Characterizing The Role Of Macrophage Polarization In Non-Surgical Periodontal Therapy in Humans

BY

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THESIS

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This thesis is dedicated to my wife Dr. Juhi Uttamani, my parents and my in-laws, for their never-ending support and guidance. A special mention to my wife for being a source of constant cheer and helping me stay calm throughout my difficult times…

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APC	Antigen-presenting cell
BOP	Bleeding on probing
CAL	Clinical attachment loss
CCL	Chemokine ligand
CD	Cluster of differentiation
ECM	Extracellular matrix
EF – Tu	Elongation Factor Tu
EBV	Epstein Barr Virus
FBS	Fetal bovine serum
GM-CSF	Granulocyte/ Macrophage Colony Stimulating Factor
HHV	Human Herpes Virus
HCMV	Human Cytomegalovirus
IL	Interleukin
IRB	Institutional Review Board
JAK	Janus-activated kinase
LAD -1	Leukocyte adhesion deficiency type 1
LFA-1	Lymphocyte function associated receptor 1
MAPK	mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MΦ	Human macrophages
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ND	Not determined
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
Pg	*Porphyromonas gingivalis*
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear leukocyte/neutrophil
PI3K	phosphoinositide 3-kinase
RANKL	Receptor activator of nuclear factor kappa-B ligand
RPL2	Ribosomal protein L2
RpoB	RNA polymerase subunit B
SD	Standard Deviation
STAT	signal transducer and activator of transcription
TLR	Toll-like receptor
Th	T helper cell
TNF α	Tumor necrosis factor alpha
TGM2	Transglutaminase 2

Summary

The inflammatory microenvironment in periodontal disease is governed by the infiltration of myeloid cells. Our previous studies demonstrated that macrophages respond to live periopathogens (Actinobacillus actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg)) and their LPS by polarizing towards the pro-inflammatory M1 phenotype. We hypothesize that macrophage polarization controls the extent of periodontal disease and contributes towards the resolution of inflammation post-therapy. The objective of this study was to evaluate the markers of macrophage polarization before and after periodontal therapy. Gingival biopsies were excised from five subjects with generalized severe periodontitis, undergoing routine non-surgical therapy. A second set of biopsies were excised after 4-6 weeks to assess the impact of therapeutic resolution at the molecular level. As controls, gingival biopsies were also excised from seven periodontally healthy control subjects undergoing crown lengthening surgery. As a marker of inflammatory status, we examined total RNA from gingival biopsies for pro- and anti-inflammatory markers associated with macrophage polarization by qRT-PCR from these subjects. Real-time PCR based analysis was used to assess the impact of therapy on the load of Aa and Pg. The mean probing depths before therapy were 9 ± 1.52 mm and after therapy were 6.85 ± 0.89 mm. This corroborated with the reduced levels of periopathic bacterial transcripts in the gingival biopsies after therapy. Lower expression levels of inflammatory markers TNF-α, CXCL10, STAT1, miR-155 were observed after therapy as compared to the diseased samples. On the contrary, higher expression of anti-inflammatory markers including STAT6, TGM2, IL10, CCL22 were observed post-therapy as opposed to the biopsies from diseased gingiva. Decrease in the levels of M1 macrophage markers and the converse increase in M2 macrophage polarization markers correlates with the clinical improvement in periodontal disease. Clinical Relevance: Imbalance in M1 and M2 polarized macrophages by assessment of their markers can provide relevant clinical information on the successful response of periodontal therapy and can be used to target non-responders with exaggerated immune responses.

CHAPTER 1: INTRODUCTION

1.1 Periodontal Disease: Immunopathogenesis

Periodontal disease is a chronic infectious inflammatory disease that can sever the homeostasis of the periodontal apparatus leading to progressive destruction of the tissues surrounding teeth (Darveau 2010). Being the second most common oral disease, it accounts for a nationwide prevalence of almost fifty percent (Eke et al., 2015). The oral environment forms a niche, harboring a plethora of organisms including bacteria, viruses and fungi. Studies have demonstrated a change in the microbial profiles accompanying the increased bacterial load with lack of oral hygiene. Periodontal health can be challenged by a dysbiotic microbial biofilm on the hard tissue surfaces, inducing inflammatory changes in the gingiva. The tissue damage following periodontal disease is a result of the host elicited immune responses to the periodontal pathogens (Hajishengallis et al., 2011).

In susceptible patients, the incipient dysbiosis that causes a breach in the gingival barrier triggers TLRs on various cells including epithelial cells, fibroblasts, tissue resident immune cells, thus, activating the non-specific host response pathways in response to the invading microbes. This leads to production of pro-inflammatory mediators such as cytokines, chemokines, prostaglandins, reactive oxygen species, as well as complement activation. The histamine - complement system mediated vasodilation and increased permeability accounts for the exaggerated crevicular fluid exudate and vasculitis. The chemotactic transmigration of PMNs to the site of infection follows activation of other innate immune cells like monocytes and macrophages (MΦs) that cause phagocytosis and opsonization, in an effort to eliminate bacteria (Page & Schroeder, 1976). If uncontrolled, the secretion of pro-inflammatory molecules by these cells and the enhanced release

of proteinases such as elastase and collagenase results in collagen degradation and bone resorption. The chronicity of the advanced lesions characterized by predominance of T and B-lymphocytes that is bridged by the antigen presenting cells like dendritic cells (DC); is clinically synonymous with irreversible apical migration, detachment of the junctional epithelium along with RANKL dependent bone degradation (Socransky et al., 1984).

1.2 Pathogenic Contributors in Periodontal Disease

Oral ecosystems comprising of commensal microbiota contribute towards maintaining healthy equilibrium. Ecological shifts under the influence of characteristic milieu such as pH, temperature, endogenous nutrients, surface availability for biofilm formation, accompanied by the patient-modifiers, including but not limited to smoking, diet and stress; allows the pathogens to manifest (Arweiler & Netuschil, 2016). Virulence factors derived from bacterial DNA, plasmids or bacteriophages integrated into the bacterial chromosome can influence host-cellular functions by tissue invasion and replication/expansion, thereby triggering host defense mechanisms and disease symptoms. The classic microbiota of the members of red complex i.e. *P. gingivalis, T. forsythia, T. denticola and A. actinomycetemcomitans* (Socransky et al., 1998); have been well characterized to drive the aggressive destruction of the hard and soft tissues through the production of potent virulence factors in periodontal disease.

As a keystone periodontal pathogen, *P. gingivalis* possesses distinct virulence molecules/structures such as proteases (e.g. gingipains), fimbriae, haemaglutinnin, LPS, etc. that are essential for interacting with the host cells and thereby instigating immune-mediated inflammatory destruction (Abdi et al., 2017). In order to ensure a continuum of host-derived nutrient supply, *P. gingivalis* has evolved to prolong (MΦs) and C5 complement activation,

inside-out signaling and Ca^{2+} release; enabling adequate binding of the microbe onto host cells. Apart from the proteolytic cleavage of immunomodulatory proteins in the host, gingipains also facilitate adhesion with oral epithelial cells and promotes colonization of the plaque biofilm by serving as ligands for co-aggregation with other oral bacteria such as the members of orange complex, i.e. *F. nucleatum*, *P. intermedia*, etc. (How et al., 2016). The high virulence potential of *A. actinomycetemcomitans* is exemplified by the fact that this bacterium can sense the environmental changes and alter the transcription of genes as needed for its survival within the host. Adhesion to oral surfaces, host immune suppression, induction of inflammation and tissue destruction is governed by several key factors such as fimbriae, adhesions (Aae, Omp 100), bacteriocins (Actinobacillin), Fc binding proteins (OmpA), leukotoxin, collagenase, LPS, etc. (Henderson et al., 2010). Adhesins, invasins and outer membrane proteins not only mediate tenacious biofilm formation, they are also associated with cellular invasion. The preferential binding of cytolethal distending toxin (CDT) and leukotoxin (LtxA) of *A. actinomycetemcomitans* to LFA-1 receptor in host, causes membrane disruption of the host cells; rendering *A.a* to be one of the most destructive periodontal pathogens (Slots & Ting, 1999). Amongst, the other potential inhabitants of periodontal lesions include viruses of the Herpesviridae family, including cytomegalovirus, EBV, HSV-1 that appear to play a role in the pathogenesis of periodontitis by subverting molecular signaling pathways thereby priming inflammatory immune cells. The dysregulated environment thus created could facilitate viral proliferation, causing clinical pathology. In an immunosuppressed host exhibiting periodontal tissue damage, the examples of viral-mediated synergistic responses include neutrophilic activation of type-1 interferon, release of proinflammatory cytokines and MMPs and activation of the osteoclastic pathway (Slots 2017). The pattern of herpes viral infection i.e. its latency/activation and tissue tropism, could be attributable

to the hallmark signs of periodontal diseases such as its episodic progression and localized destruction nature due to transient immunosuppression.

1.3 Innate Immune Cellular Players in Periodontal Disease

The interaction between the periodontal pathogens and gingival epithelial cells is one of the first cellular events orchestrating pro-inflammatory cytokine release in response to endotoxins and bacterial products. The recognition of microbial products by pattern recognition receptors (PRRs) expressed on various gingival resident cells such as epithelial cells, fibroblasts, (MΦs), neutrophils, and dendritic cells further propagates translation of signaling pathways that promote pro-inflammatory cytokines and chemokine expression (Abdi et al., 2017). The coordinated recruitment of neutrophils, that comprise >95% of leukocytes in the gingival crevice is vital to periodontal health. With gingival barrier breach, neutrophils are stimulated to exit the gingival microvasculature, chemotactically transmigrate towards the vascular endothelium via exogenous chemo-attractive signals (like bacterial LPS) and activated complement products. Neutrophils, once at the site of infection/injury release proteolytic enzymes and antimicrobial peptides, IL-1β, TNF-α, IL-6 and IL-8, reactive oxygen species, etc. in an attempt to curb (phagocytose) the invading bacteria (Dennison & Van Dyke, 1997). Virulence factors of several periodontal pathogens exhibit anti-neutrophil capabilities, inhibiting neutrophil transmigration into the periodontal tissues or are cytotoxic to the recruited neutrophils. Neutrophils are central to the pathogenesis of periodontitis and hypo- as well as hyper-responsive neutrophils exhibit the potential to foster collateral periodontal tissue damage. Further, being short lived innate immune sensing cells, if do not enter tissues or undergo apoptosis within 1 to 2 days, they are subsequently cleared by (MΦs).

MΦ are promptly recruited to the sites of periodontal infection and encounter antigens from invading organisms. Belonging to the mononuclear phagocyte system, MΦs are infiltrated by several tissue factors to metamorphose as well as constantly replenish, from the circulating monocytes (Harokopakis et al, 2006). These recruited MΦ are exposed to a variety of host derived inflammatory products and pathogen molecules, at the infection site. Studies, including the ones from our lab (Fig 1) have shown the elevated presence of macrophages in gingival tissues from chronic periodontitis subjects, along-with greater secretion of pro-inflammatory cytokines like IL1, IL8, TNF-α, etc. by MΦ in chronic periodontitis patients (Fokkema 2012, Keller et al., 2009).

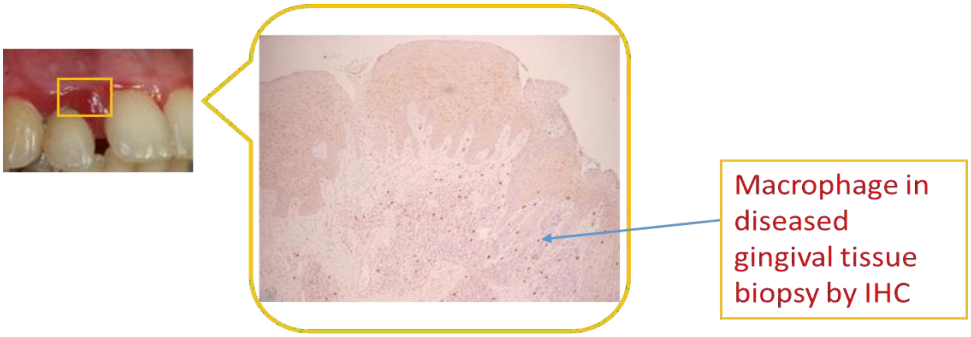

Figure 1: Macrophages visible in high numbers in the connective tissue of diseased gingival biopsy (blue arrow from a chronic periodontitis subject) as observed by CD68 immunostaining.

Studies have demonstrated increased induction of prostaglandin (PGE2), a pro-inflammatory lipid mediator associated with periodontal bone resorption and intensified inflammatory activity in (MΦs) upon stimulation with Pg LPS (Chapple et al., 1998). In the diseased periodontium, (MΦs) are shown not only to encompass a major source of inflammatory molecules like macrophage inflammatory protein (MIP)-1a, IL1, TNF-α and IL-8, but are also crucial precursors to osteoclastic bone resorption. MIP-1a and IL-8 regulate activation of phagocytic cells and lymphocytes during inflammatory episodes. The cytokine mediated bone matrix degradation via MΦ is considered essential for the attachment of premature osteoclasts

providing that differentiate to form multinucleated cells and subsequently resorb bone (Berglundh & Donati 2005).

1.4 Macrophage polarization

The classic chronic inflammatory state in periodontal disease is characterized by prolonged inflammation in conjunction with healing events (viz. angiogenesis and fibrosis), while at the same time favoring the sustained dysbiotic biofilm owing to its enriched nutritional microenvironment (Socransky et al., 1984). The phagocytic abilities of both tissue-resident (MΦs) and monocyte-derived recruited MΦ alike, are essential in innate defense as well as in the regulation of acquired immunity. Studies report differential transcriptional profiling in the tissue resident versus recruited MΦ from circulating monocytes, signifying that location and environmental triggers stimulate their polarization (Das et al., 2015). MΦ can be activated in response to a plethora of stimuli, such as periodontal pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and other inflammatory cells to acquire distinct functional subsets of differing phenotypic polarizations. Their cellular plasticity thus enables MΦ to respond to distinct environmental signals in different regulatory, phenotypic, functional (cytokine release), and metabolic rearrangements (Zhou et al., 2019). Based on the type-1/type-2 T helper cell polarization concept, phenotypically polarized MΦ can differentiate into M1 (classical) or M2 (alternative) phenotypes (Garlet & Giannobile 2018). Exemplifying opposing activities, M1 MΦ promote bacterial killing and a proinflammatory environment by increased production of IL-6, TNF-α, inducible nitric oxide synthase (iNOS), etc. , while M2 MΦ are anti-inflammatory in nature favoring resolution of inflammation and wound-healing.

Preliminary studies from our lab have shown that the priming of naive monocytes to host cytokines, such as interferon gamma (IFN-γ) or IL-4, can give rise to different MΦ phenotypes (Naqvi et al., 2016). This has considerable consequences for how MΦ detect, phagocytose and kill bacteria. MΦ polarization in pathologic conditions such as chronic periodontitis most appropriately represents a continuum, as opposed to two distinct scenarios involving heterogeneity of the effector cells to a spectrum of polarization states that do not fit to the oversimplified M1/M2 classification (Garaicoa-Pazmino et al., 2019). The local microenvironment dependent abundance of MΦ within tissues is finely controlled through the axis of M-CSF and GM-CSF, enabling appropriate responses to the microbial challenge or repair following an injury. The GM-CSF signaling pathway which encompasses the JAK-STAT, MAPK, PI3K pathways, ultimately trigger NFκB activation (Self-Fordham et al., 2017).

Several activated interconnected pathways also tightly regulate MΦ polarization functions. The balance between activation of STAT1 and STAT6 play a decisive role with the preponderance of LPS/microbial driven STAT1 activation promoting M1 MΦ polarization, while STAT6 promotes M2 MΦ polarization by increased IL-4/IL-13 and IL-10 signaling (Yu et al., 2016). “M1-like” states lead to cytotoxic function (killer) and “M2-like” phenotypes can induce the functional capacity of repair, inducing angiogenesis, remodeling. Gene expression analysis have shown that M1 MΦ release high levels of proinflammatory cytokines and chemokines such asTNF-α, IL6, CXCL10, etc. On the contrary, M2 MΦ produce high levels of IL10, CCR2, and CCL22 with higher expression of TGM2, arginase-1, etc. highlighting their relevance in tissue remodeling (Keller et al., 2009).

MicroRNAs (miRNAs) are small, single stranded non-coding RNAs that have emerged as important biologic regulators, capable of modulating gene expression at the post-transcriptional

level (Kulkarni et al., 2016). Accumulating evidence including studies from our lab suggest a relevant role for several miRNAs in leukocyte differentiation, innate signaling as well as MΦ activation (Kulkarni et al., 2017; Mendell & Olson 2012). Our lab has extensively studied the inhibitory myeloid cell functionality upon over-expression of miR-24, miR-30b, and miR-142-3p on LPS-stimulated monocyte, MΦ, and DC resulting in the reduction in pro-inflammatory cytokine secretion (Naqvi et al., 2016). In the context of MΦ polarization, several authors have reported certain key miRNA players such as miR-155, miR-146a, miR-127, 125b, etc. to be involved in modulating MΦ activation state (Self-Fordham et al., 2017; Zhang et al., 2016; Ying et al., 2015). Overexpression of miR-155 drives MΦ to M1 phenotype, by regulating Akt-dependent M1/M2 MΦ polarization. Additionally, it has also been demonstrated that miR-155 can suppress M2 phenotype by downregulating the expression of IL-13Rα1. Likewise, over-expression of miR-146a has been reported to cause a significant decreased production of TNF-α in M1 MΦ, with increased production of M2 marker genes such as Arg1 by targeting Notch signaling pathway (Huang et al., 2016). Divergent miRNA expression occurring in periodontal disease can influence monocyte-to-MΦ differentiation, thereby affecting MΦ functionality in the disease pathogenesis.

1.5 Study Significance and Specific Aims

MΦ are capable of exhibiting inherent functional plasticity via initiating the innate responses and shaping the adaptive arm of immunity allowing them to thus, adeptly respond according to a myriad of pathogenic stimulus in the oral cavity. The inflammatory microenvironment in periodontal disease is governed by infiltration of myeloid cells along-with the local stromal MΦ help to augment immune cell activation and prevent tissue damage. Periopathogens (such as *P. gingivalis* and *A. actinomycetemcomitans*) alter host immune response

by perturbing expression of pro- and anti-inflammatory milieu causing activation of M1 MΦ phenotype that are primarily involved in Th1 responses, lymphokine production, and degradation of intracellular pathogens. Periodontal therapy that results in reduction of bacterial load that may instigate a pro-resolution environment favoring M2 MΦ polarization, triggering Th2 responses such as immunotolerance, and tissue remodeling, etc.

Based on our preliminary studies demonstrating that human MΦ respond to live Pg or Aa and their LPS by polarizing towards the pro-inflammatory M1 phenotype (Naqvi et al., 2016), we hypothesized that non-surgical periodontal therapy creates a M2 MΦ dominant micro-environment favoring tissue repair. There have been reports studying the expression of M1, M2 markers in human gingival tissues of patients with chronic periodontitis (Zhou et al., 2019). However, to the best of our knowledge there has been no longitudinal study evaluating the influence of periodontal therapy on MΦ polarization. This is important given the dynamic interaction of immune mediated signaling governing the inflammatory component as well as tissue healing in the cyclical nature of periodontal disease.

The studies proposed here are designed to shed light on the pathogenesis of periodontal disease in response to non-surgical therapeutic intervention, in the context of changes in the MΦ profiles. Based on our preliminary analysis, the expression of bacterial gene transcripts from Pg and Aa that were most consistently expressed in gingival biopsies of chronic periodontitis subjects across various geographically distinct cohorts were examined to reflect their changes post-therapy. Next, we identified the impact of periodontal therapy on M1, M2 MΦ gene markers as well as the expression levels of M1, M2 associated cytokines/chemokines. Studies proposed here aim to elaborate on the impact of non-surgical periodontal therapy, which has not been previously reported, on MΦ polarization in humans. The ultimate goal of which being identification of

therapeutically beneficial drug targets that will enhance current modalities for the treatment of periodontitis. Thus, this proposal has multiple innovative aspects that have not been previously investigated. The overall hypothesis and specific aims are:

Hypothesis: MΦ polarization contributes to periodontal pathogenesis and conversely, contributes towards the resolution of inflammation post-therapy.

Aim 1: Evaluate the clinical parameters and correlate with the perio-pathogen load in human gingival biopsies after non-surgical periodontal therapy by examining the expression profiles of Aa, Pg encoded genes.

A. Acquire gingival tissue biopsy samples from human subjects diagnosed with severe chronic periodontitis before and after non-surgical periodontal therapy.

B. Quantitate the bacterial load by assessing periodontal pathogen gene transcript levels to compare/contrast with pre and post-therapy outcomes.

Aim 2: Evaluate the profiles of M1 and M2 MΦ associated markers in human gingival biopsies before and after non-surgical periodontal therapy.

A. Evaluate the expression of M1 markers (STAT1, miR-155) and M1 associated cytokine/chemokines (TNF-$\boldsymbol{\alpha}$, CXCL10) after non-surgical periodontal therapy and correlate with pre-therapy levels.
B. Evaluate the expression of M2 markers (STAT6, TGM2) and M2 associated cytokine/chemokines (CCL22, IL-10) after non-surgical periodontal therapy and correlate with pre-therapy levels.

The presence of MΦ exhibiting the combined polarization phenotype by concurrently expressing M1 and M2 markers have been recently reported (Zhang et al., 2019). Our aim was to fill existing knowledge gaps by examining the profiles of polarized MΦ (M1 and M2) after non-surgical periodontal therapy to provide further insights of their role in modulating oral mucosal immune responses as well their active participation in wound repair. This transition between M1 and M2 polarization can ultimately determine the pathogenesis of periodontal disease. We anticipate that these changes can provide insightful findings on the role pathogen stimulated immune responses in the development of oral diseases.

CHAPTER 2: METHODOLOGY

2.1 Study Design

Subject selection and sample collection

The present investigation was an observational, cross-sectional pilot study approved by the Institutional Review Board and the Ethics Research Committee at the University of Illinois at Chicago, College of Dentistry (IRB Protocol# 2017-1064). The study was conducted according to the ethical principles of the Helsinki Declaration. Subjects presenting to the Postgraduate Periodontics Clinic at the UIC College of Dentistry for routine periodontal care from March 2018 to May 2019 were recruited for this study. All participants were informed of the aims of the study and signed the informed written consent form prior to entering the study. The convenience sample from the present study comprised twelve individuals from a multiethnic group, of both sexes, and divided into two groups: periodontally healthy (Control, H; n = 7) or chronic periodontitis (Experimental, D; n = 5).

For the diseased group, a biological sample (gingival tissue including the gingival epithelium, col area and underlying connective tissue) were obtained at baseline (D, pre-therapy) and 4-6 weeks after non-surgical periodontal therapy (T, post-therapy) from the same subject. The second biopsy was taken from a separate, preselected site. For the control group (H), samples were derived from healthy gingival tissues normally discarded during routine crown lengthening procedures. Crown lengthening is a common surgical procedure that removes a collar of gingival tissue to reestablish proper tooth length and width for placement of a tooth filling, fabrication of a crown, or to address esthetic concerns due to excess gingival tissues. Inclusion criteria included

male and female patients ages 18–65 years and in good systemic health. Exclusion criteria included chronic disease (diabetes, hepatitis, renal failure, clotting disorders, HIV, etc.), smokers, antibiotic therapy for any medical or dental condition within a month before screening, and subjects taking medications known to affect periodontal status (e.g., phenytoin, calcium channel blockers, cyclosporine).

Periodontal status was assessed using periodontal probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), and radiographic evaluation of crestal bone loss. All examinations were performed with a manual periodontal probe (PCPUNC-15, Hu-Friedy, Chicago, IL, USA) by two investigators (V.K and J.R.U.), with high inter-examiner correlation coefficient kappa '0.85'. The presence or absence of chronic periodontitis was described by our previous studies (Perri et al., 2012). Briefly, subjects with generalized severe chronic periodontal disease displayed probing depth ≥ 6 mm, ≥ 4mm of attached gingiva, bleeding on probing and radiographic evidence of bone loss (AAP Parameter on Chronic Periodontitis, 2000). Health periodontal patients displayed probing depths ≤ 3 mm, with no bleeding on probing and no radiographic evidence of bone loss (American Academy of Periodontology, 2015).

The biopsy sample was harvested using intrasulcular and inverse bevel incisions approximately 2 mm from the free gingival margin at the crest of the interproximal papillae extending horizontally capturing the interproximal col area. For the diseased cohort, a gingival tissue sample was obtained prior to non-surgical periodontal therapy ("deep cleaning") and 4-6 weeks after therapy. The post-therapy sample was collected from a second, periodontally involved site away from the pre-therapy biopsy site. For the control group, samples were collected during a single crown lengthening procedure with no additional samples collected. Figure 2 depicts the

details on the number of subjects recruited for the study followed by biopsy sample collection / processing.

2.2 RNA Isolation and RT-PCR

Specimens were immediately placed in RNAlater (Qiagen, Gaithersburg, MD, USA) and stored at −80°C. Tissue samples were lysed using the TissueLyzer (Qiagen) and total RNA isolated using the miRNeasy (Qiagen) kit according to the manufacturer's instructions. Briefly, after homogenization, the samples were incubated on ice for 10 min. Afterwards, the lysis/binding buffer of acid phenol: chloroform was added. The contents were mixed for 60 s and centrifuged for 15 min at 12,000 × g. Supernatant solutions were then transferred to another Eppendorf tube with the addition of 1.25 volume of 100% ethanol followed by the filter-column based manufacturer's protocol for total RNA extraction. Samples were quantified (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA) and RNA quality assessed (Bioanalyser 2100 RNA 6000 Nano Kit, Agilent Technologies, Waldbronn, Germany) with an A260/A280 ratio ≥1.8.

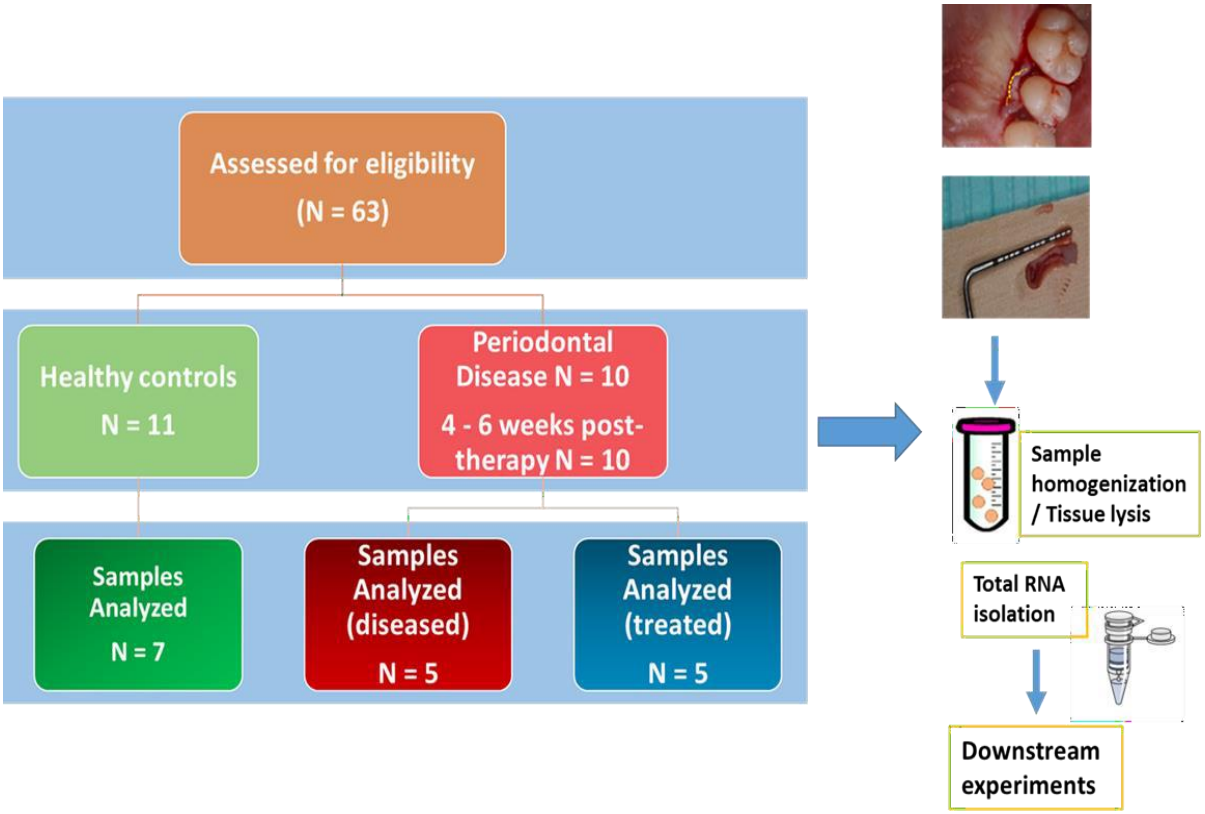

Figure 2: Subject Recruitment and Biopsy obtainment followed by sample processing for downstream analysis

For mRNA expression analysis, cDNA was synthesized from 250 ng total RNA using first strand cDNA synthesis kit (Invitrogen Co., Carlsbad, CA, USA). Real-time PCR based analysis was used to assess the impact of therapy on the load of periodontal pathogens: *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* by testing bacterial gene products of the two most highly expressed candidate genes for each. Briefly, transcript levels of primers designed for gingipain RgpA, RNA polymerase sub-unit β of *P. gingivalis* and Elongation Factor Tu, Ribosomal protein L2 of *A. actinomycetemcomitans* were evaluated by RT-PCR (Table 1). Using gene specific forward and reverse primers, the expression levels of STAT1, STAT6, TGM2, CXCL10, CCL22, IL10 and normalization control GAPDH were examined by qRT-PCR (Sigma

Aldrich, St. Louis, MO, USA). The Ct values of replicates were analyzed to calculate relative fold change using the delta-delta Ct method.

Additionally, for mature miRNA-155 quantification, miScript primers and miScript II RT Kit were purchased from Qiagen. 100 ng total RNA was reverse transcribed according to manufacturer's instruction. The reactions were performed using miRNA specific primer, universal primer (Qiagen) and SYBR Green (Roche, Indianapolis, IN, USA). RNU6B was used as endogenous control. The Ct values of replicates were analyzed to calculate relative fold change using the delta-delta Ct method and the normalized values plotted as histograms with standard deviations (SD). The Ct values of replicates were analyzed to calculate relative fold change using the delta-delta Ct method. The primers used for our experiments are listed in Table 1.

Gene	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
STAT1	ACCCAATCCAGATGTCTATG	GAGCCTGATTAAATCTCTGG
STAT6	TACTGAAGACTCAGACCAAG	GATGATTTCTCCAGTGCTTTC
TGM2	CTTCATTTTGCTCTCAACG	AGGATCCCATCTTCAAACTG
IL10	GCCTTTAATAAGCTCCAAGAG	ATCTTCATTGTCATGTAGGC
TNFa	CCCTTTATTACCCCCTCCTTCA	ACTGTGCAGGCCACACATTC
CCL22	GTGGTGTTGCTAACCTTC	GGCTCAGCTTATTGAGAATC
CXCL10	AAACGAGTTAGCAAGGAAAG	TCATTGGTCACCTTTTAGTG
GAPDH	ACAGTTGCCATGTAGACC	TTTTTGGTTGAGCACAGG
Bacterial gene transcripts: *P. gingivalis* (Pg) *A. actinomycetemcomitans* (Aa)		
Pg (PG2024) RgpA gingipain	AGTTCAATCCTGTAAAGAAC	TCTGCTGCGAGCACAACCTT
Pg (PG0395) RNA pol β	CAGTATGCTCAAGCGTAAGGA	CCAGGTAGTCAGTCTTACCA
Aa (AA 918) EF – Tu	GCAAATGGACGGTGCTATCT	CCCGGGAAGTCATATTGAGA
Aa (AA 778) Ribosomal_L2	TACAGATCATTGCCCGTGAA	GCTTTACCCAATACGCGAAG

Table 1: Primer sets used for M1, M2 macrophage markers and bacterial gene transcripts

2.3 Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The results were presented as SD or ±SEM from three independent replicates, and experiments were conducted at least three times. The p values were calculated using a Student *t* test and/or ANOVA for more than two groups. A p value <0.05 was considered significant. Age distribution in the healthy controls and disease groups were assessed by Mann-Whitney U test. Gender distribution across the groups was evaluated by McNemar $\chi 2$ test.

CHAPTER 3: RESULTS

3.1 Population Characteristics

The sample size of this pilot study consisted of 12 individuals. Accounting for the pre-treatment and post-treated samples the total number of 17 biologic replicates for the three groups were as follows: Healthy Controls 'H' n = 7, Diseased 'D' n = 5, Treated 'T' n = 5, respectively. There were no statistically significant differences noted in age (p=0.49) or gender (p=0.55) across the groups in our cohort. The mean age of individuals in the healthy group being 43 ± 11.3 years (range: 32–60) while the mean age in the chronic periodontitis/treated group was 52 ± 9.31 years (range: 38–59). The mean PD in the control group was 2.75 mm. For individuals with generalized chronic severe periodontitis, mean PD was 9 mm pre-therapy, which was reduced to approximately 7 mm after the non-surgical periodontal therapy. Representative clinical images of the healthy controls, periodontitis subjects before and after therapy is depicted in Figure 3. Periodontal clinical parameters of study groups and the subject demographics, are shown in Table 2. A statistically significant reduction in the PD, CAL and BOP was observed post-therapy.

Figure 3: Representative clinical images of healthy cohort and chronic periodontitis subjects before and after non-surgical periodontal therapy

Patient Variables	Controls (n = 7)	Chronic Periodontitis (n = 5)	Non-Surgical Therapy* (n = 5)	P - value
Age (years)	43 ± 11.13	52 ± 9.31		
Gender (M/F)	3/4	3/2		
Number of teeth present	28 ± 2.9	25 ± 3.7		< 0.05
Mean PPD (mm)	2.75 ± 0.63	9 ± 1.52	6.85 ± 0.89	<0.001 *<0.01 (post-therapy)
Mean CAL	3.15 ± 0.12	9.35 ± 1.28	5.59 ± 0.35	<0.001 *<0.01 (post-therapy)
% BOP	7 ± 2.4	74 ± 4.89	25 ± 7.35	<0.001 *<0.01 (post-therapy)

Table 2: Subject demographics and clinical parameters: The total sample size of the study was 17 samples included from the 3 groups: Healthy N = 7, Diseased N = 5 and Treated N = 5. There were no statistically significant differences in age and gender between the groups. Reduction in the mean probing depth, bleeding and clinical attachment loss were observed in the diseased individuals as measured 4-6 weeks after non-surgical periodontal therapy ($p<0.05$) highlighted in red square.

3.2 Reduced Levels of Pg and Aa Bacterial Gene Transcripts in Gingival Biopsies After Non-Surgical Periodontal Therapy Corroborates with Improvement in Clinical Parameters

To investigate the impact of non-surgical periodontal therapy response on the bacterial load at the molecular level, we compared expression of bacterial gene transcripts in gingival biopsies derived from periodontally healthy, diseased, and treated subjects. Our preliminary analysis indicated RgpA gingipain (PG 2024), RNA poly β (rpoB)/ (PG 0395) to be one of the most highly expressed *Porphyromonas gingivalis* encoded transcripts enriched in chronic periodontitis. Likewise, Elongation Factor – Tu (EF – Tu)/ AA 918 and Ribosomal protein L2 (RPL2)/ AA 778 were two of the most significantly enriched transcripts encoded by *Aggregatibacter actinomycetemcomitans*, as observed by RNA sequencing analysis (RNAseq). This observation

was consistent across four different, geographically distinct cohorts of chronic periodontits subjects (unpublished data).

Hence, we attempted to examine the expression levels of RgpA, rpoB, EF-Tu and RPL2 in the gingival biopsies collected from subjects diagnosed with chronic periodontitis prior to- and after non-surgical periodontal therapy (4-6 weeks post-treatment) comparedto healthy controls. Our data confirmed significantly increased expression of RgpA (Ct = 26.7 ± 1.5), and rpoB (Ct = 28.8 ± 1.7) transcripts from Pg (Figure 4) and EF-Tu (Ct = 25.5 ± 2), and RPL2 (Ct = 24 ± 1) transcripts from, Aa (Figure 5) in diseased biopsy specimens compared to healthy controls and post-treatment samples. Both Pg gene transcripts were not detected in healthy specimens while Aa gene transcripts had lower expression for EF-Tu (Ct = 31.5 ± 2) and RPL2 (Ct = 29.9 ± 1.4). For the gingival biopsies taken after 6 weeks post non-surgical therapy, the levels of the bacterial gene transcripts approximated as that seen in healthy controls, for both periodontal pathogens. Specifically, transcripts for Pg were either not detected or detected at significantly reduced expression levels (i.e. RgpA (Ct = 34.5 ± 1.1), rpoB (Ct = 35 ± 1.6)) Transcripts for Aa EF-Tu (Ct = 29.5 ± 1.5) and RPL2 (Ct = 28 ± 1) were also detected at reduced levels in treated samples. These results clearly show that the transcripts of periodontal pathogens demonstrated significantly greater expression in gingival biopsies derived from periodontally diseased subjects and its expression was reduced after periodontal therapy, approximating to that observed in healthy control group.

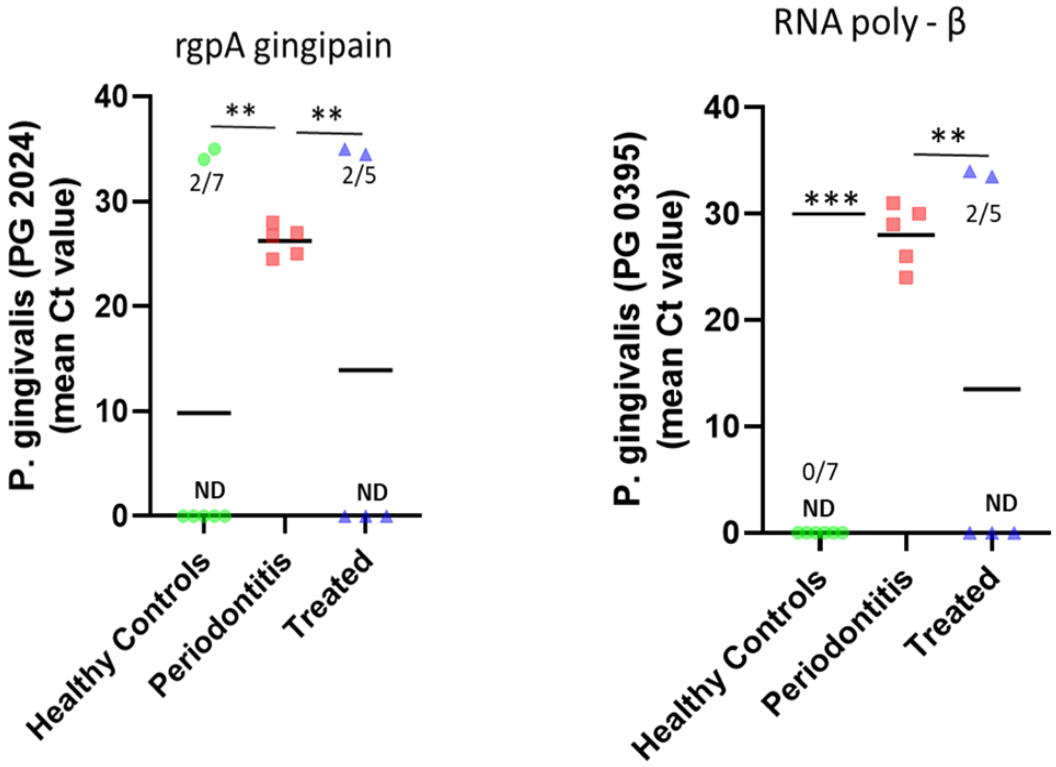

Figure 4: Reduced levels of Pg encoded gene transcripts (RgpA gingipain and RNA polymerase subunit-b) in gingival biopsies after non-surgical periodontal therapy. Quantitative PCR analysis showing with scatter plot showing mean Ct value expression of RgpA and RNA pol b in the gingival biopsy samples of periodontally diseased (n = 5) and 6 weeks post-therapy (n = 5) as compared to those of healthy controls (n = 7). Student's t-test was used to calculate p-values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is presented as ±SEM of three independent experiments.

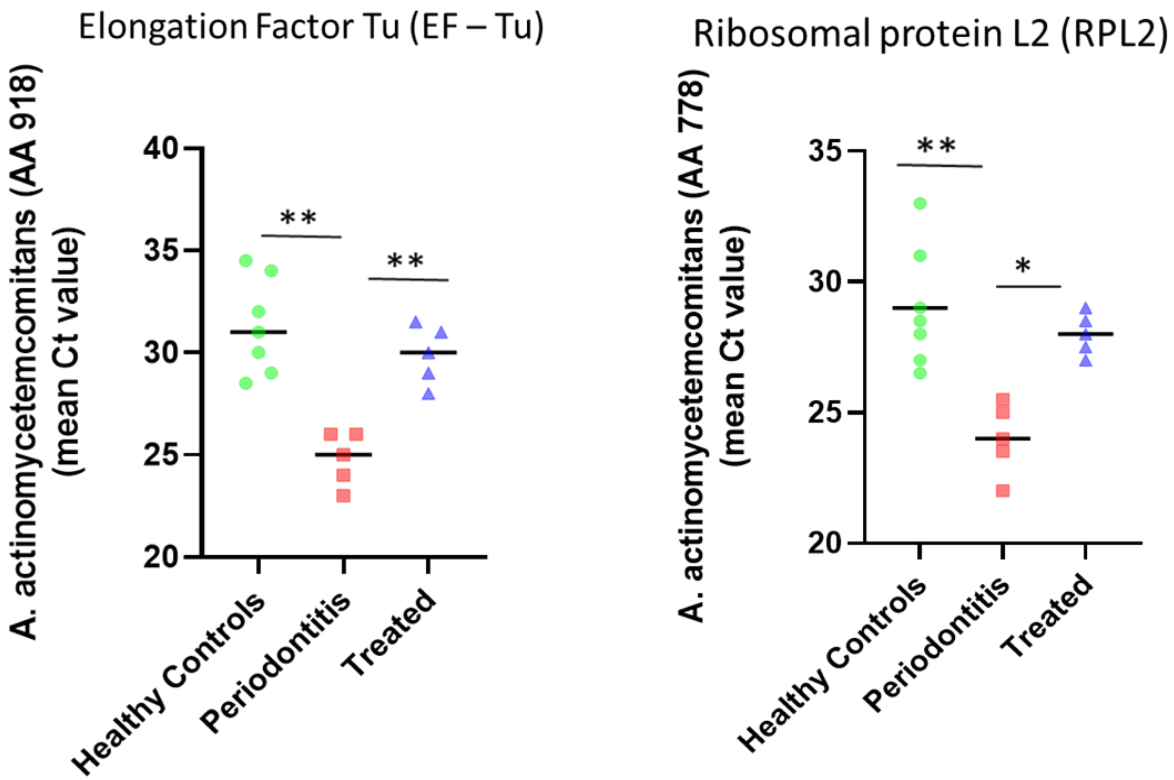

Figure 5: Reduced levels of Aa encoded gene transcripts Elongation Factor- tu and Ribosomal Protein L2, in gingival biopsies after non-surgical periodontal therapy. Quantitative PCR analysis showing with scatter plot showing mean Ct value expression of EF-Tu and RPL2 in the gingival biopsy samples of periodontally diseased (n = 5) and 6 weeks post-therapy (n = 5) as compared to those of healthy controls (n = 7). Student's t-test was used to calculate p-values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is presented as ±SEM of three independent experiments.

3.3 **Downregulation of M1 Markers (STAT1, miR-155) and M1 Associated Cytokine/ Chemokines (TNF-α, CXCL10) After Non-Surgical Periodontal Therapy**

To investigate whether non-surgical periodontal therapy can impact MΦ polarization, we evaluated changes in expression of well characterized M1 markers STAT1 and miR-155, known to promote M1 MΦ phenotype (Wang et al., 2014; Zhang et al., 2016). We compared expression of STAT1 and miR-155 in gingival biopsies derived from periodontally healthy, diseased, and treated (4-6 weeks post-treatment) subjects. We observed significantly higher expression of STAT1 (Fold

change = 4.6; Ct = 23.4 ± 2.0 vs 29.2 ± 1.6) and miR-155 (Fold change = 4.2; Ct = 22.3 ± 2.0 vs 26.9 ± 1.4) in diseased gingival samples (Figure 6) compared to healthy controls, respectively. The expression levels of STAT1 (Fold change = -1.12; Ct = 28.9 ± 1.3) and miR-155 (Fold change = 1.81; Ct = 25.9 ± 2.1) in gingival biopsies taken after 6 weeks post non-surgical therapy were significantly decreased, similar to levels observed in the healthy cohort (Figure 6).

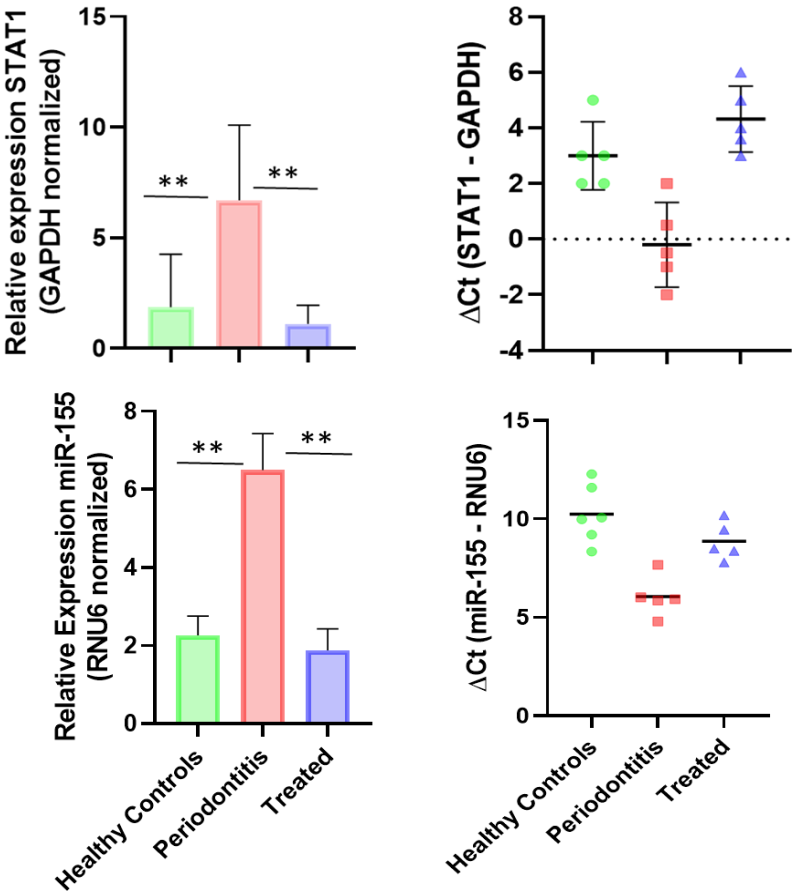

Figure 6: Downregulation of M1 markers (STAT1, miR-155) after non-surgical periodontal therapy: Histograms showing relative expression of STAT1 and miR-155 expression level by Quantitative PCR analysis in gingival biopsy samples of periodontitis patients (n = 5), after therapy (n = 5) as compared to those of healthy controls (n = 7). Results are normalized to those of controls and are represented relative to expression of GAPDH and RNU6, respectively. Student's t-test was used to calculate p-values. *$p < 0.05$, **$p < 0.01$. Data is presented as ±SEM of three independent experiments.

Likewise, upon assessing M1 related pro-inflammatory cytokine / chemokine expression, we found higher expression of TNFa (Fold change = 4.4; Ct = 23.4 ± 2.0) and CXCL10 (Fold change = 9.3; Ct = 23.4 ± 2.0) (Figure 7) in samples from chronic periodontitis subjects as compared to healthy controls. This expression significantly reduced in post-treatment biopsies with TNFa (Fold change = 0.6; Ct = 23.4 ± 2.0), CXCL10 (Fold change = -1.4; Ct = 23.4 ± 2.0), respectively. These results clearly show that M1 MΦ markers are downregulated in gingival biopsies derived after non-surgical periodontal therapy from periodontally diseased subjects.

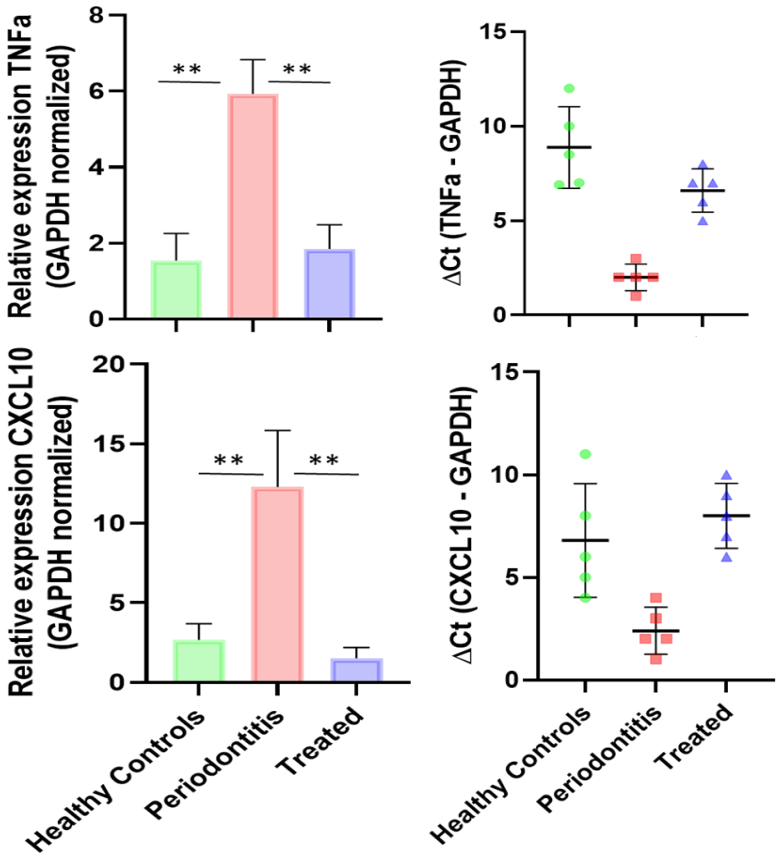

Figure 7: Downregulation of M1 associated cytokine (TNFa) and chemokine (CXCL10) after non-surgical periodontal therapy. Histograms showing relative expression of TNFa and CXCL10 expression level by Quantitative PCR analysis in gingival biopsy samples of periodontitis patients (n = 5), after therapy (n = 5) as compared to those of healthy controls (n = 7). Results are normalized to those of controls and are represented relative to expression of GAPDH. Student's t-test was used to calculate p-values. *$p < 0.05$, **$p < 0.01$. Data is presented as ±SEM of three independent experiments.

3.4 Upregulation of M2 Markers (TGM2, STAT6) and M2 Associated Cytokine/ Chemokine Secretion (CCL22, IL-10) After Non-Surgical Periodontal Therapy

To theorize the antagonistic expression levels of M2 MΦ activation markers in response to periodontal therapy, we evaluated expression profiles of TGM2 and STAT6, which have been well characterized to promote the M2 phenotype (Martinez et al., 2014; Wang et al., 2014). We also examined the expression of M2 associated effector chemokines/ cytokines (CCL22, IL10) in response to therapy. We inspected the RNA expression levels of STAT1, TGM2, IL10, and CCL22 in the gingival biopsy specimens from periodontally diseased cohorts pre- and post-treatment. In contrast to the elevated expression of M1 markers as previously observed, we found diminished expression of M2 markers STAT6 (Fold change = -1.2; Ct = 28.8 ± 1.29) and TGM2 (Fold change = -1.14; Ct = 33.5 ± 1.05) in the diseased specimens as compared to healthy controls STAT6 (Ct = 22.8 ± 0.65), TGM2 (Ct = 26.8 ± 1.5), as verified by quantitative PCR (Figure 8). Conversely, periodontal therapy recuperated STAT6 (Fold change = 2.4; Ct = 25.7 ± 2) and TGM2 (Fold change = 2.18; Ct = 29.1 ± 1) expression levels. Likewise, upon assessing M2 associated anti-inflammatory cytokine / chemokine expression, we found higher expression of IL10 (Fold change = 1.04; Ct = 28.4 ± 1.12) and CCL22 (Fold change = 1.13; Ct = 29.4 ± 2.0) (Figure 9) in post-treatment samples. This expression significantly curtailed in the pre-treatment diseased biopsies with IL10 (Fold change = -1.8; Ct = 33.1 ± 1.5), CCL22 (Fold change = -1.5; Ct = 33.4 ± 2.0), respectively. Collectively, these demonstrate that M2 MΦ or the repair-associated markers are upregulated in gingival biopsies derived after non-surgical periodontal therapy from periodontally diseased subjects.

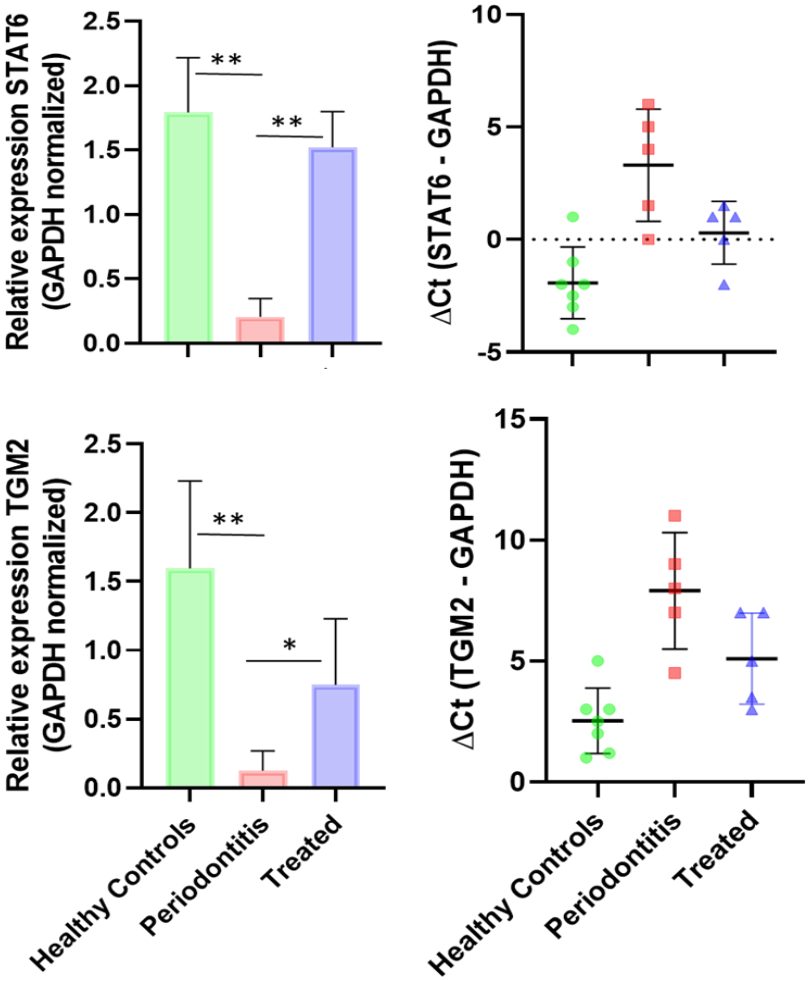

Figure 8: Upregulation of M2 markers (STAT6, TGM2) after non-surgical periodontal therapy: Histograms showing relative expression of STAT6 and TGM2 expression level by Quantitative PCR analysis in gingival biopsy samples of periodontitis patients (n = 5), after therapy (n = 5) as compared to those of healthy controls (n = 7). Results are normalized to those of controls and are represented relative to expression of GAPDH. Student's t-test was used to calculate p-values. *$p < 0.05$, **$p < 0.01$. Data is presented as ±SEM of three independent experiments.

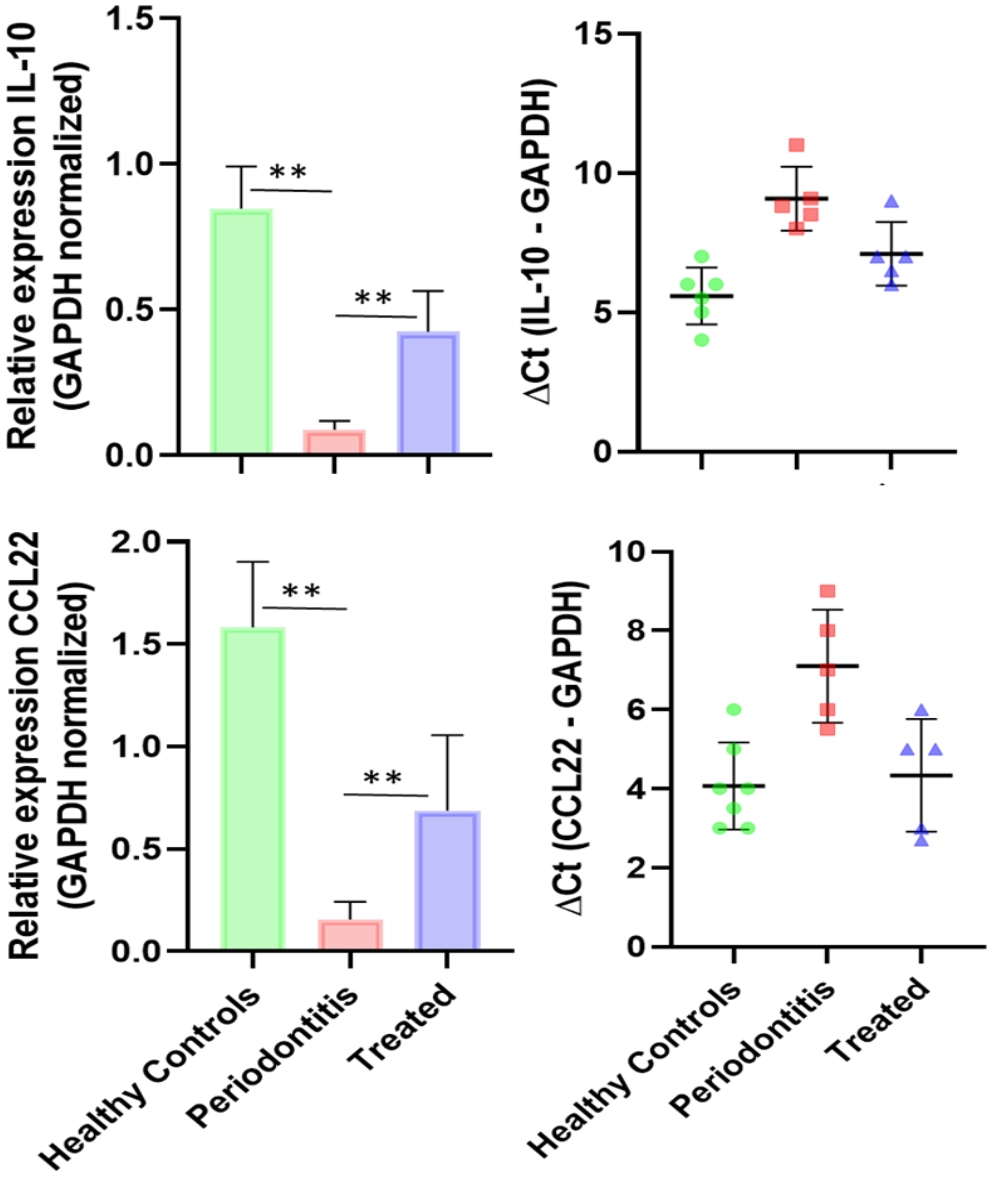

Figure 9: Upregulation of M2 associated cytokine / chemokine (IL10, CCL22) after non-surgical periodontal therapy. Histograms showing relative expression of IL10 and CCL22 expression level by Quantitative PCR analysis in gingival biopsy samples of periodontitis patients (n = 5), after therapy (n = 5) as compared to those of healthy controls (n = 7). Results are normalized to those of controls and are represented relative to expression of GAPDH. Student's t-test was used to calculate p-values. *$p < 0.05$, **$p < 0.01$. Data is presented as ±SEM of three independent experiments.

4 Discussion

Chronic periodontitis is a polymicrobial, inflammatory disease that commences with periodontal pathogen-induced immune responses, affecting the supporting tissues of the teeth including the periodontium and the alveolar bone (Kulkarni et al., 2014). Based on the concept of immunomodulation, MΦ play important roles as effector cells in mediating Th1 driven and Th2 derived immune responses. It is not incorrect to state that the chronicity of periodontal inflammation is an aggregated set of events with a continued proinflammatory environment accompanied with cyclic bursts of tissue healing patterns, including cell apoptosis, wound healing, cell proliferation, and tissue repair (Offenbacher et al., 2008). Upcoming literature evidence suggests the profound role of MΦ polarization in the gingival tissue from chronic periodontitis subjects (Zhang et al., 2019; Garaicoa-Pazmino et al., 2019). However, to the best of our knowledge there has been no study undertaken to longitudinally characterize MΦ polarization after non-surgical periodontal therapy in humans.

To address this shortcoming, we designed a case-control pilot study using a representative cohort of 17 subjects ranging from 19 to 80 years old presenting with no clinical history of uncontrolled systemic disease or use of drugs that could have an impact on periodontal pathogenesis or influence therapeutic outcomes. We also excluded pregnant subjects as well as patients with a history of uncontrolled diabetes, use of antibiotics, and smokers. To explore a more accurate picture of periodontal pathogenesis and minimize potential confounding factors (e.g. age, gender), we focused on a cohort of patients presenting chronic generalized severe periodontal

disease with age and gender matched controls. We evaluated the global MΦ polarization through assessing changes observed between M1 and M2 MΦ markers in the gingival tissue samples of periodontally diseased subjects before and after therapy and compared outcomes with healthy controls. In agreement with previous studies, the treatment of subjects in the chronic periodontitis cohort by scaling and root planning in promoting a clinically favorable environment, resulted in significant improvements in probing depths, clinical attachment levels and bleeding on probing parameters (Bhansali et al., 2014; Lindhe et al., 1984).

Studies have reported higher prevalence of *P. gingivalis* and *A. actinomycetemcomitans* in subjects with chronic periodontitis (Teixeira et al., 2009). Longitudinal studies assessing the clinical and microbiological evaluation of individuals undergoing periodontal therapy, have observed reduction in bacterial loads in the subgingival sites post-therapy (Doungudomdacha et al., 2001). However, since both Pg and Aa are capable of invading host tissues and cells (e.g. gingival epithelial cells), their influence on the outcomes of periodontal treatment and perio-pathogen load is still not clear. Our preliminary data from RNAseq analysis identified the gingipain RgpA and RNA polymerase sub-unit beta encoded by Pg and Elongation Factor – Tu, Ribosome protein L2 encoded by Aa, to be the transcripts that were highly expressed in gingival samples obtained from periodontitis subjects. This expression was consistent across three different patient cohorts across distinct geographic locations. Hence, we assessed the changes in these bacterial transcripts after non-surgical periodontal therapy. Gingipains that are involved in predisposing attachment and colonization of *P. gingivalis* (Moutsopoulos et al., 2012) and aiding its co-aggregation with other periodontal pathogens in the biofilm. They were not detected in the healthy cohort and after periodontal therapy. Likewise, rpoB, responsible for bacterial RNA synthesis was also not detected in healthy subjects and exhibited fairly undetermined levels after

therapy. Genes encoded by Aa, namely EF-Tu and RPL2 were detected in all three groups. However, their expression levels were minimal in healthy subjects and after non-surgical periodontal treatment. This observation could be owing to the fact that *A. actinomycetemcomitans*, an opportunist oral commensal member, has been detected in at-least one-third of healthy subjects (Sharma et al., 2017). EF-Tu, one of the most abundant proteins in bacterial cells, and RPL2 which is the primary RNA binding protein, can confer antibiotic resistance and alter translation frequency, thus aiding in the defense of this organism by synergistic interactions in the biofilm (Raja et al., 2014). Thus, the reduction in bacterial load by way of reduced perio-pathogen encoded transcripts corroborated with clinical improved outcomes.

Periodontal therapy triggers periodontal tissue repair and/or regeneration, requiring a change in the cellular milieu and a micro-environment supportive of resolution of inflammation. Our therapy clinically validated the assessment of M1/M2 MΦ polarization markers during the resolution of periodontal inflammation. The increased levels of M1 markers and pro-inflammatory cytokines are in concordance with other reports evaluating the role of MΦ polarization (Zhang et al., 2019). Bona-fide evidence of *in vivo* MΦ alternative activation, which elicits a strong Th2 response, was evidenced by increased IL10 and CCL22 expression, in a more comprehensive functional perspective after non-surgical periodontal therapy (Figure 10). CCL22, also known as macrophage-derived chemokine (MDC) is mainly produced by MΦ upon the stimulation with microbial products, is upregulated by Th2-type cytokines to enhance the chemotactic migration and recruitment of DCs and Th2 cells (Richter et al., 2014). In addition to the host immune responses in retaliation to the imbalances in periodontal microbiota; environmental and epi-genetic factors (e.g. miRNAs) are also responsible in directing the fate of MΦ M1, M2 switch in periodontal disease (Self-Fordham et al., 2017).

Our findings highlight the immuno-modulatory role of M1/M2 MΦ polarization in periodontal disease pathogenesis and likewise, the impact of periodontal therapy in causing a switch towards M2 MΦ phenotype. This MΦ plasticity is essential to combat pathogen/antigen clearance as well as instigate tissue repair.

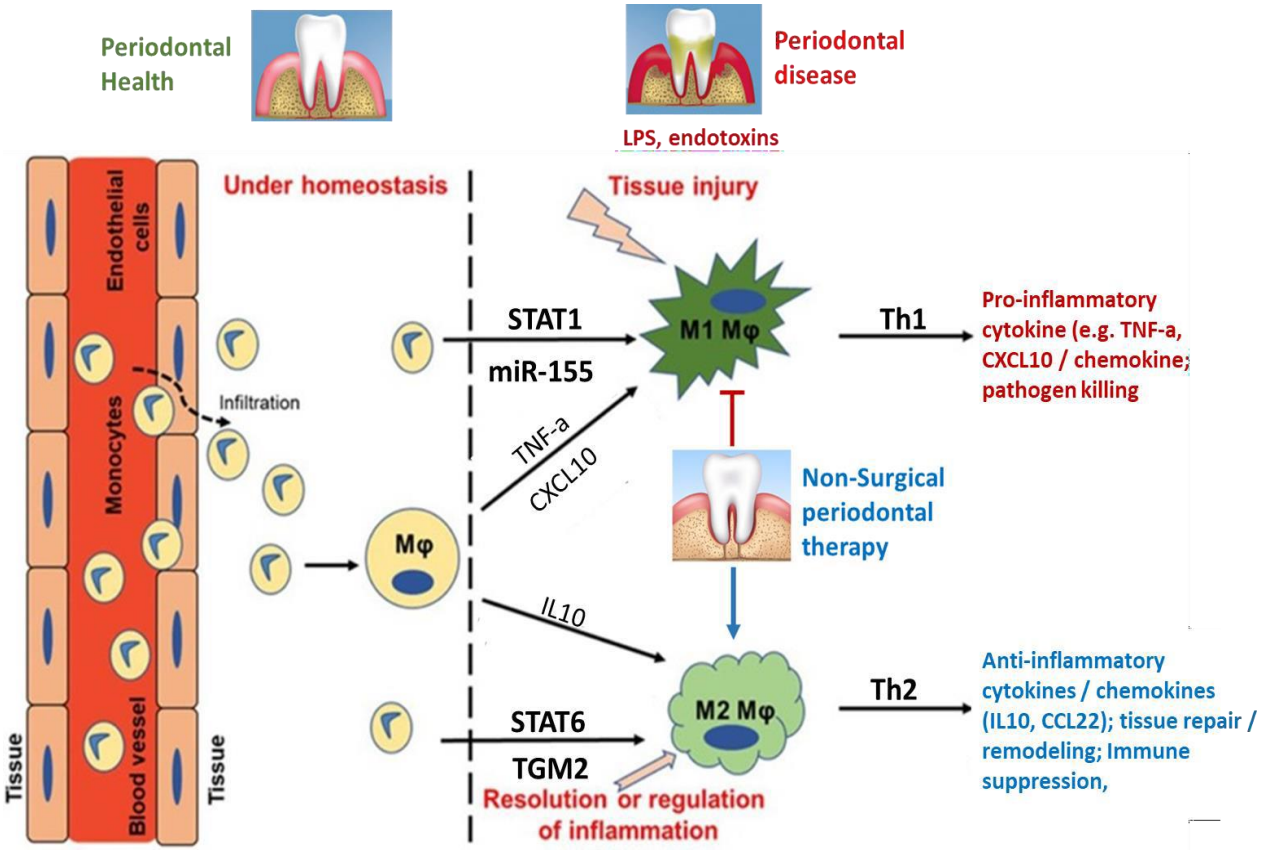

Figure 10: Summary schematic for the characterization of macrophage polarization after non-surgical periodontal therapy. The gingival barrier breach by invading dysbiotic pathogens from plaque bacteria (viz. Pg, Aa) cause infiltrating monocytes as well as tissue resident MΦ to polarize towards M1 Φ phenotype via STAT1 activation and is also controlled by miR-155. This is important for pathogen killing and tissue defense. Non-surgical periodontal therapy reduces the bacterial load and drives MΦ towards M2 Φ phenotype by STAT6 activation and increased TGM2 expression. This in-turn can stimulate tissue remodeling and repair as evidenced by improvements in clinical parameters (reduced PD, BOP; gain in clinical attachment).

5 Conclusion

The tissue equilibrium achieved between microbes in the plaque biofilm in periodontal health may be easily disrupted in states of chronic inflammation such as periodontal disease and lead to host-immune mediated tissue damage. An innovative and noteworthy finding was the restoration of M2 marker expression levels post-therapy to similar levels observed in periodontally healthy subjects, as well as reduction in the M1 MΦ marker expression levels that corroborated with clinical improvements; further affirmed the immune mediated restoration and/or maintenance of periodontal tissue homeostasis.

Our results highlight the significant role for immune governed mechanisms by MΦ plasticity and polarization in oral inflammation. The deranged immuno-modulatory responses could occur via over-activation of either pathways. It is the inherent plasticity that adeptly changes according to the tissue micro-environment, which determines M1 to M2 switch and vice versa.

M1 associated markers STAT1, miR-155 or the effector cytokines / chemokines TNF-a or CXCL10 that were responsive to periodontal therapy can be used to target specific subset of population with exaggerated immune responses. Conversely, M2 MΦ associated markers STAT6, TGM2 or the effector cytokines / chemokines IL10, CCL22 that were elevated after therapy, can be utilized to in evaluating response to therapy. Early detection and intervention utilizing effective biomarkers can help minimize or prevent periodontal destruction. Decrease in the levels of M1 Mφ markers and conversely, the increase in M2 Mφ polarization markers correlate with clinical improvement in periodontal disease.

Clinical Relevance: Assessment of M2 MΦ markers may provide clinically relevant information to evaluate patient response to periodontal therapy and may be useful to target non-responders with exaggerated immune responses.

Study Limitations and Future directions:

This is one of the first translational studies to longitudinally assess tM/M2 MΦ profiles before and after therapy in periodontitis subjects and can serve as the first step in examining current treatment strategies to include investigations on the role of MΦ immune mediated regulatory networks governing periodontal disease pathogenesis. The limited sample size of this study calls for expanding and validating the findings of this study in larger and different cohorts (greater sample size). Additionally, since these findings of this study are from gingival tissue biopsies which is heterogeneous in terms of its cellular populations, validation of the expression profiles of M1 and M2 related markers in macrophages challenged with live periodontal pathogens, in-vitro, would be beneficial. In situ detection of macrophage markers in healthy, diseased and post-therapy gingival biopsies could confirm our findings (e.g. Immunohistochemistry).To be able to translate MΦ mediated periodontal disease modulation for therapeutic purposes, future studies examining the M1/M2 axis after periodontal therapy different populations with larger sample size across differing geographical locations are warrantied. Animal models using the M1, M2 MΦ markers tested to be able to replicate the changes observed using ligature induced periodontitis models and after removal of stimulus, that would respond to an *in-vivo* therapeutic correlation are needed. The local/systemic delivery options need to be investigated in detail.

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