in the pathogenesis of periodontal disease has been documented. However, the development of proteomics
techniques allows us to identify and quantify large numbers of proteins. Two main quantitative proteomics techniques are available: isotope labelling and label-free techniques. Though the results obtained with the isotope labelling technique are considered to be more accurate, this method is time-consuming and costly, and limits the number of samples that can be analysed. The label-free techniques have become significantly more accurate, and allow researchers to identify and quantify large numbers of proteins. The spectral counting approach, a label-free proteomics technique, is based on counting the number of peptides assigned to a protein in a mass spectrometry (MS) experiment. In brief, more abundant proteins will produce a higher abundance of MS/MS spectra, which is reflective of the protein levels in the sample (109).

Utilizing proteomics in periodontal disease research may reveal changes in the protein profile (proteome) during disease progression and the identification of disease-specific biomarkers (110). Whole saliva and gingival crevicular fluid (GCF) samples were used for proteome analysis during gingivitis (111-113) and CP (110,114-117). While GCF and tissue samples provide site-specific information, salivary proteome analysis provides a comprehensive approach to oral health status, as whole saliva proteins are secreted from numerous sources (salivary glands, mucosal cells, immune cells, serum and bacteria). Blood proteins levels were reported to increase in both CP and gingivitis patients (113,114), confirming the inflammatory nature of both diseases.

The purpose of the current investigation was to identify changes in proteome profile during the development, progression and resolution of gingivitis in an EG model. Findings of specific cytoprotective proteins and their correlation with clinical parameters and oral neutrophil numbers may suggest different protective mechanisms during gingivitis that prevent tissue destruction and attachment loss, and will generate further studies of disease biomarkers and new therapeutic approaches.
3.3 Materials and methods

3.3.1 Study design

Twenty-one day experimental gingivitis (EG) model was used to investigate protein dynamics at health (day 0 of experimental gingivitis) and gingivitis (day 21 of experimental gingivitis). Study design and goals were presented to all participants, and consent was obtained in writing. The study was approved by the Scientific and Ethics Review Boards at the University of Toronto (protocols #24295/#24567) and conducted in the Graduate Periodontics Clinic at the Faculty of Dentistry from September – December 2012. Five periodontally healthy participants (2 males, 3 females, age range 20-36) completed EG trial. All study participants were systemically healthy and non-smokers. Participants completed baseline periodontal exam and professional scaling by a registered dental hygienist, followed by 7 days of enhanced oral hygiene (pre-study hygiene phase). On day 0 of EG, full periodontal exam was completed, and participants were asked to refrain from any oral hygiene procedures (including brushing, flossing, use of mouthwash, and gum chewing) for the remainder of the trial. EG inflammatory phase was concluded after 21 days. Participants received scaling and oral hygiene instructions. Participants were followed for two weeks during healing phase (See figure 3.1 for trial flow chart). During the trial, participants were followed weekly. Full periodontal exam and sample collection was completed at each visit.

EG participants were used both as healthy control group (EG0 - day 0 samples) and gingivitis group (EG21 – day 21 samples).
Figure 3.1 – Experimental gingivitis trial design flow chart (days).
3.3.2 Oral neutrophil quantification

Oral neutrophil quantification was completed utilizing hemocytometer technique, as previously described (118). Rinse samples for oral neutrophil cell counts were collected prior to any periodontal instrumentation to avoid sample contamination with blood. Patients were asked to rinse with 10mL of 0.9% isotonic sodium chloride solution (Baxter, Toronto, ON) for 30 seconds, and then expectorate into a 50mL polypropylene tube (Sigma-Aldrich, St. Louis, MO). A 500-μl sample was separated into an Eppendorf tube and fixed with 50μl 37% formaldehyde (Sigma-Aldrich, St. Louis, MO). Samples were kept in 4° C until analysis. All cell counts were completed by the same examiner (GMA). Samples were centrifuged at 1139 relative centrifugal force for 5 minutes (Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was removed and the pellet was resuspended in 100μL of phosphate buffered saline (PBS. Sigma-Aldrich, St. Louis, MO). 1μL acridine orange (Sigma Chemical, Burlington, ON, Canada) was added to the cell suspension. Acridine orange is a fluorescent nucleic acid marker. Its interaction with DNA and RNA allows the identification of neutrophils under fluorescence microscope. Following acridine orange staining, samples were incubated, light protected, for 15 minutes at room temperature. A 10μL aliquot of this suspension was loaded on to a hemocytometer (Bright-Line; Hausser Scientific, Horsham, PA, USA), and the neutrophils were visually counted using fluorescence microscopy (Leitz Orthoplan Microscope; Leitz, Wetzlar, Germany). Neutrophils were counted and quantified based on the standard protocol for hemocytometer use.
Figure 3.2 – Oral neutrophil isolation and investigation. Oral rinses with of 0.9% saline solution were used to isolate oral neutrophils. Sequential filtering through 40μm, 20μm and 11μm filters removed debris and epithelial cells from the rinse. This resulted in 98% purity of oral neutrophil samples. Neutrophil RNA was further isolated for microarray and qRT-PCR assays and proteins isolated for western blot analysis of AO production. Oral neutrophils isolated through this method can also be used for functional assays such as stimulated ROS production.
3.3.3 Whole saliva sample collection

Stimulated whole saliva samples were collected prior to any periodontal instrumentation to avoid sample contamination with blood. Chewing has been demonstrated to increase GCF flow, therefore subjects were asked to chew on a 5X5cm parafilm (1.4g, Parafilm M, Brand, Wertheim, Germany). This allowed for an increased GCF component in the analysed whole saliva samples (119). All EG sample collection took place in the morning, at the same time for each subject. Stimulated saliva in the first 30 seconds was discarded (swallowed). Patients were then asked to expectorate stimulated saliva into a 50ml polypropylene tube (Sigma-Aldrich, St. Louis, MO) until 15ml were collected. Saliva samples were placed on ice until aliquoting, which was completed within 3 hours of sample collection. Samples were aliquoted into 1ml portions. Three 1ml samples were kept at -80°C until analysis and analyzed as whole saliva samples. Six 1ml samples were centrifuged at 14,000g over 20 minutes (Eppendorf Centrifuge 5415R, Eppendorf, Hauppauge, NY). Supernatant was separated from pellet, and both were kept at -80°C until analysis. The remaining 6ml were stored at -80°C and were used as a reservoir for additional testing (120).

3.3.4 Sample preparation

Our goal was to establish a disease-specific proteome profile and a trend that characterizes the inflammatory and healing phases of gingival inflammation rather than individual investigations, therefore samples for each of the study groups were pooled together (121). As previously described (117), pooled samples were denatured and reduced for two hours by buffer containing 4M urea, 10mM dithiothreitol, and 50mM ammonium bicarbonate, pH 7.8.
Following dilution with 50mM ammonium bicarbonate and the addition of 2% w/w sequencing-grade trypsin (Promega, Madison, WI), tryptic digestion was carried out for 18 hours at 37°C.

### 3.3.5 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

Samples were dried and re-suspended in acetonitrile/formic acid buffer (2.4% and 0.1% respectively), then subjected to reverse LC-ESI-MS/MS. Peptide separation and mass spectrometric analyses were completed using a nano-HPLC Proxeon (Thermo Scientific, San Jose, CA), which was linked to LTQ Velos mass spectrometer (Thermo Scientific, San Jose, CA). Electrospray voltage and the temperature of the ion transfer capillary were 1.8 kV and 250°C, respectively.

The obtained MS/MS spectra were searched against human protein databases (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA). Search results were filtered for a false discovery rate of 1% employing a decoy search strategy utilizing a reverse database. An additional inclusion criterion for positive identification of proteins was the same protein passing the filter score at least in two different MS analyses from a total of three MS analyses per condition.

### 3.3.6 Relative Proteome Quantitation and Statistical analysis

For quantitative proteome analysis, three MS raw files from each pooled group were analyzed using SIEVE technology (Version 2.0 Thermo Scientific, San Jose, CA, USA) as previously
described (121). Relative Proteome quantitations for day 0 vs. day 21 of EG, were carried out. EG0 samples were compared to each of the study groups. Initial alignment step was carried out using a single MS raw file belonging to the EG0 group. This file was selected as the reference file and all of other files were adjusted accordingly. Following the alignment, the feature detection and integration (or framing) process was performed through the “Frames from MS2 Scans” feature, using the MS level data. This framing process employs only MS mass-to-charge ratio (m/z) values that were associated with MS2 scan only. The parameters used consisted of a frame m/z width of 1500 ppm and a retention time width of 1.75 min. Peak integration was performed for each frame and these values were used for statistic analysis. Next, peptide sequences obtained from the database search using SEQUEST algorithm were imported into SIEVE. Peptides were grouped into proteins and a protein ratio and p-value were calculated. SIEVE uses a weighted average of the peptide intensities for the protein calculation. By using the weighted average, peptides with lower variance in their intensity measurements have a higher weight on the overall protein ratio. This is done to decrease variance in protein level quantities based on variance of the peptides that compose the proteins (121).
3.4 Results

3.4.1 Clinical findings – experimental gingivitis

As expected (6), increases in clinical inflammatory parameters and biofilm accumulation were evident in all EG participants by day 21, including Bleeding on Probing (BOP), and Gingival Index (122). Changes in clinical parameters were detected by day 7, and increased significantly by day 14 reaching a peak on day 21 and returned to baseline levels by day 35. BOP was significantly higher on days 14 and 21 compared to day 0 and 35 (day 0, 12.8 ± 1.6 %; day 7, 22.2 ± 4.4 %; day 14, 36.5 ± 4.6 %; day 21, 38.1 ± 5.1 %; day 28, 30.9 ± 5.2 %; day 35, 19.8 ± 1.4 %) (Figure 3.3). Similarly, gingivitis increased progressively during the inflammatory phase and decreased in the healing phase (day 0, 4.4 ± 1.3; day 7, 8.8 ± 0.7; day 14, 11.2 ± 0.4; day 21, 11.6 ± 0.24; day 28, 10 ± 0.7; day 35, 8.8 ± 0.4) (Figure 3.4).
Figure 3.3 – Bleeding on Probing trend in experimental gingivitis. Bleeding on probing (BOP) was measured at baseline (day 0) and on days 7, 14, 21, 28 and 35 during EG. Mean ± SEM; n = 5; One way ANOVA with Turkey-Kramer HSD, * p < 0.05 vs. day 0, # p < 0.05 vs. day 21.
Figure 3.4 – Gingival Index trend in experimental gingivitis. Gingival index (122) was measured at baseline (day 0) and on days 7, 14, 21, 28 and 35 during EG. Mean ± SEM; n = 5; One way ANOVA with Turkey-Kramer HSD, * p < 0.05 vs. day 0.
3.4.2 Oral neutrophil quantification

A significant increase in oral neutrophil numbers was found on day 21 of the inflammatory phase of gingivitis compared to baseline (Figure 3.5). Oral neutrophil counts decreased in the resolution phase by day 35, reaching levels close to baseline (day 0, 198 ± 40 x 10⁴; day 7, 322 ± 32 x 10⁴; day 14, 363 ± 27 x 10⁴; day 21, 722 ± 44 x 10⁴; day 28, 242 ± 73 x 10⁴; day 35, 220 ± 79 x 10⁴).

3.4.3 Protein identification EG0 vs. EG21

Data analysis revealed that 119 proteins showed significant level changes (at p<0.05) during EG. Twenty-one percent of these proteins (25 proteins) demonstrated a significant increase of at least 2 fold by day 21 compared to baseline (Table 3.1). Serum albumin levels identified in whole saliva samples significantly increased during the inflammatory EG phase (2.3 fold increase). Furthermore, collagen fragment abundance increased significantly (7.35 fold increase), in line with the histologic findings previously described (9).

Significant increases in several cytoprotective proteins were observed during the inflammatory phase, including proteins involved in inflammatory regulation (Annexin A1 and Vitamin D binding protein – 4.15 and 2.78 fold increase, respectively), antibacterial (Lactotransferrin – 3.5 fold increase), antioxidants (β-globin and Thioredoxin – 3.75 and 2.29 fold increase, respectively) and protease inhibitor proteins (Cystatin SN, Cystatin S – 2.33 and 2.14 fold increase, respectively) (Figure 3.6). Abundance of these cytoprotective proteins went back to baseline levels in the resolution phase of EG. Eighteen of the 25 salivary proteins that increased by >2 fold by day 21, followed a trend of reduction in the resolution phase. Aryl
hydrocarbon receptor repressor, cDNA FLJ3590 fis and lactotransferrin increased further by day 35 (Figure 3.7).

Oral neutrophil numbers correlated moderately with salivary AO β-globin and thioredoxin ($R^2 = 0.649$ and 0.564 respectively) and strongly with collagen alpha-1(XXVII) chain and G-protein coupled receptor 98 ($R^2 = 0.883$ and 0.884 respectively) in the inflammatory and resolution phases of gingivitis (Figures 3.8 and 3.9).
Figure 3.5 – Oral neutrophil quantification during experimental gingivitis. Oral neutrophils isolated from oral rinses collected on days 0, 7, 14, 21, 28 and 35 of EG were quantified using a hemocytometer. Cell numbers were normalized to the rinse volume collected. Mean ± SEM; n = 5; One way ANOVA with Turkey-Kramer HSD; * p < 0.05.
Table 3.1 – Proteins with significant increase of >2 fold during the inflammatory phase of EG.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein name</th>
<th>Fold change</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3KTH9</td>
<td>cDNA FLJ38275 fis, highly similar to GAS2-like protein 3</td>
<td>2.31</td>
<td>0.004</td>
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<tr>
<td>B4DSR5</td>
<td>Kinesin-like protein KIF3B</td>
<td>2.23</td>
<td>0.013</td>
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<tr>
<td>C3PTT6</td>
<td>Pancreatic adenocarcinoma unregulated factor</td>
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<td>0.012</td>
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<tr>
<td>C9JAJ5</td>
<td>Putative uncharacterized protein LOC349136</td>
<td>2.18</td>
<td>0.036</td>
</tr>
<tr>
<td>D3DRN4</td>
<td>KIAA1539, isoform CRA_a</td>
<td>4.72</td>
<td>0.003</td>
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<tr>
<td>E7EMQ1</td>
<td>Carbonic anhydrase 6</td>
<td>2.23</td>
<td>0.039</td>
</tr>
<tr>
<td>E7ETI5</td>
<td>G-protein-coupled receptor 98</td>
<td>3.41</td>
<td>0.018</td>
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<tr>
<td>E9PUJ5</td>
<td>Transmembrane protease serine 13</td>
<td>2.21</td>
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<td>Apolipoprotein A-I</td>
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<td>H0YD40</td>
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<td>Albumin</td>
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<td>P02774</td>
<td>Vitamin D-binding protein</td>
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<td>Lactotransferrin</td>
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<td>Annexin A1</td>
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<td>P10599</td>
<td>Thioredoxin</td>
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<td>Q9BZG5</td>
<td>Androgen receptor</td>
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</table>
Figure 3.6 – Cytoprotective proteins significantly increased during experimental gingivitis (day 21 versus day 0). Whole saliva samples were analyzed by LC-ESI-MS/MS and relative proteome analysis performed based on peptide sequences obtained from the database search using SEQUEST algorithm after peak integration from acquired frames and SIEVE-assisted recognition of proteins form peptide sequences.
Figure 3.7 – Time course of salivary protein levels for select proteins that increased by > 2 fold on day 21 versus baseline. With exception of aryl hydrocarbon receptor repressor, cDNA FLJ3590 fis and lactotransferrin all proteins with > 2 fold increase in the inflammatory phase were reduced by day 35, 2 weeks after oral hygiene was resumed.
Figure 3.8 – Oral neutrophil recruitment in the inflammatory and resolution phases of gingivitis was moderately correlated with salivary thioredoxin and β-globin levels. The graphs illustrate average fold changes on days 7, 14, 21, 28 and 35 relative to day 0.
Figure 3.9 – Oral neutrophil recruitment in the inflammatory and resolution phases of gingivitis was strongly correlated with salivary collagen alpha-1 (XXVII) chain and G-protein coupled receptor 98 levels. The graphs illustrate average fold changes on days 7, 14, 21, 28 and 35 relative to day 0.
3.5 Discussion

The comparison of proteome profile in EG0 and EG21 demonstrated that several salivary proteins increase by at least 2 fold during the inflammatory phase of gingivitis (Table 3.1). Among these proteins, we identified 10 cytoprotective proteins (Figure 3.6). The overall increase in these proteins involved in tissue protection and inflammatory control, suggests that activation of protective pathways, including antibacterial activity, regulation of the inflammatory process, antioxidants, and protease inhibitors characterizes host responses of healthy individuals to increasing bacterial burden on the gingiva (Figure 3.10). The moderate correlation between oral neutrophil numbers and β-globin and thioredoxin AO in the inflammation and resolution phases of gingivitis may be due to the pooled sample technique used. Another possible explanation may be that these specific AO may not be the primary source of protection against neutrophil-mediated oxidative damage in periodontal tissue inflammation.
Figure 3.10 – Suggested salivary cytoprotective mechanisms involved in gingivitis.
3.5.1 Antibacterial proteins: Lactotransferrin

Lactotransferrin (also known as lactoferrin, LTF) is an iron-binding protein (123,124), found in serum and secreted fluids, and also present in specific neutrophil granules (125). Several roles have been suggested for LTF. Its antibacterial properties result from bacteriostatic activity, directly related to its iron-chelating capacity; and bacteriocidal activity, as a result of a direct interaction between the protein and bacteria where the N-terminal region of LTF (known as lactoferricin) can disrupt or possibly even penetrate bacterial cell membranes (123). Anti-biofilm activity of LTF was also demonstrated, as it inhibits biofilm formation and reduces the established biofilm of oral bacteria at physiological concentrations (124,126). It has been speculated that LTF inhibits biofilm formation and disrupts existing biofilm by preventing bacterial adhesion or stimulating bacterial motility.

Similar to our current findings, significant increase in LTF abundance was previously demonstrated in GCF samples during EG (111). The reported antibacterial and anti-biofilm properties of LTF can explain its increased abundance during EG, as an innate protective response to bacterial accumulation. Due to these protective properties, LTF was suggested as a potential treatment for CP (126).

3.5.2 Inflammatory regulation: Annexin A1 and Vitamin D binding protein

As discussed above, inflammation can lead to tissue damage in periodontal diseases. Our results showed an increase in two inflammatory-regulating proteins during EG: Annexin A1 and Vitamin D binding protein. Annexins are a family of calcium and phospholipid binding proteins (127), which dampen the inflammatory response via inhibition of neutrophil activation
Annexin A1 levels in GCF were previously demonstrated to be stable during a 21 days EG model, opposing our current findings (112). One possible explanation is that we used whole saliva samples not GCF samples. Therefore, it is possible that salivary sources contribute to annexin A1 production and its involvement in regulation of gingival inflammation.

Vitamin D binding protein (DBP) is a multifunctional protein found in plasma. Its ability to bind to vitamin D (calcitriol) and derived metabolites, and to transfer them to target cells plays a major role in the involvement of vitamin D in inflammatory regulation. Vitamin D was shown to be involved in both the innate and adaptive immune systems, with vitamin D deficiency being linked to many inflammatory disorders, including periodontal diseases. It has been suggested that vitamin D may act similarly to cytokines, and regulate the inflammatory process by several mechanisms: stimulating phagocytosis and antibody-presenting actions to enhance the initial immune response. As the inflammatory process progresses, vitamin D plays a role in inhibition of T-cell proliferation and thus inflammation resolution (129). DBP was demonstrated to have significant neutrophil chemotactic activity in vivo where DBP knock-out mice demonstrated significant decreases in neutrophil recruitment to the site of inflammation when compared to the wild type group. Exogenous addition of DBP was shown to restore neutrophil response (130). Our observation of increased DBP levels in the inflammatory phase of EG may suggest an increased abundance of vitamin D during onset of inflammation. Indeed, vitamin D levels were found associated with reduced inflammation in gingivitis (131).

3.5.3 Antioxidants: β-globin and Thioredoxin

Increases in oxidative stress, where reactive oxygen species (ROS) levels exceed AO levels, have been shown to directly contribute to periodontal inflammation and connective tissue breakdown in periodontal disease. The AO defense systems have an important role in
balancing physiological oxidative stress (19). Total antioxidant capacity (TAOC) levels were found to be significantly lower in periodontitis patients compared to healthy controls (101). Our current findings demonstrate an increase in AO proteins - β-globin and thioredoxin during onset of gingivitis, suggesting an additional protective mechanism during this reversible phase of periodontal disease.

The globin superfamily of proteins includes hemeproteins that can be found in all known forms of life. Its role in O\textsubscript{2} transport in vertebrate erythrocytes is recognized as a relatively recent adaptation from its more primitive functions in non-erythroid cells, including iron metabolism regulation, intracellular oxygen transport, oxygen sensing, NO scavenging, and hydrogen peroxide scavenging (132). Several human studies demonstrated that hemoglobin overexpression reduces oxidative stress, suggesting its cytoprotective role as an AO (133,134). Increased β-globin levels were demonstrated in mice macrophages treated with lipopolysaccharide and interferon-γ (135). In-line with our current findings, salivary proteome analysis of samples collected from gingivitis patients revealed increases in both α- and β-globin compared to healthy controls (113).

Thioredoxin is an intra-cellular protein that, together with thioredoxin reductase and NADPH, is part of the thioredoxin system. This system has been shown to play a key role in many intra-cellular pathways including H\textsubscript{2}O\textsubscript{2} as a means to reduce oxidative stress (136). The cytoprotective effects of thioredoxin were demonstrated in transgenic mice overexpressing human Trx1 which are resistant to oxidative stress conditions, and are more resistant to inflammation (137). In humans, increased extracellular thioredoxin levels were reported for various systemic conditions, including rheumatoid arthritis (138). Our current results demonstrate an increase in thioredoxin during EG, suggesting a protective role during this reversible inflammatory phase.
3.5.4 Protease Inhibitors: Cystatin Family

Several proteolytic enzymes have been demonstrated to play a key role in periodontal tissue destruction. The main source for these enzymes are the neutrophils that populate the periodontal pocket and produce them as part of the non-oxidative killing function (139). We identified a significant increase in the protease inhibitor proteins Cystatin SN and Cystatin S during the inflammatory phase of EG.

The cystatin proteases consist of 12 intra- and extracellular protease inhibitors (140). They demonstrate high potency in inhibiting cysteine proteases - a large superfamily of proteases. Cysteine proteases play a key role in many physiological processes, however over-expression and increased activity was associated with immune-pathophysiological conditions and may mediate proteolysis and tissue damage (140,141). They were shown to be produced by perio-pathogenic bacteria, neutrophils and macrophages in periodontitis lesions (142,143). Cystatins SN and S were first identified in saliva, and have been identified in other secreted body fluids. Their production in secretory glands suggests their role as cytoprotective inhibitors of exogenous cysteine peptidases (140).

Cystatin S levels in GCF have been demonstrated to be stable during the 21 days EG model, which once again is in contrast to our current findings (112). In a different study, GCF proteome analysis reported decrease in cystatin S abundance in GCF samples obtained from gingivitis patients (144). As discussed above, salivary glands contribute to cystatin S production, which can explain the differences in our analysis of whole saliva samples, as opposed to GCF samples. In support of our findings, cystatin activity was demonstrated to increase in whole saliva samples obtained from gingivitis patients (145), as well as in inflamed gingival tissues (146).
The increase in cytoprotective proteins during the inflammatory phase of EG, suggests that production of proteins with potential to dampen the effector proteins involved in the inflammatory process is important during onset of inflammatory diseases including gingivitis (Figure 3.10). Similar findings were reported in a human tears study, which demonstrated increase in cystatin S and lactotransferrin levels in tears collected from patients with autoimmune conditions (141). Interestingly, reduced levels of several of these cytoprotective proteins were previously reported in patients diagnosed with CP.

Reduced cystatins (S, SN, SA) levels were previously reported in whole saliva samples obtained from CP patients when compared to healthy controls (114,147,148). Reports of LTF levels in CP were inconsistent: whole saliva proteome analysis demonstrated a 2-fold increase in LTF levels compared to healthy controls (116). Contrary to this report, low levels of LTF were seen in whole saliva samples obtained from patients diagnosed with Aggregatibacter actinomycetemcomitans-associated periodontal disease (149), and in whole saliva samples obtained from patients diagnosed with generalized AgP compared to healthy controls (150). The same study also showed increased vitamin D binding protein levels in generalized AgP compared to health (150).

Several theories may explain the reduced levels of protective proteins during CP: as the inflammatory process progresses, increased levels of endogenous and exogenous ROS and proteases may lead to damage of the cytoprotective proteins. A second theory suggests that once bacterial and inflammatory overload reach a specific threshold, the cytoprotective mechanisms are reduced. This threshold can vary between subjects, and may be responsible for the observed variability in the development of periodontal disease among subjects. The findings of the present study demonstrate that in the initial inflammatory phase of gingivitis levels of salivary cytoprotective proteins are increased and that they decrease as inflammation resolves.
3.6 Conclusion

As an exploratory investigation, our study has an inherent limitation of sample size. We were however able to demonstrate a significant increase in several cytoprotective proteins in the inflammatory phase of gingivitis. Further, we found that levels of these proteins decreased in the healing phase of gingivitis. The increase in cytoprotective proteins in onset of gingivitis may prevent periodontal tissue destruction and clinical attachment loss in patients at risk of developing periodontitis due to other host and environmental factors. Future studies to investigate whether these proteins can serve as biomarkers for gingivitis progression to periodontitis are necessary.
3.7 Acknowledgments

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Authors’ contribution:

C.S. – Assisted with graphics and data analysis

E.M. – Proteomics analysis, Western University, ON, Canada

Y.X. – Proteomics analysis, Western University, ON, Canada

W.L.S. – Proteomics analysis, Western University, ON, Canada

M.G. – Research supervisor

3.8 Conflict of Interest Statement

Authors report no financial or commercial relationship to any organization that may be affected by this study.
Chapter 4

Refractory Periodontitis Population Characterized by a Hyperactive Oral Neutrophil Phenotype


4.1 Abstract

Background: Neutrophils, in addition to being the primary protective component of the innate immune system, also contribute to periodontal destruction through production of ROS, which can cause damage to connective tissues and extra-cellular matrix following neutrophil activation. We have previously shown that peripheral blood neutrophils of patients diagnosed with refractory CP are hyper-responsive to stimulation. In order to test the hypothesis that oral neutrophil hyperactivity correlates with periodontal disease severity, we utilized a flow cytometric approach to isolate and analyze oral neutrophil ROS production in a refractory CP patient population.

Methods: Oral rinse samples and venous blood were obtained from 13 patients diagnosed with refractory CP. Following isolation of neutrophils from both samples, dihydorhodamine 123 was used as a fluorescent probe for Phorbol 12-Myristate 13-Acetate (PMA) mediated ROS production assessed by flow cytometry. For each patient, oral ROS production levels were expressed as a percentage of their baseline to maximal peripheral blood neutrophil ROS production.
Results: Two distinct groups of refractory CP patients were identified based on levels of PMA stimulated oROS production. The patient group with high oROS production had significantly more clinical attachment loss compared to the patient group with low oROS production.

Conclusions: Our findings demonstrate that a group of refractory CP patients with increased clinical attachment loss present with a hyperactive oral neutrophil phenotype characterized by increased potential for ROS production. Identification of this oral neutrophil phenotype could allow clinicians to identify patients who are more susceptible to disease progression despite conventional periodontal therapy.
4.2 Introduction

Conventional treatment for most cases of periodontitis is focused on removal of bacterial plaque and calculus – the primary environmental etiologic factors for inflammatory periodontal diseases. Mechanical debridement, combined with proper home care by the patient as well as elimination of other risk factors such as smoking, is generally associated with healing and satisfactory maintenance of periodontal health (2). However, clinicians are challenged by the fact that some periodontitis patients respond poorly to conventional periodontal therapy in spite of otherwise adequate treatment and improved home care. This intriguing and incompletely understood response to therapy leads to a diagnosis of refractory CP (26). Refractory CP patients present with continuing disease progression and attachment loss that does not correlate with plaque levels, microbiology assessments, and compliance to maintenance programs (27-29). Several studies indicated that up to 17% of patients with periodontitis will continue to experience attachment loss following treatment (30,151). It has been shown that refractory patients lose a significantly higher number of teeth during the maintenance phase compared to non-refractory periodontitis patients (29,30). Therefore, early diagnosis of refractory CP is essential in preserving dentition and maintaining periodontal health in this high risk group of patients.

The observation that refractory CP patients do not respond adequately to periodontal therapy suggests that the host immune response plays a significant role in progression of tissue breakdown in refractory CP (152). Neutrophils play a vital role in maintaining periodontal health through control of biofilm pathogenicity but they are also involved in the pathogenesis of periodontitis by releasing ROS and other toxic products, and producing pro-inflammatory cytokines (19,153,154). Neutrophils produce high levels of ROS upon stimulation in an attempt
to neutralize pathogens that invade host structures. Neutrophil ROS are generated through the process of respiratory burst as described in Chapter 2. ROS can contribute to periodontal attachment loss by direct damage to the extracellular connective tissue (both mineralized and unmineralized) (19).

Most studies on ROS production by neutrophils in periodontitis were carried on peripheral blood neutrophils. ROS production by peripheral blood neutrophils was found to be increased in patients diagnosed with CP indicating a hyperactive neutrophil state in these patients (66-69). Previous work in our lab demonstrated a hyperactive neutrophil phenotype in peripheral blood samples obtained from refractory CP patients (155). Interestingly, it was also demonstrated that neutrophil hyperactivity (as measured by peripheral blood ROS levels) is not altered by periodontal treatment (66,70). This suggests that a hyper-responsive neutrophil phenotype may be a pre-existing systemic risk factor for periodontitis.

Oral neutrophil ROS activity in periodontitis has been investigated to a less extent compared to peripheral blood neutrophils. Increased stimulated levels of ROS in GCF of CP patients compared to healthy controls were previously reported (156). It was also demonstrated that whole saliva samples obtained from periodontitis patients have significantly higher levels of 8-hydroxy-deoxyguanosine (8-OHdG) compared to healthy control subjects (82). As a marker for oxidative DNA damage, 8-OHdG levels suggest an increased ROS activity.

A simple method for isolation of oral neutrophils from oral rinse samples was described in 1989 (157). The method was based on rinsing the oral cavity with isotonic solution, followed by filtering out epithelial cells from the rinse sample using a nylon mesh. This resulted in a neutrophil-rich solution with over 95% neutrophils and 85% viability. Using the procedure described, several studies examined and characterized oral neutrophil ROS production in healthy subjects (157-161). It was demonstrated that oral neutrophils have higher ROS levels at
baseline when compared to peripheral blood neutrophils, and that they are able to respond to various types of stimuli (157,158,161). This higher ROS production at baseline is not surprising, given that oral neutrophils are constantly exposed to oral flora. However, the significance of oral neutrophils ROS generation in the pathogenesis of periodontitis is poorly understood.

The goal of the present study was to determine the correlation between oral neutrophil responsiveness to stimulation and periodontitis severity in refractory CP patients. As mentioned above, previous work in our lab demonstrated a hyperactive neutrophil phenotype in peripheral blood samples obtained from refractory CP patients (155). Characterizing oral neutrophil responsiveness to generate ROS is important to our understanding of local neutrophil contribution to pathogenesis of refractory CP. It may also serve as a diagnostic test to identify refractory CP patients. In this study we investigated oral neutrophil responsiveness to stimulation as measured by ROS production in a group of refractory CP patients, and correlated these results with history of periodontal breakdown.
4.3 Materials and Methods

4.3.1 Study population

Thirteen patients were enrolled in this study (6 males, 7 females, age range 32-73). All participants were diagnosed with refractory CP following regular follow-up care and subsequent monitoring of disease progression in the Refractory Disease Unit, Dental Research Institute at University of Toronto. All refractory CP patients included in the study had experienced progressive attachment loss despite adequate maintenance therapy. The documented history of surgical treatment and/or antimicrobial therapy was accessed through RDU following consent from patients. Patients received regular subgingival scaling and prophylaxis every 3 months. All study participants had non-contributory medical history, were non-smokers with the exception of one light smoker (<10 cigarettes per day) (162). The study was approved by the Scientific and Ethics Review Boards at the University of Toronto and conducted from January - June 2010.

4.3.2 Study design

All patients underwent complete intraoral examination on the day of sample collection. Oral rinse sample collection was performed as previously described (159). Briefly, participants were asked to rinse with 5 ml of isotonic sodium chloride solution 0.9% for 30 seconds prior to any instrumentation in order to avoid initiating bleeding that might interfere with the results. Patients were then asked to expectorate the rinse sample into a 50 ml falcon tube. Rinse samples were used for oral ROS evaluation.

Periodontal examination was conducted and the following clinical parameters were assessed at six sites per tooth: Probing Depths (PD), Bleeding on Probing (BOP), and visible plaque index (163). Tooth mobility, furcation involvement and recession were also noted. PDs
were measured using a standard controlled force automated pocket probe, which ensured that periodontal pockets were measured with equal force between patients and appointments. MGB was responsible for all clinical data collection.

All participants provided non-fasting venous blood samples for blood assays and cytology tests. From these samples, 3 ml of venous blood was used for peripheral blood ROS evaluation. For each patient, peripheral blood and oral neutrophil samples were prepared for flow cytometry testing within 2-4 hours of sample collection. Samples were kept at 4° C till analyzed. All flow cytometry was completed on the day of sample collection.

4.3.3 Peripheral blood neutrophil isolation

Blood samples were drawn into sodium citrate vacutubes at the blood lab at Mount Sinai Hospital, Toronto, ON. Neutrophils were isolated using a one-step neutrophil isolation solution (155). Briefly, the blood sample was layered carefully over 3 ml of this solution and the mixture was centrifuged at 527 relative centrifugal force (RCF) for 30 minutes at 4° C. Neutrophils were harvested from the lower of 2 bands, and washed by centrifugation with phosphate buffer saline (PBS) at 1083 RCF for 5 minutes at 21° C. Cells were resuspended in 1ml of PBS. Cell count was obtained using coulter counter. This method resulted in greater than 95% neutrophils with greater than 95% viability (155).

4.3.4 Oral cellular component isolation

Based on an established protocol (158,159), rinse samples were vortexed and filtered through a sterile 40 μm nylon mesh. Filtered samples were centrifuged at 1083 RCF for 5 minutes at 21° C. The supernatant was discharged and the pellet was resuspended in 1 ml of
PBS. It has been verified that ROS measurements in oral rinse samples present similar values whether the rinse sample is simply filtered or a full neutrophil isolation procedure is conducted (159).

We verified presence and viability of neutrophils from blood and oral rinse samples by using a fluorophore-conjugated antibody against CD-11b, a specific granulocyte-monocyte cell surface marker (164). Back-gate analysis on forward by side scatter plots was used to confirm neutrophil selection based on morphologic characteristics that differentiate neutrophils from monocytes.

4.3.5 Measurements of cellular ROS

Peripheral blood and oral rinse samples from all patients were stained with dihydorhodamine 123 (155,164). Dihydrorhodamine 123 (DHR) is oxidized into rhodamine 123 (RH) during the superoxide production process by reactive oxygen species such as hydrogen peroxide and \( \text{O}_2^- \) (165). It was shown that DHR may be used as a fluorescent marker for the generation of \( \text{O}_2^- \) in peripheral blood neutrophils, therefore indicating the level of neutrophil activity (165,166).

Blood samples were counted and measured to contain \( 1 \times 10^6 \) peripheral blood neutrophils. Following cellular isolation, 300 ml of the cellular PBS solution was used for staining. After washing the samples in \( 4^\circ \text{C} \), the cellular pellet was resuspended in 500 \( \mu \text{l} \) of a 2 \( \mu \text{M} \) per liter concentration of DHR dissolved in Hank’s Balanced Salt Solution (HBSS) and incubated at \( 37^\circ \text{C} \) for 20 minutes on a shaking platform. For each patient, a second set of both blood and oral neutrophils were stimulated using PMA. PMA was used as a neutrophil stimulation agent for this trial since it maximally induces ROS production directly through Protein Kinase C (167). Samples were stimulated with 0.5 \( \mu \text{l} \) PMA at a concentration of \( 10^{-5} \text{ M} \).
(for final concentration of 1 nM) and incubated at 37° C for 15 minutes on a shaking platform. CD-11b levels at baseline and after PMA stimulation were measured in all samples using flow cytometry. ROS levels were measured in all samples by flow cytometric analysis.

4.3.6 Statistical analysis

High and low responders were compared using both t-tests and the Mann-Whitney test.
4.4 Results

4.4.1 Method validation

Identification of oral neutrophils by flow cytometry was confirmed through the use of the anti-CD11b fluorophore-conjugated antibody and back-gate analyses of selected cell populations. Further, DiffQuick stained micrographs of cytospinned oral neutrophils isolated from oral rinses confirmed the high (>95%) purity of our sample. Data collected from refractory CP patients was used to verify that ROS levels can be measured in both blood and in oral neutrophils by flow cytometry. Results showed an increase in ROS levels after PMA stimulation for both blood and oral neutrophils. For the majority of patients their ROS production range of blood neutrophils (resting to maximal activation) was always wider than for oral neutrophils (Figure 4.1).
Figure 4.1 – Representative oral and blood neutrophil ROS production in response to PMA stimulation in High-responders refractory CP patients. (A) Peripheral blood ROS levels at baseline (1) and after stimulation (2). (B) Oral ROS levels at baseline (1) and after stimulation (2). The increase in ROS is represented by the graphs’ shift to the right. Relative oral ROS production was determined based on the peripheral blood ROS values: (A1) Peripheral blood unstimulated value set as baseline (0%). (A2) Peripheral blood stimulated value set as maximum stimulation (100%). (B1) Oral unstimulated value (Baseline ROS%) was calculated as % of the peripheral blood baseline to maximally stimulated ROS production range (A2-A1). (B2) Oral stimulated value (Stimulated ROS%) was calculated as % of the peripheral blood baseline to maximally stimulated ROS production range (A2-A1). (B2-B1) The activation potential of oral neutrophils was calculated as difference between oral stimulated ROS% and oral baseline ROS%. D – Negative stained cells; C – Positive stained cells; G – Total cell population, MCN – Mean Channel Number.
4.4.2 Low and High responders

The range between baseline and stimulated ROS values for blood neutrophils was used as an activation range by which we evaluated oral neutrophils ROS levels. Unstimulated peripheral blood values were set as 0%, while maximum stimulated peripheral blood values were set as 100%. Oral neutrophil ROS values at baseline and following PMA activation were calculated as a percentage of the peripheral blood neutrophils’ baseline to maximally-stimulated ROS production range (Figure 4.1) and reported as oral neutrophils ROS percentage (oROS%) (Table 4.1). The difference between stimulated and baseline oROS% was also calculated, indicating the activation potential of oral neutrophils (Figure 4.1, Table 4.1).

Based on the stimulated oROS% (value (B2) in figure 4.1) as well as the oral activation potential ((B2-B1) in figure 4.1), two distinct patient groups were evident. Five patients showed stimulated oROS% lower than 45% and activation potential lower than 13%. This group is referred to as “Low responders”. Eight patients showed stimulated oROS% higher than 45% and activation potential higher than 19% (Table 4.1). This group is referred to as “High responders”. The two groups did not differ significantly in age. High responders presented significantly higher stimulated oROS% when compared to Low responders (p=0.002), suggesting that the activated oral neutrophils of these patients could produce ROS levels closer to their maximal potential (Table 4.1). High responders also presented significantly larger differences between stimulated and unstimulated values for oral neutrophils (p=0.002), indicating a higher activation potential.
Table 4.1 – Low Responders and High Responders oral ROS%

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th># Remaining Teeth</th>
<th>Oral PMN baseline ROS%</th>
<th>* Oral PMN stimulated ROS%</th>
<th>** oPMN Activation Potential(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject 1</td>
<td>F</td>
<td>33</td>
<td>28</td>
<td>3.78</td>
<td>9.79</td>
<td>6.01</td>
</tr>
<tr>
<td>Subject 2</td>
<td>M</td>
<td>40</td>
<td>28</td>
<td>25.01</td>
<td>20.73</td>
<td>-4.28</td>
</tr>
<tr>
<td>Subject 3</td>
<td>F</td>
<td>32</td>
<td>28</td>
<td>20.11</td>
<td>26.24</td>
<td>6.13</td>
</tr>
<tr>
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<td>30</td>
<td>20.01</td>
<td>32.33</td>
<td>12.32</td>
</tr>
<tr>
<td>Subject 5</td>
<td>M</td>
<td>37</td>
<td>28</td>
<td>33.73</td>
<td>41.15</td>
<td>7.42</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>40.4</td>
<td>28.4</td>
<td>20.5</td>
<td>26.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>11.42</td>
<td>0.89</td>
<td>10.9</td>
<td>11.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Subject 6</td>
<td>M</td>
<td>60</td>
<td>23</td>
<td>12.04</td>
<td>45.36</td>
<td>33.32</td>
</tr>
<tr>
<td>Subject 7</td>
<td>F</td>
<td>73</td>
<td>29</td>
<td>22.97</td>
<td>53.38</td>
<td>30.41</td>
</tr>
<tr>
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<td>40</td>
<td>30</td>
<td>38.03</td>
<td>57.42</td>
<td>19.39</td>
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<tr>
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<td>M</td>
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<td>27</td>
<td>35.37</td>
<td>60.16</td>
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</tr>
<tr>
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<td>F</td>
<td>50</td>
<td>28</td>
<td>41.49</td>
<td>73.4</td>
<td>31.91</td>
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<tr>
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<td>28</td>
<td>57.87</td>
<td>82.13</td>
<td>24.26</td>
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<tr>
<td>Subject 12</td>
<td>M</td>
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<td>25</td>
<td>71.14</td>
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<td>36.79</td>
</tr>
<tr>
<td>Subject 13</td>
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<td>49</td>
<td>26</td>
<td>7.23</td>
<td>137.01</td>
<td>129.78</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>51.38</td>
<td>27</td>
<td>35.8</td>
<td>77.1</td>
<td>41.3</td>
</tr>
<tr>
<td>Standard Deviation</td>
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<td>11.25</td>
<td>2.27</td>
<td>21.77</td>
<td>31.2</td>
<td>36.2</td>
</tr>
</tbody>
</table>

Patients' oral ROS% data is shown. Low Responders are marked in light grey. High Responders are marked in dark grey. Mean values for high and low-responders are marked in green and red respectively. (* P value = 0.002, ** P value = 0.002). PMN - Neutrophil
4.4.3 Clinical findings

No statistical significant differences were found for PD >4mm, BOP, visible plaque Index, number of remaining teeth, and age between the two groups (Table 4.1, Figure 4.2). In contrast, Clinical Attachment Loss (CAL) was significantly higher in the High responders group compared to the Low responders group. A significantly higher mean percentage of sites with CAL >5 mm was found in High responders (p=0.003). Although not statistically significant, High responders also trended towards a higher mean percentage of sites with CAL >4 mm (p=0.08).
Figure 4.2 – High and Low Responders clinical findings: Clinical findings for High and Low responders. Low responders are marked in black and HE in grey. Mean ± SEM, n = 5 Low responders and 8 High responders. Mann-Whitney U test. * p < 0.05. A-PI, Ainamo plaque index; BOP - bleeding on probing; CAL - clinical attachment loss; PD - probing depth.
4.5 Discussion

Neutrophil hyperactivity during periodontal diseases has been previously reported. These neutrophil function studies used cells isolated from peripheral blood samples, assessing neutrophil counts (69,168), phagocytic activity (155), chemotactic activity (169-171), or specific enzyme levels (172,173). Intra-cellular ROS levels are also commonly used as a neutrophil activity marker, and an increase in peripheral blood neutrophil ROS levels in periodontitis patients has been demonstrated by our group and others (66-69,155,172-175). However, ROS production by oral neutrophils has not been characterized in periodontitis patients. Levels of 8-hydroxy-deoxyguanosine (8-OHdG) levels were found increased in whole saliva samples obtained from periodontitis patients compared to healthy control subjects (82). This marker for oxidative DNA damage suggests increased ROS activity during periodontal disease, however, no correlation to clinical parameters, disease activity or response to therapy was reported.

Peripheral blood and oral neutrophil bactericidal activity in health and periodontitis was described in the past. Wilton compared peripheral blood and GCF neutrophils obtained from periodontally healthy individuals, and reported a significant reduction in phagocytic activity of GCF neutrophils but similar bacterial killing abilities (176). Similar comparisons carried in periodontally-healthy subjects reported differing levels of activity between salivary, peripheral blood and GCF neutrophils (177,178). The same comparative approach was used with periodontitis patients to assess GCF ROS production (156). It was demonstrated that following PMA stimulation, periodontal patients presented enhanced GCF ROS production compared to healthy controls. Our goal here was to correlate oral neutrophil ROS production with periodontal disease history and severity.

The present study provides a novel assessment of oral neutrophil response to stimulation of ROS production in patients previously diagnosed with refractory CP. For each
patient, we determined maximal oral neutrophil ROS production as a percentage of the peripheral blood baseline to maximally stimulated ROS production range (Figure 4.1). It was previously shown that oral neutrophil functionality is impaired when compared to peripheral blood neutrophils (176,178). It was suggested that the functional potential of neutrophils is maximal in their naive state, prior to any stimulation. Our findings are consistent with this hypothesis, as the stimulated ROS value of peripheral blood neutrophils was higher than the stimulated ROS value of oral neutrophils. We believe that the maximal oROS% is a measure of potential for exaggerated response to stimulation of ROS production by oral neutrophils in a given patient. Based on the stimulated oROS%, we identified a High responders group and a Low responders group of patients within our refractory disease cohort. The notion that oROS% is a measure of oral neutrophil hyper-response potential is supported by our finding that High responders patients presented with more severe bone loss, associated with a significantly higher mean percentage of sites with CAL >5mm (22%). These findings indicate a more severe phenotype in High responders compared to the approximately 5% of sites with CAL >5mm seen in the Low responders group (p=0.003). Considering the heterogeneity of the patient population and the cross-sectional nature of the data, it is important to note that plaque index and age, which are regarded as contributing factors for progressive periodontal tissue destruction in refractory CP, were not significantly different between the two groups. Similarly, BOP and PD, which may be considered markers of cumulative past disease activity were not significantly different between the two groups.

Heterogeneity in clinical presentation and disease progression of refractory CP patients was reported as early as 1985, although the definition of refractory CP per se has changed over the past three decades. Two different refractory disease types were initially described - immediate refractory disease onset following treatment, and a refractory pattern that develops
after a quiescent period (179). Microbiologic heterogeneity has been identified among refractory CP patients, and it has been suggested that refractory CP is not a single entity but a group of several periodontal diseases differentiated by potentially unknown etiologies (151,180). Another group found that 3 of 21 refractory CP patients accounted for over 50% of the sites with significant attachment loss (27). This finding emphasizes the likelihood of more than one type of refractory CP. Consistent with our data, High responders and Low responders groups of refractory CP patients have been classified according to oral neutrophil chemotactic activity and cell counts. High responders patients were shown to have 10-fold higher neutrophil counts in GCF samples when compared to Low responders patients (181). The same study also reports that 5 out of 9 patients demonstrated an unusual response pattern in both healthy and diseased sites, while the other 4 patients showed the same response pattern only at diseased sites. Altogether these findings suggest an intrinsic immune-mediated pathogenesis in certain refractory CP cases.

As is evident from previous studies, patients diagnosed with refractory CP may present with differing levels of disease progression and severity, as well as variable neutrophil activity. The significant increase in stimulated oral ROS production within the High responders group suggests an oral hyper-responsive neutrophil phenotype, which is likely to contribute to periodontal tissue destruction as demonstrated by the clinical findings in High responders patients. Our identification of two refractory CP groups of patients based on stimulated oROS% that correlated with disease severity suggests possible mechanistic explanation for heterogeneity in periodontitis manifestation and it may allow us to identify refractory CP patients at increased risk of periodontal breakdown.
4.6 Conclusion

This study presents a novel assessment of oral neutrophil maximal potential for ROS production, as measured by ROS generation in neutrophils of refractory CP patients. The oral neutrophil ROS potential allowed us to identify an extreme refractory CP patient population with more severe periodontal tissue breakdown. We propose that oral neutrophil potential to generate ROS may serve as useful tool in assessment and prediction of disease severity in refractory CP patients. The observed oral neutrophil hyper-responsiveness may result in excess production of ROS, which has been shown to lead to severe loss attachment.

Future investigations will include gene expression analysis in peripheral blood and oral neutrophils of refractory CP patients, as well as the evaluation of oral neutrophils in CP and AgP patients before and after treatment. Longitudinal prospective observation may indicate whether patients with hyper-responsive neutrophils (or High responders) are refractory to treatment and more susceptible to periodontal breakdown. This could result in early diagnosis of refractory CP patients, followed by targeted frequent follow-up appointments and development of novel individualized therapeutics for these patients.
4.7 Footnotes

‡ Baxter Corporation, Toronto, ON
§ Florida Probe, Gainesville, FL
ǁ 1-step polymorphs, Axis-Shiled PoC, Oslo, Norway
¶ Sigma-Aldrich, St. Louis, MO
‖ Beckman Coulter Inc., Brea, CA
** Fisher Scientific Canada, Ottawa, ON
†† BioLegend, San Diego, CA
‡‡ Invitrogen, Carlsbad, CA
§§ Diff-Quik Stain Set, Siemens, Newark, DE
4.8 Acknowledgements

The authors would like to give special thanks to Wilson Lee and Chun Xiang Sun (Matrix Dynamics Group, University of Toronto) for their help with flow cytometry tests and laboratory work, Mathew Laski (University of Western Ontario) for his help during the preliminary work for the study, and Laurel Duquette of the Statistical Consulting Service, University of Toronto for statistical advice. This work was funded by The Canadian Institutes of Health Research (CIHR, Ottawa, ON) (Glogauer), the Periodontal Department at the University of Toronto and the Alpha Omega Foundation of Canada (Toronto, ON). GA is supported by scholarships granted by CIHR and the Harron scholarship (Faculty of Dentistry, University of Toronto).

Authors’ contribution:

M.B.G – Clinic director, Periodontal department, Faculty of Dentistry, University of Toronto

M.G. – Research supervisor

4.9 Conflict of Interest Statement

Authors report no financial or commercial relationship to any organization that may be affected by this study.
Chapter 5

Nrf2 down-regulation is associated with high oxidative damage in periodontal lesions and severe chronic periodontitis

G.M. Aboodi, C. Sima, F.S. Lakschevitz, C. Sun, M.B. Goldberg, M. Glogauer. Nrf2 down-regulation is associated with chronic periodontitis and results in periodontal tissue destruction in animal model. *(Manuscript under review)*

5.1 Abstract

**Background:** The balance between reactive oxygen species (ROS) and antioxidants (AO) plays an important role in periodontal tissue health. We demonstrated that high ROS production by oral neutrophils in patients with chronic periodontitis (CP) refractory to conventional therapy is associated with severe loss of tooth attachment to periodontium. To get further insight into the role of oral neutrophil oxidative stress in periodontal tissue destruction we investigated the neutrophil oxidative state in CP patients. We prove the hypothesis that reduced AO production through down-regulation of Nrf2 pathway in oral neutrophils, despite enhanced recruitment in the oral cavity is associated with severe CP.

**Methods:** Oral neutrophil samples from 8 CP patients and 6 healthy controls were used to assess gene expression patterns related to AO production. Microarray, qRT-PCR and western blot analyses were used to measure expression of critical recruitment mediators, Nrf2 pathway factors, SOD1 and CAT levels in oral neutrophils. Murine ligature-induced periodontitis model was used in Nrf2/- mice to assess the roles of local AO in inflammation-mediated periodontal tissue degradation.
**Results:** The Nrf2-mediated oxidative stress response pathway was downregulated in oral neutrophils of severe CP patients compared to healthy controls. Levels of neutrophil superoxide dismutase 1 (SOD1) and catalase (CAT) were decreased in severe CP despite upregulation of CXCL8 (*IL-8*), CXCR1 (*IL-8 receptor 1*) and triggering receptor expressed on myeloid cells 1 (*TREM1*), and increased recruitment in the oral cavity. Nrf2−/− mice had more severe alveolar bone and attachment loss in response to periodontitis-inducing subgingival ligatures compared to wild types. Levels of 8-hydroxy-deoxyguanosine (8-OHdG) were increased in epithelium and connective tissue of periodontal lesions of Nrf2−/− mice indicating high oxidative damage.

**Conclusions:** We report for the first time Nrf2 pathway downregulation in oral neutrophils of patients with severe CP. The more severe phenotype in Nrf2−/− mice compared to wild types during experimental periodontitis confirms the critical role of Nrf2 in reducing oxidative damage in periodontitis lesions.
5.2 Introduction

Periodontal diseases are a group of highly prevalent inflammatory diseases affecting up to 70% of US population (11). Chronic periodontitis (CP), the most common form of periodontal disease, is characterized by progressive destruction of tooth-supporting structures as a result of altered host-biofilm interactions in the gingival crevice and unresolved inflammation. Although pathogenic biofilms are required for disease onset, host factors drive inflammation-mediated resorption of alveolar bone and damage of connective tissue attachment. Neutropenia, altered neutrophil recruitment into periodontium, and impaired bacterial killing lead to severe forms of periodontitis (182). On the other hand, hyper-responsiveness and excess ROS production likely contribute to relapse after CP treatment. Untreated CP is associated with considerable morbidity and represents a risk factor for numerous systemic conditions with underlying low-grade inflammation. In addition to persistent inflammation, oxidative stress is believed to be a link between CP and metabolic syndrome (16).

One mechanism suggested to contribute to periodontal tissue breakdown in CP is the increase in endogenous-produced oxidative stress observed in periodontitis lesions (19,183). We have previously shown that CP patients refractory to conventional therapy present with more severe loss of tooth attachment when their oral neutrophils produce high levels of ROS, suggesting increased oxidative stress in periodontal tissues through excess neutrophil-derived ROS in these patients. On the other hand, the protective role of antioxidants (AO) in CP has been investigated by total antioxidant capacity (TAOC) measurements in whole saliva and gingival crevicular fluid (GCF) samples. TAOC levels were found to be significantly lower in CP patients when compared to healthy controls (97,101), although the mechanism responsible for oral AO decrease in CP has yet to be investigated.
Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates gene transcription of a large group of antioxidant and phase-2 detoxifying enzymes playing a central role in cellular defense against oxidative and electrophilic insults (89,90). In response to oxidative stress cytoplasmic Nrf2 is released from Kelch-like ECH-associated protein 1 (Keap1) and binds to antioxidant response elements (ARE) in the promoter region of many antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), NAD(P)H:quinone oxidoreductase, glutathione S-transferase, heme oxygenase-1, glutathione peroxidase (GPx), glutamate cysteine ligase, and peroxiredoxin (89). In addition, since NF-κB-mediated production of cytokines and chemokines such as TNF-α, IL-6, IL-8 (CXCL8) and CCL2 is likely to be redox-regulated, Nrf2 may play critical roles in control of inflammatory injury. Nrf2 was shown to play important roles in rheumatoid arthritis, gastrointestinal, renal, brain, skin and pulmonary inflammation, and atherosclerosis (92). In this study we investigated the oxidative state of local and systemic neutrophils from patients with severe CP. It was found that down-regulation of the Nrf2 pathway in oral neutrophils is associated with increased recruitment in the oral cavity and severe CP. Nrf2-/- mice had increased local oxidative damage, alveolar bone and attachment loss at sites with periodontitis.
5.3 Materials and Methods

5.3.1 Study design

Two study groups were recruited for the study: patients diagnosed with severe CP (4) (8 subjects, 2 males, 6 female, ages 37-74) and periodontally healthy controls (6 subjects, 4 males, 2 female, ages 24-38). Severe CP was defined according to Eke et al criteria (11) as the presence of 2 or more interproximal sites with ≥ 6 mm AL (not on the same tooth) and 1 or more interproximal site(s) with ≥ 5mm probing depth. All subjects were systemically healthy, non-smokers. CP patients were recruited at the periodontics graduate clinic at the Faculty of Dentistry, University of Toronto, Toronto, ON. Samples collection was completed prior to periodontal treatment. Subjects provided a written informed consent to participate, and the study was approved by the Research Ethics Board at the University of Toronto (Protocol # 24567).

Oral rinse and venous blood samples were collected from each subject. Oral rinse samples were collected as previously described (75): Subjects were asked to rinse with 5mL 0.9% isotonic sodium chloride solution (Baxter, Toronto, ON) for 30 seconds before any instrumentation to avoid bleeding that might interfere with the results. Subjects were then asked to expectorate into a sterile 50-mL falcon tube. This was repeated 6 times with 3 minutes intervals, for a total of 30mL sample collection. The same participants provided 10mL of non-fasting venous blood.

5.3.2 Neutrophil isolation

Neutrophil isolation procedures from both oral rinse and blood samples were previously described (75). Isolation procedures were initiated within 2 hours of sample collection. Blood
neutrophils were isolated using a one-step neutrophil isolation solution (1-step polymorphs, Axis-Shield PoC, Oslo, Norway). Blood sample was layered carefully over the isolation solution and were centrifuged at 527 relative centrifugal force (RCF) for 30 minutes at room temperature. Neutrophils were collected from the lower of two bands, and washed by centrifugation with Hank’s Balanced Salt Solution, no calcium, no magnesium (HBSS-/-, Invitrogen, Grand Island, NY). Hypotonic lysis was performed to remove erythrocyte contamination. Following a second wash, cells were re-suspended in 1mL of HBSS-/. Oral neutrophils were isolated through a series of filtrations through 40μm (Fisher Scientific, Loughborough, UK), 20μm and 11μm nylon mesh (EMD Millipore, Billerica, MA). The filtered samples were then centrifuged at 527 RCF for 5 minutes at 4°C. Cells were re-suspended in 1mL of HBSS-/. Both isolation procedures resulted in >95% neutrophils with >95% viability, as previously demonstrated (75). Cell quantification was completed using a Coulter counter (Beckman Coulter, Brea, CA).

5.3.3 RNA Isolation

Total RNA isolation from both oral and blood neutrophils was completed within the same day of sample collection, using mirVana isolation kit (Ambion, Austin, TX). Isolation procedure followed manufacturer suggested protocol, and was previously described (75). RNA samples were kept at (-80)°C until analyzed.

5.3.4 Gene expression microarray

Gene expression microarray was completed at the Centre for Applied Genomics, Hospital for Sick Children Research Institute, Toronto, ON. Prior to gene expression microarray analysis, RNA samples were analyzed on a bioanalyzer using an RNA kit (2100 Bioanalyzer using...
an RNA 6000 PicoLabChip Kit, Agilent Technologies, Santa Clara, CA). All samples subjected to further analysis presented with an RNA integrity number >8. Microarray analysis was completed for oral and blood neutrophils of 4 CP and 4 periodontally healthy subjects using the Illumina Human 12WG Expression BeadChip (48,000 gene transcripts). The microarray data complies with Minimum Information About a Microarray Experiment (MIAME) guidelines (184). Data set was deposited at Gene Expression Omnibus (National Center for Biotechnology Information), accession number: GSE 435525.

5.3.5 Microarray data analysis

The raw microarray data was preprocessed using R loaded with Lumi R package (http://www.r-project.org), and followed by background correction, completed with BeadStudio, Illumina software. The quantile normalization method implemented with R loaded with Lumi R package was used to normalize the data. Differentially expressed genes were identified using linear models for microarray data. Genes were selected controlling for false discovery rate at the level of 0.01 and by fold change of 2. Data analysis was completed only for genes which were significantly up or down regulated at least 2-fold (at p<0.05). Pathway analyses were performed using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). Results from the analysis of the same dataset (same subjects) were previously reported (76).

5.3.6 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

In order to validate the observed microarray data, superoxide dismutase 1 (SOD1) and catalase (CAT) gene expression were validated utilizing qRT-PCR. Both SOD1 and CAT are
downstream products of the Nrf2 pathway. qRT-PCR was completed for 2 subjects from each of the study groups, on the same RNA samples used for microarray analysis. qRT-PCR was performed in triplicates (CFX96 Real-Time System, Bio-Rad, Hercules, CA), as previously described (75). Total RNA was reverse transcribed into complementary DNA (cDNA) using murine leukemia virus reverse transcription (Superscript II, Invitrogen), and oligo-dT\textsubscript{18} VN primer (ACGT, Toronto, ON) in a 20mL reaction system. A sample without template RNA, and another without reverse transcription were used as negative controls. Reaction mixture containing 5mL template cDNA, 15mL master mix, 1mL forward and 1mL reverse primer (both 10 mM stock), 10mL deoxyribonucleotide triphosphates, Sso7d (dsDNA binding protein) fusion polymerase, MgCl\textsubscript{2}, DNA binding dye (SsoFast EvaGreen Supermix, Bio-Rad), and 3mL RNase-free distilled water was prepared. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, 5’-GGAGCGAGATCCCTCCAAAAT-3’; reverse, 5’-GGCTGTTGTCACTTCTCATGG-3’) was used to normalize the expression data of the tested genes (SOD1: forward, 5’-GGTGGGCCAAAGGATGAAGAG-3’; reverse, 5’-CCACAAGCCAAACGACTTCC-3’; CAT: forward, 5’-TGGAGCTGGTAACCCAGTAGG-3’; reverse, 5’-CCTTGGCTTGGAGTATTTGGTA-3’).

5.3.7 Western blot analysis

SOD1 and CAT protein abundance was investigated through western blot analysis. Oral and blood neutrophils were isolated from 3 control subjects and 4 CP subjects for SOD1 analysis, and 8 control subjects and 7 CP subjects for CAT analysis. Isolated cells were quantified, and 10\textsuperscript{6} cells per sample were lysed with sodium dodecyl sulfate buffer. 20mL of each sample were loaded on a 12% polyacrylamide gel (for SOD1 identification), and 10% polyacrylamide gel (for CAT identification). After electrophoresis, the gels were transferred to nitrocellulose filters (Amersham-GE, Baie d’Urfe, QC) by electro-blotting. The filters were then incubated for 1 hour.
in a blocking buffer (5% nonfat milk powder in Tris-Buffered Saline and Tween (TBST)). The membranes were maintained overnight in primary antibodies (SOD1: Cell Signaling Technology, Danvers, MA; CAT: EMD Millipore, Billerica, MA) in TBST with 5% Bovine serum albumin. Antibody concentrations followed the manufacturer’s recommendations. Membranes were washed 3 times for 10 minutes with TBST, then incubated for 1 hour in the appropriate secondary antibody (in 5% nonfat milk powder/TBST, at room temperature), according to manufacturer’s recommendations. Membranes were washed again (3 X 10 minutes with TBST). Membranes were developed with Western Lightning solution (Perkin Elmer, Akron, OH), and the resulting chemiluminescence (CL) was exposed to film. β-actin abundance was measured as control, with the appropriate antibody. Band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). For each protein, oral intensity/oral β-actin intensity, as well as blood intensity/blood β-actin ratios were calculated. The oral/blood band intensity ratio was then calculated for each patient. Data was expressed as a percentage (%), and reflects oral abundance as a % of the blood levels of each of the investigated proteins.

5.3.8 Nrf2-/- mice

Though Nrf2 was demonstrated to be the main mediator for cellular antioxidant defence, knockout mice are viable and present with a normal phenotype under normal conditions (185,186). Nrf2 -/- mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6J mice were used as wild type (WT) control. Mice were handled in accordance with the Guide for the Humane Use and Care of Laboratory Animals, and all the experiments were approved by the Animal Care Committee, Faculty of Dentistry, University of Toronto. Null mice were verified prior to breading using PCR, following Jackson Laboratories’ instructions. All mice were 6-10 weeks of age for each of the experiments completed.
5.3.9 Bone marrow neutrophils isolation

Bone marrow (BM) cells were harvested from mice femur and tibia and isolated with percoll gradient separation, as previously described (187). BM neutrophils were washed and re-suspended in 1mL of α-MEM (Life Technologies, Carlsbad, CA) and overlaid on Percoll gradients (85%/65%/55%. Sigma-Aldrich, St. Louis, MO), and were centrifuged at 900 RCF for 30 minutes at 4°C. Following centrifugation, the lower band was collected and washed with PBS. Erythrocytes were eliminated by addition of 5 ml of Pharm Lysis buffer (BD Biosciences, Mississauga, ON). Samples were washed and cells were re-suspended in 1mL Phosphate buffered saline (PBS. Sigma-Aldrich, St. Louis, MO). Cell quantification was completed using a Coulter counter (Beckman Coulter, Brea, CA).

5.3.10 Bone marrow neutrophils western blot analysis

CAT protein levels were investigated in isolated BM neutrophils of 3 WT and 4 Nrf2 null mice. Western blot protocol was similar to that described above, and the same CAT antibody was used (CAT: EMD Millipore, Billerica, MA). β-actin abundance was measured as control, with the appropriate antibody. CAT band intensity was normalized to β-actin, and quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

5.3.11 Bone marrow neutrophils extracellular ROS measurements

Extracellular ROS levels were measured by isoluminol-amplified chemiluminescence assay (66,188). \(10^5\) isolated BM neutrophils from 6 WT and 4 Nrf2 null mice, were re-suspended in 20μL PBS+/+ (with calcium and magnesium). Cells, 20μL isoluminol (3 mmol/L) with 6μL (=6 units) horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) were added to pre-blocked (PBS
containing 1% Bovine serum albumin, overnight, 4°C) white 96 well plate (BD, Franklin Lakes, NJ,). Well with no cells was used as negative control, stimulated cells were used as positive control. Cells were stimulated with $10^{-6}$M PMA (Sigma-Aldrich, St. Louis, MO). PBS+/+ was added to each well for final volume of 200μL. Plates were incubated in 37°C for 30 minutes. Fluorescence spectrophotometer (Fisher Scientific) was used for light emission recording in relative light units. All samples were measured in duplicates.

5.3.12 Bone marrow neutrophils chemotaxis assay

Neutrophil chemotaxis was measured using a Zigmond chamber, as previously described (187). Isolated BM neutrophils from 3 WT and 3 null mice ($10^6$ neutrophils per 1mL) were allowed to attach to bovine serum albumin-coated glass coverslips at 37°C for 15 minutes. The cover slip was inverted onto a Zigmond chamber. 100μL HBSS was added to the left chamber, and 100mL HBSS media containing fMLP ($10^{-6}$M) was added to the right chamber. Neutrophil movement was recorded using time-lapse video microscopy. The microscope (Nikon Eclipse E1000) was equipped with differential interference contrast optics and a 320 objective. Images were captured at 15 seconds intervals for 15 minutes with a Nikon Coolpix 995 camera. Data analysis was completed using ImageJ software.

5.3.13 Ligature-induced Periodontitis

As previously described (182), a split-mouth experimental periodontitis model was used in order to investigate alveolar bone loss in Nrf2 null, heterozygous and WT mice (10, 6 and 11 males, respectively). Mice were anesthetized with a mixture of 100
mg/kg body weight ketamine and 10 mg/kg body weight xylazine. 9-0 silk suture was placed in the gingival sulcus around the left second maxillary molar (M2), with the right side serving as the control. Mice were euthanized on day 21 following ligature placement. Maxilla’s were harvested and defleshed in a colony of dermestid beetles at Royal Ontario Museum (Toronto, ON), and freezefumigated for 7 days at (-20)° C.

5.3.14 Alveolar bone loss measurements

Dry skulls were stained with methylene blue (1% in water), and alveolar bone loss was assessed by morphometry under stereomicroscope (Nikon Eclipse E1000). Images of the buccal aspects of the right (healthy) and left M2s were taken at X5 magnification using a mounted video camera (PixeLINK, Ottawa, ON). Horizontal bone loss was measured from the cemento-enamel junction (CEJ) to the alveolar bone crest at 3 points per tooth: mid-, mesio-, and disto-buccal using ImageJ software.

5.3.15 ROS-mediated DNA damage measurements by immunohistochemistry

Immunohistochemistry was completed at the Toronto Centre for Phenogenomics, (Mount Sinai Hospital, Toronto, ON). Maxillas were harvested from Nrf2 null and WT mice (3 males per group) following 21 day ligature-induced periodontitis. Samples were fixed in 4% paraformaldehyde for 7 days, and were then subjected to decalcification in 10% EDTA solution for 7 days. Samples were embedded in paraffin, and coronal sections in the maxillary M2 area were completed, and included both ligature and control sites. Coronal sections were stained with 8-hydroxy-deoxyguanosine (8-OHdG) antibody (EMD Millipore, Billerica, MA) for the identification and quantification of 8-OHdG. Paraffin-embedded sections were dewaxed and
incubated with Xylene. Rehydration completed with ethanol and distilled water (dH2O). 30 minutes incubation with 3% hydrogen peroxides in dH2O was used for blocking of endogenous hydrogen peroxidase. Blocking was completed with 20% normal horse serum for 30 minutes (Vector labs, Burlington, ON). Primary AB was used in 1:7000 dilution. Secondary AB: Horse anti-goat IgG, Biotinylated 1: 200 (Vector labs, Burlington, ON). Avidin Biotin complex incubation followed (Vector labs, Burlington, ON). Stained slides were analysed under Nikon Eclipse E1000 microscope in X40 magnification. Images were captured with a Nikon Coolpix 995 camera. Three regions of interest (ROI) were defined for each site (ligature and control sites for each sample): Alveolar bone crest (ABC), sulcular epithelium (Epi), and gingival connective tissue (CT). 8-OHdG positive cells were quantified using ImageJ software utilizing automated particle count. Intensity threshold was defined to properly identify positive stained cells, and number of particles in each ROI, as well as ROI total area, were recorded.

5.3.16 Statistical analysis

Unless otherwise specified, t-test analysis for independent samples was used.
5.4 Results

5.4.1 Nrf2 pathway is downregulated in oral neutrophils of CP patients

Untreated CP patients and healthy controls were assessed for periodontal clinical parameters prior to blood and oral neutrophil collection (Table 5.1). CP patients had significantly higher oral neutrophil numbers compared to healthy controls despite similar circulating neutrophil numbers (oral neutrophil: healthy, 1.76±0.41 x 10^6/ml and CP 3.75±0.68 x 10^6/ml; blood neutrophils: healthy, 4.90±0.92 x 10^6/ml and CP, 4.06±0.69 x 10^6/ml) (Fig. 5.1 A). Microarray analysis of neutrophil gene expression revealed a significantly higher CXCL8 and TREM1 expression by oral neutrophils in CP compared to blood neutrophils (6.41 and 3.1 fold increase respectively). Interestingly, blood and oral neutrophils of CP patients had decreased expression of FPR1 (fMLP receptor 1). There was a mild downregulation (<2 fold) of critical factors involved in ROS production in oral neutrophils in CP compared to health (Table 5.2). Ingenuity pathway analysis of microarray data predicted significant downregulation of antioxidant expression downstream of Nrf2 in CP neutrophils (blood and oral) (Fig. 5.1 B, C). Analysis of oral vs. blood neutrophil samples within each of the study groups revealed that unlike healthy controls, CP patients present a significant downregulation of the Nrf2-mediated oxidative stress response pathway in oral neutrophils. Twenty-four and six Nrf2 pathway-associated genes were downregulated or upregulated respectively (>2 fold) in oral neutrophils of CP patients when compared to blood neutrophils (Table 5.3). The same genes had similar expression in blood neutrophils of CP and healthy subjects with exception of SOD1 and AKR1A1 (Table 5.4). Superoxide Dismutase 1 (SOD1) and catalase (CAT), key regulators of superoxide and
hydrogen peroxide, were used to verify the gene expression microarray results. qRT-PCR analysis confirmed the reduced expression levels of SOD1 and CAT in CP oral neutrophils (Oral/blood ratio: healthy - CAT, 8.70 ± 1.56; SOD1, 3.18 ± 0.90; CP – CAT, 0.15 ± 0.08; SOD1, 0.45 ± 0.22) (Fig. 1D). Comparison of blood neutrophils from both groups resulted in no significant differences in the Nrf2 pathway (data not shown). Western blot analysis of CAT and SOD1 production by neutrophils of oral CP patients revealed significant reduction compared to health (CAT: healthy 1.88 ± 0.22; CP 1.09 ± 0.18; SOD1: healthy 0.8 ± 0.15; CP 0.41 ± 0.06 relative to β-actin) (Fig. 5.1 E, F).
Table 5.1 – Demographic Information and Periodontal Clinical Parameters (mean±SD).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CP (n=8)</th>
<th>Healthy (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>54.50±18.08</td>
<td>32.17±5.30</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Ethnic group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Black</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Caucasian</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Probing depth 4 ≥ mm (%)</td>
<td>28.95±3.69</td>
<td>0</td>
</tr>
<tr>
<td>Clinical attachment loss ≥ 3 mm (%)</td>
<td>40.13±13.27</td>
<td>0</td>
</tr>
<tr>
<td>Bleeding on probing (%)</td>
<td>67±32.98</td>
<td>14.88±5.57</td>
</tr>
</tbody>
</table>
A

Healthy

Periodontitis

20 μm

Neutrophils (ml/ml)

Healthy    Periodontitis

Blood

Healthy    Periodontitis

Oral

*
The image shows a bar graph and a gel image comparing SOD (normalized to β-actin) levels in Healthy and Periodontitis states for Blood and Oral samples.

- **Bar Graph:**
  - **Y-axis:** SOD (normalized to β-actin) levels ranging from 0 to 2.
  - **X-axis:** Categories include Healthy Blood, Periodontitis Blood, Healthy Oral, Periodontitis Oral.
  - There are error bars indicating variability.
  - Stars (*) indicate significant differences between groups.

- **Gel Image:**
  - **SOD1: 18 kDa**
  - **β-Actin: 42 kDa**
  - The gel images show protein bands for Healthy and Periodontitis samples in Blood and Oral.
  - The bands are compared to the molecular weight markers.
Figure 5.1 – Oral neutrophils of CP patients produce low levels of CAT and SOD1.

Representative micrographs of Diff-Quick stained cytospins of oral neutrophils isolated from oral rinses of healthy and CP patients by sequential filtering. Blood neutrophils were isolated using a one-step neutrophil isolation protocol. Oral and blood neutrophils from 8 CP patients and 6 healthy controls were counted with a coulter counter and viability confirmed by trypan blue exclusion (A). Neutrophil RNA was isolated from oral neutrophils of healthy and CP patients as described in methods. Regulation of Nrf2 antioxidant stress response pathway was predicted based on microarray gene expression data using the Ingenuity Pathway Analysis software (B, C). CAT and SOD1 were measured by qRT-PCR and normalized to GAPDH (D). CAT and SOD1 production by oral and blood neutrophils was analyzed by western blot and normalized to β-actin (E, F). One way analysis of variance, mean ± SEM. *p<0.05, n=4/group.
Table 5.2 – Oral neutrophil changes in critical chemotaxis and ROS pathway genes in CP (fold change).

<table>
<thead>
<tr>
<th>Molecule (gene name)</th>
<th>CP vs. Healthy</th>
<th>Normalized to blood</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemotaxis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement component 5a receptor 1 (C5AR1)</td>
<td>-1.38</td>
<td>1.30</td>
</tr>
<tr>
<td>Formyl peptide receptor 1 (FPR1)</td>
<td>-1.60*</td>
<td>1.36*</td>
</tr>
<tr>
<td>Triggering receptor expressed on myeloid cells 1 (TREM1)</td>
<td>-1.28*</td>
<td>3.10*</td>
</tr>
<tr>
<td>Interleukin 8 receptor alpha, CXCR1 (IL8RA)</td>
<td>-1.41*</td>
<td>1.43*</td>
</tr>
<tr>
<td>Interleukin 8 receptor beta, CXCR2 (IL8RB)</td>
<td>-1.24*</td>
<td>-1.79*</td>
</tr>
<tr>
<td>Interleukin 8, IL-8, CXCL8 (IL8)</td>
<td>-2.63*</td>
<td>6.41*</td>
</tr>
<tr>
<td>Serine/threonine-protein kinase PAK 1 (PAK1)</td>
<td>-1.26*</td>
<td>-1.48</td>
</tr>
<tr>
<td>Cell division control protein 42 homolog, Cdc42 (CDC42)</td>
<td>1.08</td>
<td>1.06</td>
</tr>
<tr>
<td>Ras-related C3 botulinum toxin substrate 1, Rac1 (RAC2)</td>
<td>1.08</td>
<td>1.06</td>
</tr>
<tr>
<td>Ras homolog gene family, member A, RhoA (RHOA)</td>
<td>-1.15</td>
<td>-1.14</td>
</tr>
<tr>
<td><strong>ROS generation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ras-related C3 botulinum toxin substrate 2, Rac2 (RAC2)</td>
<td>-1.23</td>
<td>-1.89*</td>
</tr>
<tr>
<td>Neutrophil cytosol factor 1, p47phox (NCF1)</td>
<td>-1.48</td>
<td>-1.96*</td>
</tr>
<tr>
<td>Neutrophil cytosol factor 2, p67phox (NCF2)</td>
<td>-1.35</td>
<td>1.03</td>
</tr>
<tr>
<td>Neutrophil cytosol factor 4, p40phox (NCF4)</td>
<td>-1.01</td>
<td>1.03</td>
</tr>
<tr>
<td>Cytochrome b-245, alpha polypeptide, gp22phox (CYBA)</td>
<td>-1.39*</td>
<td>-1.47*</td>
</tr>
<tr>
<td>Cytochrome b-245, alpha polypeptide, gp91phox (CYBB)</td>
<td>1.29</td>
<td>-1.63*</td>
</tr>
<tr>
<td>Myeloperoxidase (MPO)</td>
<td>1.32</td>
<td>-1.33</td>
</tr>
</tbody>
</table>

*Significant fold change.
Table 5.3 – Nrf2 pathway genes expressed by oral neutrophils in CP and healthy subjects.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change normalized to blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (PIK3R1)</td>
<td>CP: -4.67* Healthy: -1.76</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>CP: -4.13* Healthy: -1.30</td>
</tr>
<tr>
<td>DnaJ (Hsp40) homolog, subfamily C, member 9 (DNAJC9)</td>
<td>CP: -4.07* Healthy: -1.73</td>
</tr>
<tr>
<td>Protein kinase C, beta (PRKCB)</td>
<td>CP: -3.66* Healthy: -1.11</td>
</tr>
<tr>
<td>Superoxide dismutase 1, soluble (SOD1)</td>
<td>CP: -3.58* Healthy: -1.70</td>
</tr>
<tr>
<td>Microsomal glutathione S-transferase 3 (MGST3)</td>
<td>CP: -3.49* Healthy: -2.04</td>
</tr>
<tr>
<td>Ataxia telangiectasia mutated (ATM)</td>
<td>CP: -3.45* Healthy: -1.53</td>
</tr>
<tr>
<td>Endoplasmic reticulum protein 29 (ERP29)</td>
<td>CP: -3.38* Healthy: -1.63</td>
</tr>
<tr>
<td>Glutathione S-transferase mu 2 (muscle) (GSTM2)</td>
<td>CP: -3.34* Healthy: -1.46</td>
</tr>
<tr>
<td>Aldo-keto reductase family 1, member A1 (aldehyde reductase) (AKR1A1)</td>
<td>CP: -2.97* Healthy: -2.40*</td>
</tr>
<tr>
<td>Chaperonin containing TCP1, subunit 7 (eta) (CCT7)</td>
<td>CP: -2.84* Healthy: -1.71</td>
</tr>
<tr>
<td>Kelch-like ECH-associated protein 1 (KEAP1)</td>
<td>CP: -2.81* Healthy: -1.76</td>
</tr>
<tr>
<td>Copper chaperone for superoxide dismutase (CCS)</td>
<td>CP: -2.72* Healthy: -1.18</td>
</tr>
<tr>
<td>Protein kinase C, zeta (PRKCZ)</td>
<td>CP: -2.72* Healthy: -1.38</td>
</tr>
<tr>
<td>Peroxiredoxin 1 (PRDX1)</td>
<td>CP: -2.70* Healthy: -1.98*</td>
</tr>
<tr>
<td>BTB and CNC homology 1, basic leucine zipper transcription factor 1 (BACH1)</td>
<td>CP: -2.62* Healthy: -1.27</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase 7 (MAP3K7, TAK1)</td>
<td>CP: -2.54* Healthy: -1.08</td>
</tr>
<tr>
<td>Valosin containing protein (VCP)</td>
<td>CP: -2.46* Healthy: -1.37</td>
</tr>
<tr>
<td>γ-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)</td>
<td>CP: -2.42* Healthy: -1.25</td>
</tr>
<tr>
<td>Protein kinase C, eta (PRKCH)</td>
<td>CP: -2.41* Healthy: -1.16</td>
</tr>
<tr>
<td>γ-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) (MAF)</td>
<td>CP: -2.24* Healthy: -1.78*</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 14 (MAPK14)</td>
<td>CP: -2.23* Healthy: -1.18</td>
</tr>
<tr>
<td>Phosphoinositide-3-kinase, regulatory subunit 5 (PIK3R5)</td>
<td>CP: -2.06* Healthy: -1.21</td>
</tr>
<tr>
<td>Glutathione S-transferase kappa 1 (GSTK1)</td>
<td>CP: -2.01* Healthy: -1.10</td>
</tr>
<tr>
<td>Glutamate-cysteine ligase, modifier subunit (GCLM)</td>
<td>CP: 2.27* Healthy: -1.01</td>
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<tr>
<td>Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2)</td>
<td>CP: 2.53* Healthy: 1.44</td>
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<td>DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9)</td>
<td>CP: 2.71* Healthy: 1.66</td>
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<tr>
<td>Jun B proto-oncogene (JUNB)</td>
<td>CP: 3.74* Healthy: 1.27</td>
</tr>
<tr>
<td>Ferritin, heavy polypeptide 1 (FTH1)</td>
<td>CP: 6.66* Healthy: 1.71</td>
</tr>
<tr>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) (MAFF)</td>
<td>CP: 7.43* Healthy: 2.24</td>
</tr>
</tbody>
</table>
Table 5.4 – Nrf2 pathway genes expressed in CP vs. healthy subjects.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>CP vs. Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (PIK3R1)</td>
<td>1.27    -2.06</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>-1.17   -3.42*</td>
</tr>
<tr>
<td>DnaJ (Hsp40) homolog, subfamily C, member 9 (DNAJC9)</td>
<td>1.34    -1.74</td>
</tr>
<tr>
<td>Protein kinase C, beta (PRKCB)</td>
<td>-1.02   -1.57*</td>
</tr>
<tr>
<td>Superoxide dismutase 1, soluble (SOD1)</td>
<td>1.79*   -1.33</td>
</tr>
<tr>
<td>Microsomal glutathione S-transferase 3 (MGST3)</td>
<td>1.46    -1.24</td>
</tr>
<tr>
<td>Ataxia telangiectasia mutated (ATM)</td>
<td>1.09    -1.07</td>
</tr>
<tr>
<td>Endoplasmic reticulum protein 29 (ERP29)</td>
<td>1.29    -1.39</td>
</tr>
<tr>
<td>Glutathione S-transferase mu 2 (muscle) (GSTM2)</td>
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</tr>
<tr>
<td>Aldo-keto reductase family 1, member A1 (aldehyde reductase) (AKR1A1)</td>
<td>1.51*   1.11</td>
</tr>
<tr>
<td>Chaperonin containing TCP1, subunit 7 (eta) (CCT7)</td>
<td>1.08    -1.50</td>
</tr>
<tr>
<td>Kelch-like ECH-associated protein 1 (KEAPI)</td>
<td>1       -1.54</td>
</tr>
<tr>
<td>Copper chaperone for superoxide dismutase (CCS)</td>
<td>1.37    -1.52</td>
</tr>
<tr>
<td>Protein kinase C, zeta (PRKZ)</td>
<td>-1.11   -2.06</td>
</tr>
<tr>
<td>Peroxiredoxin 1 (PRDX1)</td>
<td>1.62    1.06</td>
</tr>
<tr>
<td>BTB and CNC homology 1, basic leucine zipper transcription factor 1 (BACH1)</td>
<td>-1.25   -2.20*</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase 7 (MAP3K7, TAK1)</td>
<td>-1.03   -2.27*</td>
</tr>
<tr>
<td>Valosin containing protein (VCP)</td>
<td>-1.13   -1.98*</td>
</tr>
<tr>
<td>v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)</td>
<td>-1.10   -2.10*</td>
</tr>
<tr>
<td>Protein kinase C, eta (PRKCH)</td>
<td>1.36    -1.74*</td>
</tr>
<tr>
<td>v-MAF musculoaponeurotic fibrosarcoma oncogene homolog (avian) (MAF)</td>
<td>1.21    -1.05</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 14 (MAPK14)</td>
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</tr>
<tr>
<td>Phosphoinositide-3-kinase, regulatory subunit 5 (PIK3R5)</td>
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<tr>
<td>Glutathione S-transferase kappa 1 (GSTK1)</td>
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</tr>
<tr>
<td>Glutamate-cysteine ligase, modifier subunit (GCLM)</td>
<td>1.02    2.50*</td>
</tr>
<tr>
<td>Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2)</td>
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<tr>
<td>DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9)</td>
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<tr>
<td>Jun B proto-oncogene (JUNB)</td>
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</tr>
<tr>
<td>Ferritin, heavy polypeptide 1 (FTH1)</td>
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</tr>
<tr>
<td>v-MAF musculoaponeurotic fibrosarcoma oncogene homolog F (avian) (MAFF)</td>
<td>-2.30   1.26</td>
</tr>
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5.4.2 Nrf2-/- mouse neutrophils produce low CAT levels

Nrf2-/- mice were as a model of primary AO deficiency to assess the role of neutrophil AO deficiency on extracellular ROS production and migration towards chemoattractants. Protein extracts of bone marrow neutrophils assessed by western blot showed lower CAT production by Nrf2-/- compared to wild type. (C57BL/6, 1.52 ± 0.42; Nrf2-/-, 0.76 ± 0.09 relative to β-actin) (Fig. 5.2 A). Both wild type and Nrf2-/- produce high ROS levels in response to PMA (Baseline: C57BL/6, 172.5 ± 28.7 RU; Nrf2-/-, 120.75 ± 25.3 RU; Stimulated: C57BL/6, 267.3 ± 46.7 RU; Nrf2-/-, 230 ± 50.7). Extracellular ROS levels were measured as peak relative light units using an isoluminol-amplified chemiluminescence assay (66,188). Extracellular ROS production in response to PMA stimulation was similar in Nrf2-/- and wild type neutrophils (C57BL/6, 1.53 ± 0.04; Nrf2-/-, 1.93 ± 0.18) (Fig. 5.2 B). Migration speed and directionality of neutrophils during chemotaxis were previously demonstrated to be regulated by ROS (54). Nrf2-/- neutrophil response to chemoattractants was tested using a Zigmond chamber as previously described (187). Nrf2-/- had higher migration speed along an fMLP gradient compared to wild type (C57BL6, 4.52 ± 0.34 μm/sec; Nrf2-/-, 6.86 ± 0.34 μm/sec) despite similar overall response to the chemoattractant (migrated cells: C57BL/6, 83.32 ± 0.45 %; Nrf2-/-, 84.33 ± 4.90) (Fig. 5.2 C, D).
Figure 5.2 – Neutrophils of Nrf2-/- mice produce low levels of CAT and have increased migration speed. CAT production by bone marrow neutrophils from Nrf2-/- mice was assessed by western blot and compared to wild type C57BL/6 mice (A). Extracellular ROS production by Nrf2-/- neutrophils in response to fMLP and PMA (10^{-6}M) stimulation (30 minutes) was measured by isoluminol-amplified chemiluminescence (B). Chemotaxis was measured in Zigmond chambers along an fMLP gradient (10^{-6}M). Neutrophil movement was recorded using time-lapse video microscopy. Migration speed and efficiency (percentage migrated cells) were measured by playback analysis of 15-minute recordings (C). One way analysis of variance, mean ± SEM. *p<0.05, n=4 mice/group.
5.4.3 Nrf2-/− mice have increased periodontal tissue destruction at sites with periodontitis

To get further insight into the impact of AO deficiency on periodontal tissue breakdown in periodontitis, alveolar bone loss (ABL) at sites with ligature induced-periodontitis was measured by morphometry in Nrf2-/− mice. Statistically significant ABL was observed in Nrf2-/− mice at sites with periodontitis compared to C57BL/6 mice (239.7 ± 16.4 μm vs. 162.4 ± 7.6 μm respectively). Both groups presented significant ABL at periodontitis sites compared to contralateral healthy molars and no differences were found between control sites (Fig. 5.3 A, B). Loss of attachment was measured on histological sections of diseased molars stained with Masson’s trichrome. Nrf2-/− mice had more epithelial and connective tissue attachment loss compared to C57BL/6 mice (172.6 ± 8.1 μm vs. 166 ± 2 μm respectively) (Fig. 5.3 C, D). These results demonstrate a more severe tissue breakdown in Nrf2-/− mice, indicating a cytoprotective role of Nrf2 in periodontitis.

5.4.4 Nrf2-/− mice have high 8-hydroxy-deoxyguanosine (8-OHdG) levels at sites with periodontitis

To assess ROS damage at sites with periodontitis we quantified 8-OHdG positive cells and found increased numbers around ligated molars of Nrf2-/− mice compared to healthy molars and to C56BL/6 control mice (Control: C57BL/6, 1.97 ± 0.39; Nrf2-/−, 1.49 ± 0.19; Ligature: C57BL/6, 2.08 ± 0.25; Nrf2-/−, 6.23 ± 1.05 cells/square pixels) (Fig. 5.3 E, F). Positive cells identified at the control sites are considered as physiologic background level of DNA oxidation (189). Analysis of 8-OHdG positive cell levels in different regions of interest (ROIs) in the soft tissues and along the alveolar bone surface was also completed. In the sulcular
epithelium (Epi) and gingival connective tissue (CT) around the ligature, Nrf2-/- mice had significantly more positive cells per area compared to control mice (Epi: C57BL/6, 1.77 ± 0.33; Nrf2-/-, 9.56 ± 1.38; CT: C57BL/6, 1.44 ± 0.39; Nrf2-/-, 6.11 ± 0.85). Analysis of the alveolar bone crest (ABC) region did not result in any significant differences between control and Nrf2-/- mice (C57BL/6, 2.69 ± 0.40; Nrf2-/-, 3.03 ± 0.19) (Fig. 5.3 G).
A

Control

Ligature

C57BL/6

Nrf2−/−

100 μm

100 μm

B

Bone loss (μm)

C57BL/6 Nrf2−/− C57BL/6 Nrf2−/−

Control Ligature
Figure 5.3 – In response to ligature-biofilms Nrf2-/ mice develop severe bone and attachment loss associated with high oxidative stress. Periodontitis was induced in Nrf2-/ mice using a ligature-biofilm model. Representative micrographs of buccal aspects of ligated and contralateral healthy control molars demonstrate the bone loss 21 days after induction of periodontitis. Alveolar bone loss was measured in three points from the cement-enamel junction (dashed line) to the bone crest (red arrows) (n=12 WT, 11 Nrf2-/) (A, B). Loss of connective tissue attachment was measured on Masson trichrome stained sections from the cement-enamel junction (green arrow, dashed line) to the base of the pocket (solid line) on buccal and palatal surfaces of ligated molars (n=3 mice/group) (C, D). ROS damage in periodontitis lesions surrounding the ligature (red arrows) was assessed by immunohistochemistry for 8-Hydroxydeoxyguanosine (8-OHdG), a specific marker for oxidative damage to DNA (n=3 mice/group) (E). Oxidative damage was quantified in epithelium, connective tissue and at bone crest level by counting 8-OHdG+ cells per surface area (F, G). One way analysis of variance, mean ± SEM. *p<0.05.
5.5 Discussion

The current study demonstrates for the first time that Nrf2 pathway is downregulated in oral neutrophils of patients with severe CP compared to periodontally healthy controls. Twenty-four upstream and downstream Nrf2 pathway factors were downregulated 2-5 fold in oral compared to blood neutrophils of patients with severe CP. There were also 2 fold higher numbers of oral neutrophils in severe CP compared to health. In addition, we showed that oxidative damage as a result of Nrf2 knockout results in more severe loss of periodontal tissues in an animal model of periodontitis. The Nrf2 pathway controls the expression of a wide array of essential cytoprotective genes, its activation leading to cell survival and tissue protection during oxidative stress conditions (90,91). The role of CAT in maintaining periodontal health was previously demonstrated in Acatalasemia patients who are systemically healthy, but have increased incidence of periodontitis (106). Therefore, our results indicate that reduced production of AO such as CAT and SOD1 by neutrophils in the context of increased recruitment in the periodontium aggravates tissue destruction.

The high numbers of oral neutrophils and upregulation of CXCL8, TREML and CXCR1 suggest pro-inflammatory and pro-migratory priming of these cells to sustain a continuous neutrophil influx and delay resolution of inflammation in periodontal tissues. Since neutrophils are the major producers of ROS at sites of inflammation, AO production by neutrophils is critical to protect tissues from oxidative damage. Further, ROS neutralization may play a role in preventing a continuous influx of neutrophils to allow for resolution of inflammation to proceed since neutrophil chemotaxis is a ROS-dependent process (54). Indeed Nrf2-/- neutrophils had a higher migration speed compared to wild type, suggesting a potential for enhanced inflammatory responses through fast recruitment in tissues under oxidative stress conditions.
Down-regulation of the Nrf2 pathway was previously shown to contribute to oxidative stress and inflammation in various pathologic conditions. Impaired Nrf2 function was associated with chronic kidney disease in an animal model (190). Authors concluded that impaired Nrf2 activity contributes to disease progression through increased tissue damage. Furthermore, Nrf2-/- mice had an enhanced response to lipopolysaccharide stimulation with significantly higher lung infiltration compared to WT controls, as well as high serum levels of tumor necrosis factor α (TNFα) (191). Interestingly, our study found a mild but significant decrease in FPR1 receptor by oral neutrophils of patients with severe CP despite up-regulation of CXCL8 and receptors for endogenous chemokines suggesting an altered response to subgingival bacteria and a possible neutrophil-derived autocrine feedback loop for persistent acute inflammation.

Nrf2 down-regulation in oral neutrophils of CP patients as cause for reduced neutrophil ability to neutralize ROS and withstand oxidative stress, supports previous reports of increased oxidative damage in CP. Several studies demonstrated a significant increase in intracellular ROS production by peripheral blood neutrophils in patients diagnosed with CP compared to healthy controls (66,67). However, the investigation of peripheral blood neutrophils may not provide a full understanding of the mechanisms underlying neutrophil’s role in CP pathogenesis. Recently, our group demonstrated specific gene expression in oral neutrophils obtained from healthy subjects, when compared to peripheral blood neutrophils (75). Investigation of oral neutrophil ROS production in CP is in its infancy. One study showed increased stimulated levels of ROS in gingival crevicular fluid (GCF) of CP patients compared to healthy controls but no difference in superoxide generation by blood neutrophils or in AO capacity of cell-free GCF in disease versus health (156). It is currently believed that in a subset of patients with CP, who are refractory to conventional therapy, oral neutrophils are hyperactive and produce high ROS levels associated with more severe loss of periodontal tissues (192).
Malondialdehyde (MDA), a thiobarbituric acid reactive substance used as marker of lipid peroxidation to assess oxidative damage, was found increased in GCF and saliva of patients with CP (77,78,97). Tsai et al reported 200-400 fold higher MDA levels in GCF compared to saliva but similar salivary AO capacity. However, salivary AO capacity may have a lower prediction value for CP activity then oral neutrophil AO capacity considering that most oral neutrophils reach the oral cavity through the gingival crevice (193). To our knowledge, the current study is the first to report an association between reduced oral neutrophil AO production and severe CP, and opens the question whether blood neutrophils in patients with CP are primed to a low response to specific subgingival bacteria.

One hypothesis is that systemic low neutrophil AO production may precede onset of periodontal tissue destruction and contribute to CP pathogenesis. Increased severity of tissue breakdown at sites with periodontitis in Nrf2-/− mice was associated with decreased CAT but normal extracellular ROS production by neutrophils and increased oxidative damage in periodontal tissues compared to wild type mice. Levels of 8-OHdG, a commonly used marker of oxidative DNA damage, were higher at sites with periodontitis in Nrf2-/− mice. This confirms that persistent subgingival challenge results in worse local oxidative damage tissue breakdown when there is low systemic AO production. Whether low AO production by oral neutrophils in severe CP is due to systemic priming or due to local inflammatory milieu remains unknown. One study reported high 8-OHdG levels in whole saliva and GCF of patients with CP in a Japanese population but systemic levels of 8-OHdG were not measured (194). However, GCF levels of 8-OHdG were suggested as a biomarker for DNA damage caused by oxidative stress and an indicator for CP severity (56). D’Aiuto et al have shown that severe CP patients have higher reactive oxygen metabolites in serum and reduced total serum AO capacity compared to healthy controls (195). In search for salivary biomarkers for oxidative stress and alveolar bone
loss in CP a recent study has found increased salivary 8-OHdG and MDA levels, and reduced salivary TAOC in CP patients. A correlation between salivary MDA levels and C-terminal telopeptide of type I collagen (CTX I), a biomarker for bone loss, was reported (196). These findings point to a possible systemic low AO expression in CP but temporal relationships between low neutrophil AO production and periodontal tissue breakdown need further investigation.
Figure 5.4 – Down-regulation of Nrf2 pathway is associated with severe CP. Persistent subgingival bacterial challenge (biofilms adherent to calculus, or silk in the present mouse model) results in recruitment of neutrophils in periodontal tissues and extravasation into the oral cavity through the GCF. Oral neutrophils of CP patients up-regulate CXCL8 (IL-8) and CXCR1 (IL-8 receptor) expression, and slightly down-regulate FPR1 (fMLP receptor) and NADPH oxidase subunit expression. Further, down-regulation of Nrf2 pathway components in oral neutrophils of CP patients contributes to reduction in synthesis of AO. This may result in their increased recruitment but reduced ability to control pathogenicity of the subgingival biofilm and increased oxidative stress damage in periodontal tissues in the context of high neutrophil trafficking.
5.6 Conclusion

Increasing evidence suggests an important role of AO in control of tissue injury in inflammatory diseases. Multiple lines of evidence demonstrated a critical role of neutrophil function in onset and progression of periodontal tissue destruction. Neutrophils recruit in higher numbers at diseased sites in CP patients. The present study demonstrated that several factors involved in chemotaxis were upregulated at gene expression level in oral neutrophils of patients with severe CP. Further, Nrf2 AO pathway was downregulated in oral neutrophils of these patients and Nrf2 knockout in mice resulted in severe periodontitis through increased local oxidative damage. It can therefore be concluded that reduced AO production by oral neutrophils in un-resolved periodontal inflammation may contribute to increased severity of CP through local oxidative stress.
5.7 Acknowledgments

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Authors’ contribution:

C.S. – Assisted in surgical procedure for ligature-induced periodontitis model, and data analysis

F.S.L. – Took part in sample collection and preparation for gene expression microarray

C.S. – Assisted in chemotaxis assay and western blot analysis

M.B.G – Clinic director, Periodontal department, Faculty of Dentistry, University of Toronto

M.G. – Research supervisor
Chapter 6
Discussion and Future Directions

6.1 Discussion

Oxidative stress has been long recognized as a key player in periodontal tissue destruction and disease progression. Pathogenic mechanisms mediated by ROS, previously described in different inflammatory conditions, were suggested to be involved in various forms of periodontitis. However, some important questions on ROS involvement in periodontitis onset and progression remain unanswered. It is unclear whether ROS simply regulate the mechanisms that contribute to periodontal tissue destruction such as ostoclastogenesis (197) and neutrophil extravasation (55), whether they primarily cause direct ROS-mediated tissue damage, or whether both those mechanism exist at different levels during periodontal disease. In perspective, several known aspects of periodontitis pathogenesis point to a critical role of ROS in onset and progression of this disease. The fact that destruction of periodontal tissues in periodontitis is driven by host factors, that neutrophils are the front line innate immune cells interacting with subgingival biofilm, that ROS is their main weapon used against pathogens but can be deleterious to host tissues, that they persist in high numbers in the oral cavity of patients with untreated periodontitis and can be hyper-responsive to stimulation in a subset of patients non-responsive to conventional therapy all suggest that neutrophil-derived ROS is central to the course of periodontal inflammation in all forms of periodontitis. Understanding the mechanisms that regulate a balanced neutrophil ROS production and release in periodontal tissues will aid in designing improved prevention, diagnosis and treatment schemes for CP.
The work presented in chapter 3 indicated that salivary relative AO protein abundance in inflammatory and resolution phases of gingivitis correlates moderately with oral neutrophil recruitment. We can speculate that the observed increase in salivary AO levels in the inflammatory phase would favour an anti-inflammatory state that prevents tissue destruction. This hypothesis is supported by the finding that AO and other cytoprotective proteins were reduced in saliva during the resolution phase of gingivitis. It can therefore be suggested that patients with gingivitis who are able to maintain this anti-inflammatory state are more likely to experience resolution of gingival inflammation and less likely to develop loss of clinical attachment.

In order to better understand the role of neutrophil oxidative stress in progression of periodontal inflammation and tissue destruction, I chose to examine the responsiveness to stimulation for ROS production in oral neutrophils of patients diagnosed with refractory CP. Continued CAL in treated patients who are systemically healthy and compliant to their periodontal maintenance program characterizes this form of periodontitis. As previously noted, disease progression in these patients does not correlate with plaque levels, microbiology assessments, and homecare practices. Compromised host responses in refractory CP patients were reported but the specific mechanisms involved remain unknown (26).

Using a flow cytometry approach, which can measure per event (cell) parameters, I analyzed stimulated oral neutrophil ROS production in refractory CP patients and identified two patient sub-groups: High and Low responders. High responders were characterized by increased oral neutrophil stimulated ROS production, and presented with a more severe disease phenotype. The association between oral neutrophil ROS production and disease severity in this sub-group of refractory CP patients suggests a pro-oxidative oral neutrophil phenotype that may contribute to periodontal tissue destruction in certain CP patients.
To get further insight into mechanisms that may lead to neutrophil-mediated oxidative damage in periodontitis lesions I investigated the systemic and oral neutrophil oxidative state in patients with severe CP. The characterization of the oral neutrophil phenotype was completed by gene expression microarrays in CP patients and healthy individuals. Analysis of oral neutrophil gene expression revealed several pathways with significantly different regulation in CP patients compared to health (76). My observation of Nrf2 pathway downregulation in oral neutrophils in CP patients but no significant blood/oral changes in health suggests that the hypothesis of a site-specific pro-oxidative neutrophil phenotype may be more relevant to pathology than health. Microarray results were confirmed through PCR analysis of upstream Nrf2-mediated AO – SOD1 and CAT. In support of gene expression findings, oral/blood neutrophil ratios of CAT and SOD1 protein levels were reduced in CP. The reduction in CAT and SOD1 levels were statistically significant. These results indicate that low local neutrophil AO production is associated with severe CP.

The observed low oral neutrophil AO production through Nrf2 downregulation in patients with severe CP led me to investigate the impact of Nrf2 on periodontal tissue destruction. For this I used a murine model of periodontitis in Nrf2-/- mice. Nrf2-/- mouse neutrophils produced low levels of CAT and had high migration speed towards chemoattractants compared to wild types. Furthermore, Nrf2-/--developed severe periodontitis characterized by increased CAL and alveolar bone loss compared to wild types and high local oxidative damage. Altogether, these findings indicate that local low AO production results in increased oxidative damage and more severe loss of periodontal tissues. This may be due to normal or reduced ROS production/cell but higher reduction in AO production that results in altered protection against oxidative damage (Figure 6.1).
Figure 6.1 – Net increase in local neutrophils inefficient in controlling the biofilm and ROS damage. Increased neutrophil recruitment in the oral cavity of CP patients may result in oxidative damage in periodontal tissues in the context of normal or reduced ROS production/cell but inefficient control of biofilm pathogenicity and reduced production of AO.
In conclusion, the main findings of my thesis are as follows (figure 6.2):

1. Salivary AO and other cytoprotective protein levels increase in the inflammatory phase and subsequently decrease in the resolution phase of gingivitis. A few of these cytoprotective proteins correlate strongly with oral neutrophil numbers in gingivitis.

2. Hyper-responsive oral neutrophils, characterized by increased potential for stimulated ROS production, is associated with increased CAL in certain refractory CP patients.

3. Downregulation of the Nrf2 antioxidant and cytoprotective pathway in oral neutrophils is associated with upregulation of endogenous-types of chemotaxis mediators, increased oral neutrophil recruitment and severe CP.

4. Nrf2 knockout is associated with increased local oxidative damage and severe periodontal tissue destruction as demonstrated in a murine model of periodontitis.
Figure 6.2 – Suggested roles of neutrophil-mediated oxidative damage in pathogenesis of inflammatory periodontal diseases.
6.2 Future Directions

1) Our focus on neutrophil function and its role in periodontal tissue destruction in Nrf2 null mice does not explain the full extent of the effect of the Nrf2 pathway on CP pathogenesis. Nrf2 deficiency has been demonstrated to regulate osteoclast differentiation through the increase in oxidative stress (197). This mechanism may play a role in CP pathogenesis, and contribute to alveolar bone loss. Osteoclast differentiation and function in Nrf2 null mice is currently being investigated in our lab.

2) The use of exogenous antioxidants for the treatment of oxidative stress-related pathologies has been previously suggested. Local injection of bovine SOD was shown to be effective in several inflammatory pathologies, while systemic administration in rheumatoid arthritis patients resulted in no therapeutic effect (198). It is speculated that the nonhuman origin of the enzyme and its rapid metabolism in the human body (half life < 30 min following intravenous administration) may limit its systemic therapeutic effect (199). The use of a synthetic SOD mimetic was demonstrated to be effective in reducing all inflammatory parameters in an acute inflammatory model in rats’ pleural cavity, including significant reduction in oxidative stress markers (200). In contrast, AO treatment was reported to have several limitations such as promoting cell proliferation, inhibiting immune response, and affecting mitochondrial function (201). Yet the nature of periodontal disease does allow for efficient local administration (as observed with local administration of antibiotics), and may limit undesired side-effects. Using ligature-induced periodontitis model in Nrf2 null mice, will allow us to investigate the therapeutic potential of locally-administrated exogenous antioxidants during periodontitis. This may lead to future human clinical trials to investigate the therapeutic effects of topical AO treatment adjacent to SRP.
3) Whether the observed Nrf2 down regulation in oral neutrophils in CP patients is a response to the inflammatory process or an inherent risk factor remains unanswered. Investigating changes in Nrf2-regulated AO levels in isolated oral neutrophils before and after initial periodontal treatment in patients with severe CP will allow for better understanding of the observed Nrf2 down regulation in untreated CP patients. In addition to the suggested investigation in CP patients, the investigation of changes in Nrf2-regulated AO levels in isolated oral neutrophils during EG will elucidate the possible role of Nrf2 down regulation during the reversible phase of periodontal disease, and possible regulatory mechanisms for disease progression. Together, the proposed analysis of Nrf2-regulated AO levels during different periodontal conditions will expand our knowledge of AO dynamics during the development and progression of periodontal diseases, and may identify inflammatory-mediated changes in AO production in periodontal patients.

4) Neutrophil recruitment to the site of inflammation during CP (i.e. the periodontal pocket) is a key element in periodontal disease, as can be seen in increased oral neutrophil levels during EG and in CP patients when compared to healthy controls (Figures 3.5 and 5.1A). As stated in chapter 5, microarray analysis of neutrophil gene expression revealed a significantly higher CXCL8 and TREM1 expression by oral neutrophils in CP compared to blood neutrophils (Table 5.2). Increased IL-8 and TREM1 levels were previously demonstrated in GCF samples collected from CP patients (202,203). Chemotaxis-related protein analysis in oral neutrophils isolated from CP patients pre- and post-treatment and healthy controls will provide further insight into this aspect of oral neutrophil activity and its contribution to periodontal disease progression. This will contribute to our understanding of the oral neutrophil pro-inflammatory and pro-migratory phenotypes.
References


(20) Van Dyke TE. Commentary: periodontitis is characterized by an immuno-inflammatory host-mediated destruction of bone and connective tissues that support the teeth. *J Periodontol* 2014;85:509-511.


(65) Giannopoulou C, Krause KH, Muller F. The NADPH oxidase NOX2 plays a role in periodontal pathologies. *Semin Immunopathol* 2008;30:273-278.


(73) Differential functions of neutrophil phenotypes. The neutrophil in immunity; 2012.


(179) Page RC. Comment on refractory cases. AAP news 1985;20:5.


