Identification and characterization of novel salivary biomarkers for oral inflammatory disease

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Certificate of approval

I confirm that, to the best of my knowledge, this thesis represents an original research carried out by **Ahmed Salih Khudhur** in fulfilment of the requirements for the degree of Doctor of Philosophy according to the regulations of Newcastle University.

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Abstract

Salivary biomarkers not only reflect the current status of periodontal diseases but may predict their progression and response to treatment. This study aimed to identify and characterize novel salivary biomarkers for periodontal diseases as a biomarker paradigm for an oral inflammatory disease.

Proteome profiler arrays were used to study the protein profile of whole saliva from 12 patients with chronic periodontitis and 12 healthy subjects, and to identify novel salivary biomarkers for periodontal diseases. Results revealed that there were differences in the whole saliva protein profile between health and disease status. Salivary urokinase (uPA), urokinase receptor (uPAR), and vitamin D binding protein (VDBP) were among the highly expressed proteins in periodontitis. These proteins were identified as candidate salivary biomarkers for periodontal diseases.

ELISA assays were used to quantify the candidate salivary biomarkers in 158 patients with periodontal diseases as compared to 103 healthy controls and were found to be significantly elevated in the patients ($p<0.001$). Salivary uPA levels but not uPAR and VDBP, were significantly downregulated following treatment of the patients ($p<0.01$). The uPA enzymatic activity was investigated in saliva of the patients and was found to be significantly elevated as compared to the controls ($p<0.001$). Salivary uPA, uPAR and VDBP levels and uPA activity were positively correlated with periodontal disease measures such as pocket depth.

The in vitro stimulation of human gingival fibroblasts with *P.gingivalis* lipopolysaccharide induced the cells to produce higher levels of uPA and uPAR along with uPA activity as compared to unstimulated cells ($p<0.01$).

In conclusion, salivary uPA, uPAR and VDBP in addition to uPA activity are suggested as biomarkers that may be useful in the diagnosis of periodontal diseases and can indicate their severity. Salivary uPA is a potential biomarker that can assess the response of periodontitis to treatment and may predict the disease progression.

Dedication

This humble work is dedicated to,

The man who taught me that to achieve knowledge you need to work hard and be patient. For his endless support and encouragement from the very first day I started my study.

My father Prof Salih Khudhur

The woman who brought me to life and blessed me with her unconditioned love and care that sustained me throughout my life.

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To prince charming my beloved son Yousif

To my beloved sisters

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Finally yet importantly, I am indebted and grateful to my beloved father, mother, wife and children for their patience to wait for me, their support, and encouragement during my study far away from them.

List of abbreviations

1 & 2-D PAGE	One & Two-dimensional polyacrylamide gel
2DE	Two-dimensional gel electrophoresis
3D	Three-dimensional
°C	Celsius/Centigrade
µg	Micro gram
µl	Micro litre
%BOP	Percentage of bleeding on probing
A.actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans* (old name *Actinobacillus actinomycetemcomitans*)
AAP	American Academy of Periodontology
ADAM8	A disintegrin and metalloproteinase domain-containing protein 8
ADAM9	A disintegrin and metalloproteinase domain-containing protein 9
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (von Willebrand factor-cleaving protease)

ADHS	Adult health survey
AFM	Alpha-albumin/afamin
AFP	Alpha-fetoprotein
AIDS/HIV	Acquired immune deficiency syndrome/ Human immunodeficiency virus
ALB	Albumin
ALP	Alkaline phosphatase
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMC	Amido-methyl-coumarin
APCs	Antigen presenting cells
ApoA-I	Apoliprotein A-I
AST	Aspartate aminotransferase
AUC	Area under the curve
BAFF	B-cell activating factor
BSA	Bovine serum albumin
C. rectus	*Campylobacter rectus*

Ca	Calcium
Cab	Capture antibody
CAL/LOA	Clinical attachment loss/ Loss of attachment
CD	Calibrator diluent
CDC	Centre for Disease Control and Prevention
CEJ	Cemento enamel junction
CO_2	Carbon dioxide
COX	Cyclooxygenase
CPI-PLC	Phosphatidylinositol-specific phospholipase C
CRM	Chemiluminescent reagent mix
CV%	Coefficient of variation
Dab	Detection antibody
DAC	Detection antibody cocktail
DDP	Dipeptidyl peptidase
DCD	Dermcidin
DGF/NGF	Down syndrome patients' gingival fibroblasts/ Non-Down syndrome subjects' gingival fibroblasts

DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPIV/CD26	Dipeptidyl peptidase-4 or adenosine deaminase complexing protein 2/ or cluster of differentiation 26
E. coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EFP	European Federation of Periodontology
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducer
ENA-78	Epithelial-derived neutrophil-activating peptide 78
EOP	Early onset periodontitis
F. nucleatum	*Fusobacterium nucleatum*
FBS	Foetal bovine serum
FLU	Fluorescence intensity

g	Gram
GAgP	Generalized aggressive periodontitis
GCF	Gingival crevicular fluid
G-globulin	Group specific component of serum, old name for VDBP
GI	Gingival index
GJP	Generalized juvenile periodontitis
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosyl-phosphatidyl-inositol
GSN	Gelsolin
HGF	Hepatocyte growth factor
hGFs	Human gingival fibroblasts
HI	Hyperplastic index
HRP	Horseradish peroxidase
i.e.	That is to say
ICTP	C-telopeptide pyridinoline cross-links of type I collagen
IFN-α	Interferon alpha
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
iTRAQ	Isobaric tags for relative and absolute quantitation
IU	International unit
kDa	Kilo Dalton
LAgP	Localized aggressive periodontitis
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LJP	Localized juvenile periodontitis
LPS	Lipopolysaccharide
Ltd	Limited
Ly-6	Lymphocyte antigen 6
MAF	Macrophage activating factor
MALDI-TOF-MS	Peptide mass fingerprinting and matrix-assisted laser desorption ionization time of flight mass spectrometry

MAMPs	Microbe-associated molecular patterns
MCP-1	Monocyte chemoattractant protein-1
MEMS/NEMS	Microelectromechanical systems/ Nanoelectromechanical (Nano-electro fluidic) systems
Mg	Magnesium
MGI	Modified gingival index
MILP	Mixed-integer linear optimization
MIP-1α	Macrophage inflammatory protein 1 alpha
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMP/MMPs	Matrix metalloprotease(s)
MMP-1	Interstitial/fibroblast collagenase
MMP-14	Membrane-type MMP
MMP-2	Gelatinase A
MMP-3	Stromelysin-1
MMP-8	Neutrophil collagenase

MMP-9	Gelatinase B
mRNA	Messenger RNA (Ribonucleic acid)
MS	Mass spectrometry
n	Number
Na	Sodium
NA	Not applicable
NE	Neutrophil elastase
ng	Nano gram
NHANES	National Health and Nutrition Examination Survey
nM	Nano-molar
NOS	Not otherwise specified
NS	Non-significant
NUG/NUP	Necrotizing ulcerative gingivitis/ periodontitis
OC	Osteocalcin
OD	Optical density
OHI	Oral hygiene instructions
OPG	Osteoprotegerin

OSCC	Oral squamous cell carcinoma
OSM	Oncostatin M
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
P. nigrescens	*Prevotella nigrescens*
PA/PAs	Plasminogen activator/Plasminogen activating system
PAI-1,-2	Plasminogen activator inhibitor-1,-2
PBI	Papillae or papillary bleeding index
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Probing depth/Pocket depth
PESA	Periodontal epithelial surface area
PF4	Platelet factor 4
pg	Pico gram
PGE_2	Prostaglandin E_2
PI	Pixel intensity/Plaque index (according to section used in)

PI-PLC	Phosphatidylinositol-specific phospholipase C
PISA	Periodontal inflamed surface area
PMNs	Polymorphonuclear leukocytes
POC	Point-of-care devices
PPA	Proteome profiler array
PRRs	Pattern recognition receptors
RANKL	Receptor activator of nuclear factor kappa-B ligand
RBP4	Retinol binding protein 4
RD	Reagent diluent
REC	Research ethics committee
rGFs	Rat gingival fibroblasts
RgpA-Kgp	Arginine-gingipain-and lysine-gingipain cysteine protease complex
rho	Spearman's rank correlation coefficient
ROC	Receiver operating characteristic curve analysis
ROS	Reactive oxygen species
rpm	Revolutions per minute

RPP	Rapid progressive periodontitis
RT	Room temperature
RT-PCR	Reverse transcription/ Real time -polymerase chain reaction
S_a	Amount of uPA for each sample from the standard curve of the uPA activity assay
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFM	Serum free medium
SKB	Salivaomics knowledge base or database
SOD1	Superoxide dismutase
SRP	scaling and root planing
suPAR	Soluble uPAR
S_v	Sample volume in ml added to each well in the uPA activity assay
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*

TFF3	Trefoil factor 3
TGF-α/-β	Tumour growth factor-alpha and -beta
Th	T helper cells
TIMP	Tissue inhibitors of MMPs
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue type plasminogen activator
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
v/v	Volume/volume
VD	Vitamin D
VDBP/DBP	Vitamin D binding protein
VEGF	Vascular endothelial growth factor
VN	Vitronectin
w/v	Weight/volume
WHO	World health organization

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Chapter 1 Introduction

A biomarker is a biological substance which can be measured objectively and used as an indicator for a number of activities in the human body which could be a homeostatic, pathogenic, or pharmacological response to therapy (Taba et al. 2005; Strimbu and Tavel 2010). As they both contain locally and systemically derived biomarkers, saliva and gingival crevicular fluid (GCF) can provide the basis for the diagnosis and monitoring of many oral and systemic diseases such as periodontitis, oral squamous cell carcinoma, oral lichen planus, and cardiovascular diseases (Taba et al. 2005; Ng et al. 2007; Ghallab et al. 2010; Sorsa et al. 2011; Rathnayake et al. 2013; Cheng et al. 2014; Taylor 2014). Since the 1990s, several studies on reliable salivary biomarkers for oral squamous cell carcinoma (OSCC) have been published, revealing that there are more than 100 different salivary components suggested to be potential OSCC salivary biomarkers such as interleukins (IL-1β, IL-6, and IL-8), defensin-1, α-amylase, tumour necrosis factor-α (TNF-α), matrix metalloproteinases (MMP-2,and 9) and many others (Cheng et al. 2014). Salivary interferon gamma (IFN-γ), TNF-α, and TNF receptor-2 have been found in detectable levels in patients with erosive oral lichen planus and were significantly decreased after treatment (Ghallab et al. 2010). Interleukins (IL-1β, IL-6, and IL-8), MMP- 8, tissue inhibitors of MMPs (TIMPs), TNF-α, lysozymes, peroxidases and other salivary biomarkers have been identified as potential biomarkers for periodontal diseases (Rathnayake et al. 2013; Taylor 2014). Periodontal diseases, especially chronic periodontitis and gingivitis are the most common oral inflammatory diseases affecting wide range of population in the UK, Europe, and worldwide. The early diagnosis, prevention, and management of periodontal diseases are among the priorities for any dental health care system, as the consequences of the delayed diagnosis will be an extensive loss in the supporting connective tissue and alveolar bone ending with the loss of teeth and expensive and complicated restorative treatments (Borrell and Papapanou 2005; Pihlstrom et al. 2005; Corbet and Leung 2011; Dye 2012; Petersen and Ogawa 2012; White et al. 2012; Slots 2013; Leong et al. 2014). In comparison to periodontally healthy individuals, periodontitis patients have significantly poorer oral health related quality of life with functional, psychological and social effects (Durham et al. 2013).

1.1 Periodontal diseases

Periodontal diseases are group of diverse multifactorial diseases with an inflammatory and destructive nature affecting the surrounding and supporting tissues of the teeth (Kornman 2008). Pihlstrom et al. (2005), defined periodontal disease as “any inherited or acquired disorder of the tissues surrounding and supporting the teeth (periodontium)”. Commonly, the term periodontal disease refers to the inflammation of the gingiva (gingivitis) and the surrounding periodontal tissues (periodontitis), mostly initiated as a host response to the bacteria present in the dental plaque which accumulate on the surfaces of the adjacent teeth (Pihlstrom et al. 2005).

1.1.1 Classification of periodontal diseases

Despite the fact that gingivitis and chronic periodontitis are the most common, periodontal diseases comprise a wider range of entities that requires a proper recognition and diagnosis (Highfield 2009). The need for a classification of periodontal diseases is necessary not only for the clinicians to develop the basis for a precise diagnosis and efficient management of the patients, but it is a requisite framework for the researchers in order to classify subjects into groups based on the presence or absence of the disease. The latest classification of periodontal diseases was set by the American Academy of Periodontology (AAP), and modified by an international classification workshop held at 1999 (Armitage 1999; Highfield 2009), which represents a consensus view of experts within the field of periodontology (Table 1.1).

Gingival diseases

With reference to the1999 classification, the gingival diseases or conditions can be classified into 2 main groups, the plaque and non-plaque induced (Highfield 2009). The most common form of gingival conditions is the plaque induced gingival inflammation known as gingivitis or chronic gingivitis, which is the mildest and highly prevalent form of periodontal diseases that affects 50–90% of adults worldwide depending on its precise definition (Armitage 1999; Albandar and Rams 2002; Highfield 2009). In comparison to other forms of periodontal diseases, plaque induced gingivitis is readily reversible by simple and effective oral hygiene measures. Though it is caused by dental plaque, gingivitis could be modified by local factors,

which may include teeth anatomical factors, fractures, and dental restorations. Furthermore, chronic gingivitis can be modified or exacerbated to an acute form by systemic factors such as endocrine factors, pregnancy, menstruation, puberty, hematologic dyscrasias, medications, and malnutrition. Typically, gingivitis is confined to the periodontal soft tissue 'gingiva', the junctional epithelium remains on the root surface with intact alveolar bone without loss of attachment; however, gingivitis may occur with a reduced but stable attachment level with no progressive loss of attachment (Offenbacher 1996; Armitage 1999; Albandar 2005; Page and Eke 2007; Highfield 2009).

The non-plaque induced gingival conditions comprise groups of lesions caused by specific bacteria (such as *Neisseria gonorrhoea*, and *Streptococci* species), viruses (such as Herpes virus infections), and fungi (such as *Candida* species infections and linear gingival erythema). Other gingival conditions could be genetic (such as hereditary gingival fibromatosis), as manifestations of systemic conditions such as mucocutaneous diseases (lichen planus, and pemphigus vulgaris), or allergic reactions to medications, dental restorations and food additives. Gingival traumatic lesions, which could be iatrogenic or accidental lesions due to chemical, physical, and thermal injuries. Gingival lesion as a reaction to a foreign body and other non-specified conditions (Highfield 2009).

Periodontitis

Subjects with gingivitis are more prone to have periodontal attachment loss than those with no signs of gingival inflammation. Hence, gingivitis always predates the appearance of periodontitis, with the exception of aggressive periodontitis no case of periodontitis without an antecedent gingival inflammation has been reported (Albandar et al. 1998b; Albandar and Rams 2002). When the inflammation extends from the gingiva deep into the surrounding connective tissue and alveolar bone leading to loss of these supporting structures this is known as periodontitis (Pihlstrom et al. 2005). Periodontitis is usually associated with sub-gingival pockets which may extend deeply forming crevices between the gingiva and tooth root. If left untreated, the inflammation will progress to advanced or severe periodontitis resulting in loosening of the teeth, discomfort and pain, impaired mastication, and subsequently loss of teeth (Pihlstrom et al. 2005). According to the 1999 classification, there are various forms of periodontitis including chronic periodontitis, aggressive periodontitis,

periodontitis as a manifestation of systemic diseases, necrotizing periodontal diseases, and other periodontal lesions such as abscesses of the periodontium, periodontal-endodontic lesions, and developmental or acquired conditions (Highfield 2009).

Chronic periodontitis

Chronic periodontitis is the most common form of destructive periodontal diseases (Albandar 2005), defined by Lindhe et al. (1999) as “an infectious disease resulting in inflammation within the supporting tissues of the teeth, with a progressive attachment and bone loss, characterized by pocket formation and/or gingival recession, its onset may be at any age, but is most commonly detected in adults, its prevalence and severity increases with age, and it may affect a variable number of teeth and has variable rates of progression’’. Though it is more common in adults, cases have been reported in adolescents and occasionally in children, that’s why the term adult periodontitis was replaced by the term chronic periodontitis in the 1999 classification. It was found that the term chronic is more representative for the disease as such a term is nonspecific, non-age dependent, and less restrictive (Highfield 2009).

The international workshop of 1999, listed the clinical features of chronic periodontitis (Lindhe et al. 1999; Highfield 2009), to be:

- Most prevalent in adults but can occur in children and adolescents.
- The amount of destruction is consistent with the presence of local factors.
- Sub-gingival calculus is a frequent finding.
- Associated with a variable microbial pattern.
- Slow to moderate rate of progression but may have periods of rapid progression.
- Can be further classified on the basis of extent and severity.
- Can be associated with local predisposing factors (such as tooth-related or iatrogenic factors).
- May be modified by and/or associated with systemic diseases (such as diabetes mellitus, HIV infection).
- Can be modified by factors other than systemic disease such as cigarette smoking and emotional stress.

Depending on the number of teeth sites affected, chronic periodontitis can be either localized or generalized. The case is defined as localized when the sites affected are 30% or less, and as generalized when more than 30% of sites are affected (Lindhe et al. 1999; Highfield 2009). Conventionally, the severity of the disease has been described as being slight or early when the bone loss is confined to the coronal third of the root, moderate when bone loss extends to the middle third of the root, and advanced when the bone loss extends to the apical third of the root. Moreover, the 1999 workshop categorized a further general guide for the disease severity based on the clinical attachment loss (CAL) as follows: slight = 1–2 mm CAL, moderate = 3 to 4 mm CAL, and severe = ≥ 5 mm CAL (Lindhe et al. 1999; Pihlstrom et al. 2005; Highfield 2009). A further periodontitis case definition was set by the 5th European Workshop in Periodontology which includes measuring the CAL at the proximal sites of non-adjacent teeth (Tonetti and Claffey 2005), (Table 1.2). The AAP case definition suggests measuring both the CAL and the probing depth (PD) at the interproximal sites of more than one tooth as criteria for case definition (Page and Eke 2007), (Table 1.2). Considering the possible mistakes that may happen while measuring the CAL, which may exclude cases with periodontitis or include cases without periodontitis, a threshold for the CAL was set at ≥5 mm by (Tonetti and Claffey 2005) and ≥6 mm by (Page and Eke 2007).

Severe periodontitis is defined by the AAP as a minimum of two teeth with 6 mm CAL and one tooth with a probing depth of 5 mm present (Page and Eke 2007). According to this case definition, a patient or a subject included in a study might be diagnosed with severe periodontitis by the presence of only minimal levels of attachment loss which might be due to overhanging restorations or on the distal aspect of second molars where a third molar has been extracted. Therefore, in order to generate more reliable data from which conclusions can be drawn, it is necessary to include cases which have sufficient levels of the disease. In comparison to the AAP case definition, the 5th European Workshop in Periodontology have more comprehensive inclusion criteria with respect to the extent and severity of the disease, requiring an interproximal attachment loss of ≥ 5 mm in ≥ 30% of teeth present to define the subject with severe periodontitis (Tonetti and Claffey 2005).

For an improved description of the total prevalence of periodontitis in populations, there was an update on the periodontitis case definition by the Centres for Disease

Control and Prevention (CDC) in partnership with AAP (Eke et al. 2012), (Table 1.3). The criteria for defining both the moderate and severe periodontitis remained same as previously described by Page and Eke (2007), the update was the inclusion of the case definition criteria for mild periodontitis. The previous case definitions by the 5th European Workshop and the AAP did not define mild periodontitis as they set their case definition criteria for surveying moderate and severe periodontitis only. Therefore, the moderate and severe periodontitis case definitions were not enough to determine the overall prevalence of periodontitis in populations as they missed the mild periodontitis (Eke et al. 2012). In their survey, Eke et al. (2012) found that the incorporation of the mild periodontitis criteria increased the total prevalence of periodontitis about 31% when compared to the surveys involving the moderate and severe periodontitis only. Therefore, neglecting mild periodontitis while carrying out a survey will underestimate the overall prevalence and burden of the disease. This could be more evident in populations with young adults who are more likely to have mild periodontitis which is more responsive to oral health measures and preventive dental care, but if not detected will remain at risk of progressing to the moderate and severe periodontitis that are more difficult and expensive to treat (Eke et al. 2012).

All the above-mentioned case definitions used the CAL as the primary criteria; however, there is a perception that the definition of periodontitis cannot be restricted to a single variable such as CAL. Though it represents a measure of the cumulative lifetime experience of periodontitis, CAL presents brief information about the current inflammatory status of the periodontal tissues. Hence, clinicians and researchers considered the incorporation of other measures such as the PD, bleeding on probing (BOP), recession, periodontal epithelial surface area (PESA), and periodontal inflamed surface area (PISA), along with the CAL for a more precise case definition (Hujoel et al. 2001; Page and Eke 2007; Nesse et al. 2008; Nesse et al. 2009). Furthermore, there are concerns expressed by the clinicians that the CAL measurement might be challenging, time consuming and difficult especially when the gingival margin is located coronal to the cemento-enamel junction (CEJ) as the CAL measurement in this case may be a kind of guesswork or estimation. Reports stated that clinicians are excluding the CAL measurements in favour of PD alone or PD with recession measures at the mid-facial or mid-buccal or mid-lingual aspects of the teeth, only when the recession is actually present (AAP-TF 2015; Lopez and Baelum

2015). On the other hand, the half and full mouth examination were considered in the case definition and prevalence of periodontitis, for instance the accuracy of the periodontitis prevalence determined by the National Health and Nutrition Examination Survey (NHANES) using partial mouth periodontal examinations, was compared to the full mouth 'gold standard' periodontal examinations. This comparison found that the partial examinations substantially underestimated the prevalence of periodontitis by 50% or more depending on the periodontitis case definition used, which generated high levels of misclassification of periodontitis cases (Eke et al. 2010).

1.1.2 Pathogenesis of periodontal diseases

The proper diagnosis and treatment, as well as the prevention of periodontal diseases, require a comprehensive understanding of the disease aetiology and pathogenesis. It is well known that the dental plaque with all its inhabitant species of bacteria and their products is the main cause of periodontal diseases. This dental plaque induces the inflammatory process of periodontal diseases through the disruption of normal homeostasis, and this is modified by many environmental and genetic factors (Pihlstrom et al. 2005; Zee 2009; Darveau 2010). Typically, the inflammatory process will start first in the gingiva leading to gingivitis which is reversible with the improvement of oral hygiene. If left untreated, the inflammation of the gingiva will progress to periodontitis which is a substantially irreversible damaging inflammatory process affecting the surrounding tissue (gingiva and periodontal ligament) and the supporting alveolar bone. This destructive process will lead to tissue injuries including loss of collagen fibres and attachment with cementum surface, apical migration of junctional epithelium resulting in deepened periodontal pockets and alveolar bone loss. If left untreated, periodontitis will continue with further bone destruction resulting in teeth mobility, pain, impaired function and finally teeth loss (Pihlstrom et al. 2005; Taba et al. 2005; Kornman 2008), (Figure 1.1).

Dental plaque and periodontal pathogens

Like any other surface in the body, the oral cavity, possesses a substantial microflora living in harmony with the surrounding host environment. The oral microflora encompasses hundreds of species of aerobic and anaerobic, or Gram-positive and Gram-negative bacteria which live and grow within biofilms (Pihlstrom et al. 2005). Clinically, the biofilm may be defined as a bacterial deposit which cannot be easily rinsed away and it may form on any surface within the oral cavity such as teeth,

mucosa or other solid surfaces (Hasan and Palmer 2014). The biofilm represents an organized community or communities of bacteria (comprises about 15-20% of the biofilm volume), nested in a glycocalyx matrix (comprises about 75-80% of the biofilm volume), these bacterial populations are attached to each other and to a solid surface. The major advantage of the biofilm is the protection that it provides for the colonizing bacteria from other competing microorganisms, from the surrounding environment effects such as host defence mechanisms, and from potentially toxic substances in the environment such as antibiotics, this protection allows the bacteria to stick to and to multiply on the solid surface (Socransky and Haffajee 2002). The biofilm which forms and grows on solid surfaces including teeth or restorative materials (the metal, ceramics or acrylic in appliances), is known as the dental plaque or dental biofilm. As it is formed on solid and non-shedding surfaces, the dental plaque is a stable community and it differs from other biofilms formed on shedding or desquamating mucosal surfaces (Hasan and Palmer 2014).

Loe et al. (1965), were the first to describe the role of dental plaque in the initiation and development of periodontal diseases, when they found that gingivitis will develop within four days following the build-up of the dental plaque in an experimental gingivitis model. In the primary stages, the bacterial populations in the dental plaque consist of aerobic and facultative anaerobes such as Gram-positive cocci (*Streptococci* species). Later on, Gram-positive rods appear, increase in number and finally predominate over the cocci. Subsequently, Gram-positive filaments such as *Actinomyces* species will predominate. The Gram-positive bacteria (cocci and rods) have specific surface receptors that allow the adherence of Gram-negative bacteria, which otherwise lack the ability to attach directly to the biofilm. If the dental biofilm remains, and as time goes on, there will be a shift in the microflora from Gram-positive to Gram-negative microorganisms with more diversity in the inhabitant microbial species (Hasan and Palmer 2014).

Hasan and Palmer (2014), indicated that despite the fact that the dental plaque plays a major role in the pathogenesis of periodontal diseases, whether the bacteria in the dental plaque induce periodontal diseases specifically or non-specifically, this is still a debate. Hasan and Palmer (2014), reported three hypotheses about the role of the bacteria in the periodontal diseases pathogenesis, though, these hypotheses ignored the role of the host factors and responses. The first hypothesis is the “non-specific

plaque” hypothesis, which proposes that periodontal diseases occurs due to the accumulation of bacteria regardless to their species. According to this hypothesis, preservation of standard oral hygiene will prevent periodontal diseases. Thus, the dental plaque amounts may correlate positively with the severity of periodontal diseases in cross-sectional studies; however, this correlation may be poor in longitudinal studies, this was explained by the variations in the dental plaque bacteria or even the host responses which were both ignored by this hypothesis (Marsh 2003; Marsh 2006; Hasan and Palmer 2014).

The second hypothesis is the “specific plaque” hypothesis, which proposes that a periodontal disease results from an infection with specific bacteria. This may explain why not all patients who have high amounts of dental plaque suffer from severe destructive periodontitis, which means that specific bacteria cause this form of periodontitis. This hypothesis implies that one should be concerned about the specific bacteria responsible for periodontal disease; therefore, there is a need to focus on the oral hygiene measures and treatment that eliminate the specific pathogen. According to this, it may be unnecessary to remove all the dental plaque and it may be sufficient either to remove the anaerobic bacteria or to disrupt their growth and survival which could be achieved by the root surface debridement. Moreover, when a specific pathogen is identified the treatment could be targeted at this specific pathogen using a specific antibiotic to which it is sensitive. However, despite the fact that the bacteria *Aggregatibacter actinomycetemcomitans* is specific for aggressive periodontitis; no single pathogen has been specifically associated with chronic gingivitis or periodontitis (Marsh 2003; Marsh 2006; Hasan and Palmer 2014).

The third hypothesis is the “multiple pathogen” hypothesis, which proposes that periodontal disease results from an infection with a relatively small number of interacting bacterial species. One of the major difficulties with this hypothesis lies in the identification of the most important bacterial combination. Among these bacteria, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Campylobacter* species, are found in diseased periodontal sites and have been involved in the progression of periodontitis. However, the presence of these bacteria does not prove that they are responsible for tissue damage, and some clinicians or researchers may argue that these bacteria are

more likely to be detected in deeper periodontal pockets and at the sites with more inflammation simply because they flourish in such environments (Hasan and Palmer 2014).

The previously mentioned hypotheses (non-specific plaque, specific plaque, and multiple pathogen hypotheses), outlined the theoretical role of the dental plaque bacteria in the pathogenesis of periodontal diseases, yet they ignored the role of the host response and/or the environmental effects on the dental plaque bacteria. Consequently, the “ecological plaque hypothesis” has been proposed as a result of research that investigated the dynamic relationship between the plaque bacteria and the host environment during health and disease. This hypothesis proposes that ecological or environmental changes affect the bacterial flora in plaque resulting in a shift towards a pathogenic sub-gingival microflora and progression to periodontal disease (Marsh 1991; Marsh 1994). Hence, disease can be prevented not only by directly removing the putative pathogens, but also by interfering with the host environmental factors which influence the selection and enrichment of plaque bacteria. Therefore, more holistic approaches might be considered in disease control and management (Marsh 1994; Marsh 2003; Marsh 2006). The local host environmental changes do shift the dental plaque microflora during disease, for instance during inflammatory response to sub-gingival plaque, the pH rises to become slightly alkaline and the GCF flow increases delivering potentially novel substrates for the proteolytic bacteria in the dental plaque (Marsh 1994; Marsh 2003; Marsh 2006; Marsh et al. 2011). Moreover, conventional pure culture investigations found that such environmental changes might affect the gene expression and virulence factors of dental plaque bacteria (Marsh 2006).

Studies have reported that 500-600 or even more distinct species were identified in the dental plaque from periodontal pockets of humans and about 30-100 species may be found in a single site (Moore and Moore 1994; Pihlstrom et al. 2005; Hasan and Palmer 2014). However, number of studies indicated that it is still not easy to define which microorganisms are more important than the others due to some difficulties, such as: The host may determine what species can grow as a consequence of the host’s own environment. Contamination with other species may make it difficult to obtain representative bacterial samples from periodontal pockets. The time of collecting the bacterial samples may be critical as the periodontal

disease progresses and there is a shift in the microflora from Gram-positive and aerobic to more Gram-negative and anaerobic bacteria. The presence of opportunistic infections or infections from other sites in the oral cavity may also confound the identification of bacteria. In addition to the inter-individual variations in the host immune responses to the bacteria (Tanner et al. 1996; Tanner et al. 1998; Kroes et al. 1999; Ximenez-Fyvie et al. 2000; Ramberg et al. 2003; Lepp et al. 2004; Pihlstrom et al. 2005; Hasan and Palmer 2014). The major periodontal pathogens implicated in periodontal diseases include: *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) (previously known as *Actinobacillus actinomycetemcomitans*), *Tannerella forsythia* (*T. forsythia*) (previously known as *Bacteroides forsythus*), *Treponema denticola (T. denticola), Prevotella intermedia (P. intermedia)*, *Prevotella nigrescens* (*P. nigrescens*), *Fusobacterium nucleatum (F. nucleatum)*, and *Campylobacter* species (Zambon 1996; Pihlstrom et al. 2005; Teles et al. 2006; Hasan and Palmer 2014). It has been demonstrated by molecular techniques and cluster analysis of sub-gingival plaque that certain bacterial species frequently co-exist in ‘complexes’ These analyses confirmed a strong association of *P. gingivalis* (which is one of the red complex microorganisms) with deep periodontal pockets and increased bleeding on probing in periodontal diseases (Socransky et al. 1998). Another study, reported that plaque samples from patients with periodontitis demonstrated an increased proportion of red and orange complex bacterial species as compared to plaque samples from periodontally healthy subjects (Ximenez-Fyvie et al. 2000). Furthermore, the relationships of orange and red complex bacterial species with the clinical measures of periodontitis is also mirrored for supra-gingival plaque samples (Haffajee et al. 2008). However, other studies reported the detection of the same periodontal pathogens in periodontally healthy subjects (Loomer 2004; Sanz and Quirynen 2005), highlighting the complex interplay between the bacterial challenge and the host response that is involved in periodontal disease. Though they are less common, Herpes viruses as well as *Candida albicans* and other fungi were implicated in the pathogenesis of periodontal diseases especially in immunocompromised patients (Michalowicz et al. 2000b; Robinson 2002; Kubar et al. 2004; Slots 2004; Albandar 2005; Kubar et al. 2005; Pihlstrom et al. 2005; Saygun et al. 2005; Darveau 2010; Nickles et al. 2016).

Several studies indicated the importance of human microbiome in both host health and disease; however, there is a great interest in the mechanisms by which the host-microbial homeostasis is maintained at the mucosal surfaces, the mechanisms that disturb this homeostasis, how the microbiome mediates diseases, and protection against it (Artis 2008; Darveau 2010; Littman and Pamer 2011; Hajishengallis et al. 2012). On this basis the, “keystone pathogen” hypothesis has been developed, this hypothesis implies that certain low-abundance microbial pathogens have the ability to orchestrate an inflammatory disease by remodelling normally benign microbiota into a dysbiotic one (Hajishengallis et al. 2012). Differences in the composition of periodontal microbiota (plaque) during health and disease have been demonstrated in early bacteriological studies (Socransky 1977; Moore et al. 1982). These differences in the dental plaque bacterial community might be explained by two ways (Hajishengallis et al. 2012). Firstly, these differences could be considered as an indication that specific bacteria are implicated in the aetiology of periodontitis, and that the disease associated microbiota contained novel periodontal pathogens that were either not present or barely detected during health status (Hajishengallis et al. 2012). Secondly, the differences might suggest that periodontitis is due to the dysbiosis of the dental plaque microbiota, which means there is a shift in the relative abundance of the dental plaque bacteria as compared to their abundance during health, leading to alterations in the host-microbial crosstalk (signal-transduction) sufficient to initiate periodontitis (Hajishengallis et al. 2012).

Research has led to the identification of candidate gram-negative anaerobic bacteria known as “the red complex” including *P. gingivalis, T. denticola* and *Tannerella forsythia* (Socransky et al. 1998; Haffajee et al. 2008). Research has been carried out on these three bacteria to understand the pathogenic mechanisms, virulence factors, and their interaction with the host (Holt and Ebersole 2005). In support to the “keystone pathogen hypothesis”, studies indicated that *P. gingivalis* has developed strategies to evade the host immune components (such as toll-like receptors and complement) rather than acting directly as a pro-inflammatory bacterium (Darveau 2009; Darveau 2010; Hajishengallis and Lambris 2011). Therefore, it was proposed that *P. gingivalis* impairs the innate immunity in ways leading to alteration in the growth and development of the entire dental biofilm, affecting the host-microbial balance and initiating a destructive changes in the periodontium. Accordingly, *P.*

gingivalis could be a keystone pathogen of periodontitis (Hajishengallis et al. 2012). Despite its high prevalence and association with progressive bone loss in periodontitis patients (Moore et al. 1991; Chaves et al. 2000), *P. gingivalis* being a quantitatively minor constituent of human periodontitis-associated biofilms (Moore et al. 1982; Doungudomdacha et al. 2000; Kumar et al. 2006; Hajishengallis et al. 2011), is consistent with the keystone hypothesis (Hajishengallis et al. 2012). Moreover, the specific removal of *P. gingivalis* from the periodontal biofilm (such as using complement component 5a receptor antagonist) reverses the dysbiotic changes (Hajishengallis et al. 2011), indicating that dysbiotic diseases could be treated by targeting the keystone pathogens (Hajishengallis et al. 2012).

The periodontal bacteria present in the dental plaque have many antigens to which the host immune system will react. In addition to their antigenicity, different bacterial macromolecules play serious roles in the inflammatory process of periodontitis. Among these are: leukotoxin, lipoteichoic acid, peptidoglycan, lipopolysaccharides (LPS), proteases, fimbriae and extracellular enzymes (Travis et al. 1997; Fives-Taylor et al. 1999; Guo et al. 2010; Zia et al. 2011; Hasan and Palmer 2014). These bacterial products are the bacterial virulence factors, which will destroy the host tissues either directly or indirectly by stimulating the host immune system to respond and produce inflammatory mediators, and enzymes that will damage the supporting periodontal tissues. For instance, *P. gingivalis* LPS has a direct toxic effect on the periodontal tissues and stimulates the host immune reaction via specific host receptors, and the *P. gingivalis* gingipains facilitate bacterial invasion into the tissues and contribute to periodontal tissue destruction (Genco et al. 1999; Imamura 2003; Andrian et al. 2004; Dixon et al. 2004; Gupta 2013; Wara-aswapati et al. 2013; Hasan and Palmer 2014; Kang et al. 2016).

Host response

Though bacteria and dental plaque are necessary to initiate periodontal diseases, a susceptible host is a prerequisite. The host will react to the chronic presence of dental plaque bacteria by immune-inflammatory responses that develop in the gingiva and periodontium and result in the inflammation and destruction of these periodontal tissues leading to the clinical signs of periodontal diseases (Pihlstrom et al. 2005). However, not all the classical signs of inflammation are present in periodontal diseases, for instance pain and loss of function are not present in chronic

gingivitis and at the early stages of chronic periodontitis (Hasan and Palmer 2014). Clinically, gingivitis which is a reversible inflammatory reaction of the marginal gingiva to the dental plaque biofilm, differs from chronic periodontitis which is a non-reversible and destructive host inflammatory reaction, by demonstrating the inflammatory signs of redness, oedema, higher tendency for bleeding, and tenderness (Offenbacher 1996; Kinney et al. 2007).

The histopathology of the inflammatory events during periodontal diseases was identified in the 1970s (Page and Schroeder 1976). The presence of the dental plaque biofilm initiates host inflammatory response in the periodontal tissues which is characterized by an increase in the blood flow and dilatation of the blood vessels with enhanced permeability, this will lead to influx of fluid and inflammatory cells especially the polymorphonuclear leukocytes (PMNs) and monocytes (macrophages) into the tissues at the site of infection in order to remove the invading bacteria (Page and Schroeder 1976; Page and Kornman 1997; Page et al. 1997; Kinney et al. 2007). Therefore, both the host and the virulent bacteria in the plaque biofilm, act together to destroy the periodontal tissues. Both release proteolytic enzymes which damage the periodontal tissues directly, in addition to chemotactic factors that recruit the PMNs and other inflammatory cells into the infection site. These cells which infiltrate the junctional epithelium down to the periodontal pocket following the bacteria, will also release enzymes and other products that will damage the supporting connective tissue and alveolar bone (Pihlstrom et al. 2005; Bartold and Narayanan 2006). The periodontal tissue destruction by the basically protective host immune response may be termed as ‘collateral damage’ (Preshaw and Taylor 2011).

Neutrophils are among the important components of the innate immune system, they represent the first line of host defence and play a crucial role in maintaining the periodontal tissues health especially when they are threatened by bacterial challenge from the plaque biofilm. Neutrophils protect the host via their capability to phagocytize and kill microorganisms. Therefore, the severity of periodontal diseases is raised in patients with diseases such as leukocyte adhesion deficiency, lazy leukocyte disease and Papillon-Lefèvre, Chediak-Higashi and Down’s syndromes, as well as diabetes mellitus, which impair the functional responses of the neutrophils, and may cause neutropenia and agranulocytosis leading to recurrent microbial infections (Lekstrom-Himes and Gallin 2000; Fredriksson et al. 2003; Van Dyke and

Serhan 2003; Bascones-Martinez et al. 2009; Jain and Darveau 2010; Hasan and Palmer 2014). Aside from their protective role, neutrophils mediate the tissue destruction in the pathogenesis of inflammatory diseases. In spite of the intent of the active neutrophils to engulf invading bacteria, some virulent periodontal bacteria have the ability to avoid neutrophils that may lead to continuous accumulation of the activated neutrophils in the gingival sulcus and periodontal pocket (Van Dyke and Serhan 2003; Bascones-Martinez et al. 2009). These activated neutrophils release potent lysosomal and proteolytic enzymes, cytokines, and reactive oxygen species or radicals (ROS), all can cause direct destruction of the periodontal tissues (Van Dyke and Vaikuntam 1994; Johnstone et al. 2007; Aboodi et al. 2011). Therefore, the overproduction of all the antimicrobial proteins and ROS along with the neutrophils hyperactivity, explain the destructive role of activated neutrophils in periodontal diseases (Fredriksson et al. 2003; Johnstone et al. 2007; Aboodi et al. 2011).

The innate immunity is responsible for the earlier responses, and helps to focus the later adaptive immune responses. The bacteria present in the dental plaque biofilms represent hundreds or even thousands of antigens that have the ability to stimulate the adaptive immunity by arousing both the humoral antibody-mediated and cell-mediated immune responses. Once the microbial antigens have been identified by the appropriate receptors on the innate immunity cells such as neutrophils or macrophages or dendritic cells, then interleukins and cytokines are released which attract more cells and activate the T and B cells, thereby engaging cell-mediated and humoral immune responses, consequently the host is able to orchestrate an immune-inflammatory response that reflects the bacterial challenge (Pihlstrom et al. 2005; Hasan and Palmer 2014). The antigens expressed by the bacteria are termed as microbe-associated molecular patterns (MAMPs), these MAMPs are recognized by a collection of specific receptors on the host innate immune system cells known as the pattern recognition receptors (PRRs). Among the important MAMPs in periodontal diseases pathogenesis, are bacterial LPS and fimbriae which are recognised via receptors such as soluble LPS binding protein, membrane-associated CD14 and toll-like receptors (TLRs) (Dixon and Darveau 2005; Pathirana et al. 2010; Taylor 2010).

Numerous studies reported that the MAMPs stimulate the host cells to secrete wide range of pro-inflammatory cytokines such as IL-1 α, IL-1β, IL-6, IL-8, IL-12, TNF-α and IFN-γ, and prostanoids such as prostaglandin E_2 (PGE_2), which attract more

inflammatory cells and mediate periodontal tissue destruction, in addition to the secretion of enzymes such as matrix metalloproteinases (MMPs) which directly degrade the extracellular matrix and lead to attachment loss (Okada and Murakami 1998; Sandros et al. 2000; Jotwani et al. 2003; Dixon et al. 2004; Kusumoto et al. 2004; Eskan et al. 2007; Eskan et al. 2008; Preshaw 2008; Liu et al. 2010; Hans and Hans 2011). All these studies highlight the vital role of host cell receptors in the inflammatory immune response during the pathogenesis of periodontal diseases. Hence, the activation of specific receptors by specific bacterial MAMPs directs an inflammatory immune response that is relevant to the bacterial challenge present within the dental plaque biofilm.

The cytokines and chemokines released by the innate immune cells lead the host response toward a robust cell mediated adaptive immunity. Therefore, in the early or non-progressive periodontal lesion, the inflammatory cell infiltrate comprises predominantly of T-lymphocytes and macrophages, indicating that the cell mediated response can control the disease process (Gemmell et al. 2002; Yamazaki et al. 2003; Pihlstrom et al. 2005; Gemmell et al. 2007; Kinane and Bartold 2007; Ohlrich et al. 2009). However, in the more destructive lesions, where the T-cell response cannot supress or limit the bacterial challenge, the cell infiltrate will be dominated by the B lymphocytes and plasma cells (Gemmell et al. 2002; Yamazaki et al. 2003; Pihlstrom et al. 2005; Gemmell et al. 2007; Kinane and Bartold 2007; Ohlrich et al. 2009). The B and plasma cells produce antibodies which are either protective and therefore control the bacterial infection, or non-protective resulting in connective tissue destruction and bone loss (Gemmell et al. 2002; Yamazaki et al. 2003; Gemmell et al. 2007; Kinane and Bartold 2007; Ohlrich et al. 2009).

The types of the antibodies produced by the B cells relies on the appropriate adaptive immune response to the chronic bacterial challenge, which in turn depends on a balanced production of different subsets of T cells by the host tissues (Th1 and Th2 cells) which release diverse but overlapping sets of cytokines (Gemmell and Seymour 1994; Bartova et al. 2000; Ohlrich et al. 2009). When the Th1 cells are produced, this will lead to cell mediated immune response, which activates both the macrophages to kill the invading bacteria and the B cells to produce the opsonising protective antibodies which facilitates bacterial killing, this happens in more stable periodontal lesions. Whereas, the production of Th2 cells results in humoral immunity

response, with the activation of B cells to produce non-protecting antibodies, which is associated with more progressive periodontal lesions (Gemmell and Seymour 1994; Bartova et al. 2000; Ohlrich et al. 2009).

However, there are studies that reported the predominance of Th1 cells responses or reduced Th2 cell responses in periodontal disease (Ebersole and Taubman 1994; Salvi et al. 1998; Takeichi et al. 2000). Other studies reported the involvement of both the Th1 and Th2 cells in periodontal diseases (Gemmell et al. 1999; Nakajima et al. 1999; Berglundh et al. 2002). Despite the fact that the Th1 and Th2 cells patterns provide indications about the possible mechanism by which the periodontal lesions become progressive or remain stable, a question may arise, which is: what causes some lesions to show Th1 characteristics while others show Th2 characteristics? The answer may lie in the nature of the bacterial challenge as well as the particular genetic and environmental susceptibility factors (some of these factors may be clinically identifiable and modifiable) (Ohlrich et al. 2009). Moreover, the balance of the cytokines produced by the innate and adaptive immune responses within the inflamed periodontal tissues, is an essential factor that determines whether the periodontal disease remains stable or progresses to a more destructive form (Okada and Murakami 1998).

Interestingly, chronic inflammatory reactions within local tissues lead to dysregulation or imbalance in the bone remodelling homeostasis of the adjacent bone which results in osteolytic lesion (bone loss). This may explain the enhanced bone loss and resorption during chronic periodontitis (Liu et al. 2010). During periodontitis the infiltrating inflammatory cells in addition to the resident fibroblasts within the periodontal tissues, release a number of inflammatory cytokines, of which there are stimulatory and inhibitory cytokines that influence the osteoclasts formation and activity in the inflammatory induced periodontal bone loss lesion (Liu et al. 2010).

Cytokines in periodontal diseases

The pro-inflammatory cytokines play a significant role in the pathogenesis of periodontal diseases as they are involved in the destruction of both the periodontal connective tissues and alveolar bone, mainly via the stimulation of the host MMPs (Taylor 2010; Preshaw and Taylor 2011). During the innate host response, the bacterial LPS and other antigens stimulate the monocytes, PMNs, macrophages, and

the resident periodontal cells such as fibroblasts and other cells to release the cytokines IL-1β, TNF-α and PGE_2 (Kinney et al. 2007). IL-6 is also released by the same cells and they are all involved in the stimulation of bone resorption (Ishimi et al. 1990; Okada and Murakami 1998; Palmqvist et al. 2002; Buduneli and Kinane 2011). Furthermore, the 7th European Workshop on Periodontal Diseases confirmed in a consensus paper that IL-1β, TNF-α, IL-6 and the receptor activator of nuclear factor kappa-B ligand (RANKL) as the most important cytokines to play role in the pathogenesis of periodontal diseases (Kinane et al. 2011).

IL-1β is the key inflammatory cytokine in various inflammatory diseases characterized by destruction of bone and connective tissue, for instance IL-1β is the central mediator of inflammation and connective tissue destruction in rheumatoid arthritis (Raymond et al. 2006). IL-1β is produced by the inflammatory cells infiltrate as well as the local periodontal cells in response to the bacterial challenge from the dental biofilm, and it plays essential roles in the innate and adaptive host immune events during the pathogenesis of periodontal diseases (Barksby et al. 2007). Usually, IL-1β, TNF-α, and PGE_2 act synergistically to induce many inflammatory changes and govern the neutrophils chemotaxis and emigration from the nearby circulation into the periodontium. During the adaptive immune response, IL-1β stimulates the presentation of bacterial antigens by the antigen presenting cells (APCs) thereby influencing the T-cells development and phenotype (Assuma et al. 1998; Barksby et al. 2007).

PGE_2, which is an arachidonic acid metabolite of the cyclooxygenase (COX) pathway, is a potent vasodilator and increases the capillaries' permeability which results in the inflammation signs of redness and oedema. Moreover, PGE_2 is a potent mediator of alveolar bone loss in periodontitis, and it stimulates the fibroblasts and osteoclasts to produce IL-1β and other cytokines, as well as the MMPs that destroy the supporting periodontal tissues (Offenbacher et al. 1984; Offenbacher et al. 1993; Kinney et al. 2007; Buduneli and Kinane 2011). Though it is a less potent stimulator for PGE_2 production when compared to IL-1β, but TNF-α and IL-1β act synergistically to enhance PGE_2 production (Yucel-Lindberg et al. 1999; Buduneli and Kinane 2011). Hence, the synergism of IL-1β, TNF-α, and PGE_2, ultimately stimulates the fibroblasts and osteoclasts to secrete MMPs, as well as, activates the osteoclastic

activity which will destroy supporting connective tissues and alveolar bone in periodontitis (Kinney et al. 2007; Taylor 2014).

In a similar manner to IL-1β, TNF-α, and other cytokines, IL-6 is secreted by different inflammatory cells and local periodontal cells such as gingival fibroblasts, it is believed that the IL-1β and TNF-α may stimulate the production of IL-6 (Taylor 2014). This interaction among IL-6, IL-1β and TNF-α was illustrated by a cell culture study of primary gingival fibroblasts which revealed mRNA expression of IL-6, and a dose-dependent up-regulation of IL-6 mRNA and IL-6 protein levels in response to stimulation by IL-1β and TNF-α (Palmqvist et al. 2008). On the contrary, it has been shown that IL-6 induces IL-1 receptor antagonist (Tilg et al. 1994), which may provide a sort of control over the upregulated inflammatory responses. As a pro-inflammatory cytokine, IL-6 plays an important role in the pathogenesis of periodontal diseases via its effects in the regulation, development, proliferation, and activation of the immune cells (B and T-cells), as well as, its ability to stimulate bone resorption by the activation of osteoclasts (Palmqvist et al. 2002; Bartold and Narayanan 2006; Preshaw and Taylor 2011). Moreover, IL-6 stimulates the fibroblasts to secrete MMPs, therefore, IL-6 along with IL-1β and TNF-α, are considered as the key cytokines that propagate the destructive host inflammatory response to the plaque bacterial challenge at different levels, which may explain the increased concentrations of these cytokines found in the inflamed periodontal tissues (Irwin and Myrillas 1998; Graves and Cochran 2003).

The destruction of the alveolar bone which is one of the main characteristic features of periodontitis, led to the investigation of the importance of regulatory interactions between bone metabolism and inflammation (Nagasawa et al. 2007; Buduneli and Kinane 2011; Graves et al. 2011). Several cytokines such as IL-1β and IL-6 stimulate and upregulate the RANKL expression which enhances bone resorption, the cytokines also regulate the ratio of RANKL and its natural antagonist osteoprotegerin (OPG), and this upregulation is important in determining bone resorption and turnover (Lerner 2006; Nagasawa et al. 2007; Koide et al. 2010), and this ratio is elevated in periodontitis (Buduneli and Kinane 2011; Preshaw and Taylor 2011). Additionally, cytokines such as IL-1β and TNF-α also enhance the COX-2-mediated PGE_2 production by osteoblasts (Lerner 2006; Coon et al. 2007). In turn, PGE_2 efficiently stimulates RANKL pathway and expression in osteoblasts (Li et al. 2002),

as well as osteoclast progenitor cells (Ono et al. 2005). Therefore, at persistently high levels, PGE_2 stimulates the master osteoclast activator (RANKL) expression in osteoblasts, subsequently enhances the bone resorption which occurs in destructive inflammatory diseases such as periodontitis (Buduneli and Kinane 2011).

All the activities regulated by the cytokines indicate that, periodontal disease progression depends on the balance between pro-inflammatory and anti-inflammatory responses maintained by a network of cytokines (Graves and Cochran 2003).

Matrix Metalloproteinases in periodontal diseases

MMPs are a family of zinc-dependent proteases, their main function is to degrade the components of the extracellular matrix (ECM) and basement membrane components, and they may also play other roles related to cell growth/proliferation, and inflammation. In humans the MMPs family comprise 23 structurally related but genetically distinct proteases (Jackson et al. 2010). Though they are classified according to their main substrate, but the MMPs enzymatic activities are complex and overlapping and they may digest a wide range of peptide sequences found in a number of protein substrates and some of which are targets to specific MMPs (Birkedal-Hansen 1993; Sorsa et al. 2006; Hannas et al. 2007).

In a similar manner to the cytokines, MMPs are produced by inflammatory cells such neutrophils and macrophages, and by the cells of the periodontium such as fibroblasts, keratinocytes, endothelial cells, and osteoclasts, in response to stimulation by pro-inflammatory cytokines, as well as bacterial challenge from the dental plaque (Birkedal-Hansen 1993). In addition to their proteolytic function, MMPs play roles in the tissue development, homeostasis, repair, and cell apoptosis, as well as having roles in the host immune responses including antigen processing and presentation, cells migration, processing and activating a variety of proteins such as antimicrobial peptides, chemokines, cytokines, and growth factors, thereby mediating pro- and anti-inflammatory processes (Sorsa et al. 2006; Hannas et al. 2007; Giannobile 2008; Hernandez et al. 2011; Butler and Overall 2013). The MMPs activities are regulated at multiple levels including: transcription, secretion/degranulation, activation of the zymogen, inhibition of activity, extracellular inhibitors, localization whether inside or outside the cell, as well as internalization and

clearance. Different transcription factors, co-activators and co-suppressor proteins regulate the MMPs expression. The transcriptional activation of MMPs is stimulated by a variety of pro-inflammatory cytokines, hormones and growth factors, such as IL-1β, IL-6, TNF-α, epidermal growth factor, platelet-derived growth factor and fibroblast growth factor (Sorsa et al. 2006; Hannas et al. 2007). The active MMPs are inhibited by two main inhibitors, first, the tissue inhibitors of matrix metalloproteases (TIMPs) which form bimolecular complexes with the active forms of MMPs and sometimes even with the latent MMP precursors. The second inhibitor molecule is the α_2-Macroglobulin which captures active MMPs by a unique trap mechanism. The balance between the levels of MMPs and their inhibitors is the determinant and regulator of the MMP activity, and this balance is crucial for the maintenance of tissue integrity, otherwise any imbalance or excessive production of either the MMPs or their inhibitors may lead to increased tissue degradation (Birkedal-Hansen 1993; Sorsa et al. 2004; Jacqueminet et al. 2006; Sorsa et al. 2006; Hannas et al. 2007).

It is well known that MMPs play a serious role in the pathogenesis of periodontal diseases through the regulation of periodontal tissue turnover (Uitto et al. 2003; Sorsa et al. 2004; Sorsa et al. 2006; Li et al. 2012). During periodontal diseases, the balance between MMPs and their inhibitors especially TIMPs is influenced by the dysregulation in the synthesis and secretion of MMPs due to the bacterial and host stimulation. The periodontal tissue infected with dental biofilm bacteria will be invaded by neutrophils in an attempt to remove the bacterial threat, which may explain the high levels of the neutrophils MMPs (especially MMP-8 and -9) found in the inflamed periodontal tissues and GCF (Uitto et al. 1990; Uitto et al. 2003). Sorsa et al. (1995), reported that the main collagenase in periodontitis is MMP-8 followed by MMP-9. For instance, MMP-8 levels in GCF correlate positively with the severity of periodontal disease and reduce significantly in response to periodontal treatment (Golub et al. 1990; Kinane et al. 2003; Kinney et al. 2007). In addition to the conventional non-surgical periodontal treatment, tetracyclines, especially doxycycline, which further to their antimicrobial activity, are able to inhibit the MMPs, an effect which is independent of their antimicrobial activity (Sorsa et al. 1994; Golub et al. 1998; Preshaw et al. 2004). The sub-antimicrobial dose of doxycycline approach became a successful adjunctive therapy in the management of periodontitis, which has the ability to inhibit the MMPs at many levels especially their

activities and gene expression, and this indeed confirms the importance of the MMPs in the periodontal diseases pathogenesis (Ciancio and Ashley 1998; Golub et al. 1998; Sorsa et al. 2006; Giannobile 2008).

Risk and modifying factors

Further to the plaque biofilm bacteria and their products which are the main factors that initiate the host response in periodontal diseases, as multifactorial diseases there are several local and systemic factors which may increase the risk or susceptibility for periodontal diseases and modify the host response. However, the significance of these factors in the pathogenesis of periodontal diseases may vary from one periodontal disease to another and from patient to patient. Several studies investigated the risk factors, some of which were found to have real risk modifying effects, whereas others may barely modify the host response or the disease pathogenesis (Nunn 2003; Albandar 2005; Kornman 2008). For instance, some local factors such as tooth anatomy, dental caries, and dental restorations may enhance the dental plaque accumulation or even influence the composition of the dental plaque biofilm, therefore they may potentiate the harmful effects of the plaque biofilm and its bacteria (Albandar et al. 1995a; Kinane 2001; Albandar 2002a; Nunn 2003). However, the inflammatory response to plaque accumulation may vary substantially among individuals (Tatakis and Trombelli 2004).

In addition to the local factors that facilitate plaque accumulation, poor oral hygiene has been typically associated with the high prevalence of periodontal diseases (Albandar 2002b; Hyman and Reid 2003). It is well known that thorough oral hygiene measures are efficient in preventing or reducing the level of gingival inflammation thereby reducing the risk for chronic periodontitis (Albandar 2005), nevertheless, oral hygiene practice may not be useful in preventing aggressive periodontitis as it is not related to the amount of dental plaque (Albandar et al. 1995b; Highfield 2009; Clark et al. 2017). Various studies investigated the relationship between the oral hygiene measures and periodontitis, for example a longitudinal study carried out on a Sri Lankan population revealed that the attachment loss was significantly associated with dental calculus (Neely et al. 2001). Consequently, it is obvious that poor oral hygiene will lead to chronic periodontitis as there is evidence that patients with gingival inflammation have a higher risk for progression of attachment loss and loss

of teeth than periodontally healthy subjects (Albandar et al. 1998b; Schatzle et al. 2003; Schatzle et al. 2004).

One of the most important and common risk factors for periodontal diseases is smoking. Numerous studies reported that smokers have a higher risk for loss of attachment, periodontal destruction and loss of teeth in comparison to non-smokers (Albandar et al. 2000; Tomar and Asma 2000; Albandar 2002a; Albandar 2005; Pihlstrom et al. 2005; Zee 2009; Genco and Borgnakke 2013). Studies demonstrated that the risk of having attachment loss is increased by 2-7 folds in cigarette smokers as compared to non-smokers, with a higher risk in young smokers (Grossi et al. 1994; Linden and Mullally 1994; Grossi et al. 1995; Gelskey 1999; Bergstrom et al. 2000; Bergstrom 2003). Furthermore, evidence indicated a positive dose-effect relationship between smoking and periodontal diseases and that the attachment loss and severity of periodontitis are higher in heavy smokers with long history of smoking than the light smokers or those with brief history of smoking (Grossi et al. 1995; McGuire and Nunn 1999; Bergstrom et al. 2000; Tomar and Asma 2000; Bergstrom 2003; Albandar 2005; Genco and Borgnakke 2013). The risk of smoking in relation to periodontitis has been investigated in population surveys, for example the third National Health and Nutrition Examination survey in the USA population found that 41.9% of periodontitis cases were attributed to current smoking and 10.9% of cases were due to former cigarette smoking (Tomar and Asma 2000). Another survey from Brazil estimated that about 12% of periodontitis cases might be due to smoking (Susin et al. 2004). In a similar manner to cigarette smoking, cigar and pipe smoking have been shown to have risk effects on the periodontal health as they were significantly related to both teeth and alveolar bone loss (Krall et al. 1999; Albandar et al. 2000; Johnson and Slach 2001).

Different studies investigated the mechanisms by which smoking affects the periodontal health, Heasman et al. (2006) reported that the smoking effects on the periodontal health falls in the categories of plaque biofilm bacteria, gingival blood supply, neutrophils and PMNs phagocytosis, cytokine production (such as IL-1), the CD4 and CD8+ T-cell subsets, and periodontal healing. Studies reported evidence that smoking leads to peripheral vasoconstriction, which is probably due to nicotine and that this vasoconstriction results in reduced gingival bleeding which may explain why smokers have less gingivitis and reduced gingival bleeding as compared to non-

smokers. This nicotine vasoconstriction effect may also explain the impaired gingival circulation in smokers, which might lead to decreased oxygen tension in the periodontal pocket which favours the overgrowth of anaerobic bacteria, such as *P. gingivalis* and *T. denticola* (Loesche 1994; Bergstrom and Bostrom 2001; Dietrich et al. 2004; Morozumi et al. 2004). Smoking can affect neutrophil function especially their migration and chemotaxis, these effects might be attributed to nicotine. Moreover, nicotine enhances the degranulation of the neutrophils making these cells more sensitive to bacterial challenge (Seow et al. 1994; Soder et al. 1999; Soder et al. 2002). The periodontal connective tissues and bone destruction in smokers with periodontitis were attributed to the increased levels of macrophage/neutrophil-derived TNF-α in response to nicotine (Fredriksson et al. 2002; Genco and Borgnakke 2013). Nicotine also inhibits the proliferation, chemotaxis, and attachment of the fibroblasts within the periodontal ligament, an effect that is enhanced by the nicotine binding to the root surface which consequently inhibits the periodontal regeneration, and healing (Cuff et al. 1989; James et al. 1999). Smoking increases the number of the CD4 and CD8+ T-cell subsets in the periodontal tissues leading to an extensive periodontal tissue destruction (Loos et al. 2004). However, some studies reported that the levels of IL-1β is reduced in the GCF of smokers with periodontitis, which is raised in periodontally healthy smokers. This may indicate the possibility of an imbalance in the pro-inflammatory cytokines production that may affect the periodontal diseases pathogenesis in smokers (Petropoulos et al. 2004).

In addition to oral hygiene and smoking, age is another important risk factor in the periodontal diseases pathogenesis, various epidemiological studies reported that the prevalence and severity of attachment loss increase with age (Burt 1994; Corbet et al. 2001; Albandar 2002b; Albandar and Rams 2002; Steele and O'Sullivan 2011; White et al. 2012); however, the increased risk of periodontitis associated tissue destruction in older individuals might be related to the time cumulative effect of the disease (Nunn 2003; White et al. 2012). Along with age, it has been reported that there is an association between the gender and attachment loss, and that men have higher prevalence and severity of periodontal diseases than women (Albandar 2002b; Steele and O'Sullivan 2011; White et al. 2012). The prevalence of periodontal destruction and loss of attachment in males might be related to gender-dependent genetic predisposing factors (Reichert et al. 2002). For instance, it has been found

that oestrogen plays a protective role against periodontal inflammation, experimental studies showed that postmenopausal women receiving oestrogen supplement have less gingival inflammation and bleeding, reduced attachment loss and bone destruction than postmenopausal women without oestrogen supplementation, which may explain why periodontal diseases are less prevalent in females as compared males (Norderyd et al. 1993; Payne et al. 1999; Reinhardt et al. 1999).

Socioeconomic status is also a considerable risk factor and indicator for the prevalence and severity of periodontal diseases, high measures of attachment loss and probing depth have been reported in low socioeconomic groups in comparison to high socioeconomic groups (Steele and O'Sullivan 2011; White et al. 2012; Eke et al. 2015). Though its exact role is not clear, studies reported that race or ethnicity might also influence the severity and prevalence of periodontal diseases. For instance, studies in the USA demonstrated that certain race-ethnicity groups particularly those of African and Hispanic American background, have a higher risk of developing periodontal diseases than other groups (Albandar et al. 1999; Albandar and Rams 2002). However, the race-ethnicity factors may be negligible when other factors are considered such as smoking or socioeconomic status (Poulton et al. 2002; Hyman and Reid 2003).

Numerous studies have demonstrated the association of many systemic diseases with different periodontal diseases. Diabetes mellitus is the most common systemic disease associated with periodontal diseases, studies reported that there is a strong evidence for direct relation between diabetes and periodontitis and that there is a positive correlation between diabetes mellitus with each of attachment loss, severity and extent of periodontitis (Nunn 2003; Albandar 2005; Kornman 2008; Chapple and Genco 2013). Results obtained from cross-sectional, longitudinal and prospective cohort studies indicated that patients with type 1 diabetes at all ages and adult patients with type 2 diabetes have more severe and prevalent periodontal disease than subjects without diabetes (Nelson et al. 1990; Shlossman et al. 1990; Emrich et al. 1991; Taylor et al. 1998; Soskolne and Klinger 2001; Taylor 2001; Chavarry et al. 2009; Fernandes et al. 2009; Taylor et al. 2009; Hodge et al. 2012), additionally, uncontrolled or poorly controlled diabetic patients are at high risk for periodontitis with progressive bone loss (Taylor 2001; Pihlstrom et al. 2005). The impaired wound healing, exaggerated monocyte response to dental biofilm antigens, and the impaired

neutrophil functions and chemotactic responses, all might explain the severe periodontal tissue and alveolar bone destruction in diabetic patients with periodontitis (Salvi et al. 1997; Gustke et al. 1998).

The relationship of other systemic diseases and conditions with periodontal diseases pathogenesis were also investigated. HIV/AIDS may have some effects on the progression of chronic periodontitis. Moreover, the HIV-positive and immune-suppressed patients may develop severe forms of periodontal diseases such as necrotizing ulcerative gingivitis/periodontitis which represents an indication for the reduced count of CD4+ cells to less than 200 cells/µl (Glick et al. 1994; Robinson et al. 2002; Mulligan et al. 2004; Albandar 2005; Pihlstrom et al. 2005). Studies reported the association of poor maternal periodontal health and risk for preterm birth, low birthweight, and pre-eclampsia (Offenbacher et al. 1996; Dasanayake 1998; Jeffcoat et al. 2001; Lopez et al. 2002; Sanz and Kornman 2013). However, such an associations were not found in the UK (Davenport et al. 2002; Moore et al. 2004), and it is worthy of note that some of the studies which reported the positive association of maternal periodontal diseases and the adverse pregnancy outcomes were carried out on African-American or Hispanic-American women who were at higher risk for such outcomes than other race-ethnicity groups (Pihlstrom et al. 2005). In respect to the relationship between periodontal diseases and cardiovascular disorders, the European Federation of Periodontology (EFP) and AAP Workshop on periodontitis and systemic diseases reported that “there is consistent and strong epidemiologic evidence that periodontitis imparts increased risk for future cardiovascular disease” (Tonetti and Van Dyke 2013). Despite the fact that malnutrition was historically associated with periodontal diseases especially vitamin C deficiency and scurvy, various epidemiological studies didn’t find any relation between minor vitamin deficiencies and periodontal diseases especially in Europe and the USA; however, in countries and areas such as sub-Saharan Africa were malnutrition and poverty are common, Noma (cancrum oris) which usually starts as acute necrotising ulcerative gingivitis is still prevalent in malnourished (especially kwashiorkor) individuals (Enwonwu et al. 2000; Pihlstrom et al. 2005).

In addition to the systemic diseases and conditions, the association between genetic factors and periodontal diseases were also heavily investigated. Severe periodontal manifestations associated with some of the rare genetic disorders which have

serious effects on the phagocytes, epithelia, connective tissue, and teeth (Albandar 2005; Pihlstrom et al. 2005). For example, Haim-Munk and Papillon-Lefèvre syndromes, which are rare autosomal recessive disorders, are associated with periodontitis onset at childhood and early loss of both deciduous and permanent teeth (Hart et al. 1999; Toomes et al. 1999; Hart et al. 2000). Evidence obtained from studies carried out on twins suggested that nearly half the population variance in periodontitis might be related to genetic disorders (Michalowicz et al. 1991b; Michalowicz et al. 1991a; Michalowicz et al. 2000a). Furthermore, different studies indicated that variations and polymorphisms in or near some cytokine genes such as the IL-1 and TNF-α genes, might affect the inflammatory response in patients with periodontitis (Kornman et al. 1997; D'Aiuto et al. 2004; Taylor et al. 2004).

1.2 Saliva and gingival crevicular fluid in periodontal diagnostics

The diagnosis of periodontal diseases is based on clinical diagnostic parameters that were introduced about 4-5 decades ago and used continuously as the basic model for periodontal diagnosis in daily clinical practice. These parameters include measuring the gingival inflammation, periodontal pocket depth, bleeding on probing, clinical attachment loss, plaque and calculus indices, as well as the interpretation of radiographs to quantify the alveolar bone level (Armitage 1996; Armitage 2004a). Despite the fact that these clinical parameters are common, easy to use and cost effective for the clinicians; all these conventional parameters only reflect the history of the disease rather than the current status and are not able to predict the future outcome of the disease. These limitations led the researchers to seek a new diagnostic paradigm that is able to identify susceptible subjects who are at risk to have the disease, reflect the current status of the disease, predict the clinical course of the disease and asses its response to treatment. Therefore, the use of oral fluids including saliva and GCF gained attention in the diagnosis and monitoring of periodontal diseases. In the last 2-3 decades, significant improvements have been achieved in the analysis of saliva and GCF samples for the diagnosis of periodontal and different oral and systemic diseases (Streckfus and Bigler 2002; Taba et al. 2005; Ng et al. 2006; Kinney et al. 2007; Ng et al. 2007; Taylor 2014).

1.2.1 Gingival crevicular fluid

The gingival crevice (sulcus) is bathed with a fluid known as the gingival crevicular fluid (GCF). GCF can be either a physiological (gingival interstitial) fluid or an inflammatory serum exudate that originates from the blood supply of the highly vascularized connective tissue adjacent to the epithelial lining of the gingival sulcus. As a serum exudate, GCF contains the same normal and inflammatory components of the serum such as proteins, enzymes, pro-inflammatory cytokines, prostanoids, complement components, antibodies and inflammatory cells such as PMNs and plasma cells (Griffiths 2003; Kinney et al. 2007; Gupta 2013; Barros et al. 2016; Perunovic et al. 2016; Taylor and Preshaw 2016; Wassall and Preshaw 2016). During normal physiological conditions (healthy gingiva) the volumes of the GCF are very low, but both the volumes and the inflammatory components of the GCF increase during the inflammation of the gingiva and periodontal tissues, where the GCF traverses with all its contents into the gingival crevice to overcome the microbial challenge of the plaque biofilm (Taylor and Preshaw 2016; Wassall and Preshaw 2016). Hence, GCF plays a protective role in the host-bacterial interactions by two means: First, physical via the dilution of bacteria and their products, as well as, its outflow in an attempt to wash out these bacteria and their products. Secondly, delivering all the anti-inflammatory and antibacterial components into the site of infection within the gingival crevice and periodontal pocket (Armitage 2004b; Lamster and Ahlo 2007).

GCF was discovered accidentally in the late 1950s while investigating the sub-gingival biofilm from the gingival pockets of a dog (Brill and Krasse 1958; Krasse 1996), the interest in studying this oral fluid began in the early 1960s (Egelberg 1963), which evolved rapidly during the 1970s and the first detailed study concerning the GCF was published in 1974 (Cimasoni 1974). As its contents reflects the inflammatory status of the gingiva and periodontium, along with the non-invasive collection procedure and the improvement in the analytical technologies for the very small volumes of biological fluids, the analysis of GCF gained the attention of both the clinicians and researchers and became a popular tool for the diagnosis of periodontal diseases and improved the understanding of their pathogenesis (Lin et al. 2005; Loos and Tjoa 2005; Bostanci et al. 2007; Fine et al. 2014; Barros et al. 2016; Wassall and Preshaw 2016). In addition to its non-invasive collection and the ability

to collect more than one sample simultaneously, the major advantage of the GCF analysis is that it provides site-specific information about the inflammatory status at any particular periodontal site. However, as it is site-specific, GCF analysis may not provide comprehensive diagnostic information about the periodontal disease at full mouth level unless samples are collected from numerous or all periodontal sites, which is indeed costly, time consuming and clinically impractical (Taylor 2014; Wassall and Preshaw 2016).

1.2.2 Saliva

The oral cavity is bathed with a complex biological fluid known as saliva, comprised mainly of water as well as various organic and inorganic components (Chiappin et al. 2007; Hassona and Scully 2016). Saliva is produced by three pairs of major salivary glands (parotid, submandibular, and sublingual), and hundreds of minor salivary glands scattered throughout the oral cavity on the buccal, labial and palatal mucosa (Veerman et al. 1996; Korte and Kinney 2016). Most of the salivary constituents are produced by the major and minor salivary glands; however, there are also non-salivary components that may pass to saliva from the nearby oral/periodontal tissues and circulation through diffusion, active transport and ultrafiltration (Miller et al. 2010). Water constitutes about 99% of the total salivary composition. Minerals and ions are the inorganic components of saliva. Whereas, the organic part of saliva consists of salivary and non-salivary components including mucin, GCF, serum, blood traces, proteins, antibodies, enzymes, peptides, desquamated cells, various local and systemic body secretions such as nasopharyngeal discharge, extraneous debris, bacteria and bacterial by-products and many other constituents (Schenkels et al. 1995; Zimmermann et al. 2007; Miller et al. 2010; Hassona and Scully 2016; Korte and Kinney 2016).

Saliva performs a number of important physiological functions which are crucial to maintain oral health. Besides protecting, lubricating and hydrating the oral mucosa, saliva facilitates speech and swallowing, wash out substances from the mouth, buffers pH, maintains teeth mineralization, aids in wound healing, digest carbohydrates via amylase and neutralizes some harmful dietary components, influences the oral flora, and protects against infections by its antimicrobial and inflammatory contents (Proctor 2016). The typical salivary flow rate in healthy adults ranges between 800-1500 ml/day, which means that saliva is a readily available and

abundant biological fluid. Indeed this makes the collection of saliva easy, non-invasive, painless, inexpensive, and do not require sophisticated techniques and highly trained or specialized personnel. However, variations in salivary production and flow rate may occur due to factors such as time of day or circadian rhythm, duration of collection, temperature, hydration status, individual oral and systemic health as well as the emotional status. Saliva can be collected either as unstimulated (resting) or stimulated following chewing paraffin wax or applying 2% citric acid onto the tongue (Navazesh 1993; Wu et al. 1995; Vissink et al. 1996; Kavanagh et al. 1998; Ship 2002; Burlage et al. 2005; Kariyawasam and Dawes 2005; Baum et al. 2011; Hassona and Scully 2016).

Within the last 2-3 decades the use of saliva as a clinical diagnostic fluid gained growing attention and became popular in the diagnosis of different periodontal, oral and systemic diseases (Lee and Wong 2009; Zhang et al. 2010a; Pfaffe et al. 2011; Liu and Duan 2012; Hassona and Scully 2016). However, some points should be considered when using saliva as a diagnostic fluid. For instance, inhibitors and enzymes present in saliva may affect or obscure some of the immunologically important proteins. Proteases are the most common, they are found to be elevated in saliva of periodontitis patients, and may reduce the levels of some proteins and biomarkers (Ingman et al. 1993; Ng et al. 2007). Salivary flow rate, oral hygiene status, inflammatory conditions such as gingivitis and salivary gland diseases may also affect saliva composition and complicate the measurement of some biomarkers. Even the method of saliva collection may alter the efficacy of different salivary assays (Baum et al. 2011; Genco 2012; Slowey 2013). However, the analysis of salivary biomarkers is still common and of great importance in both medicine and dentistry (Korte and Kinney 2016). Moreover, the advances in the technologies such as proteomics, genomics, metabolomics, and nanotechnology have improved the sensitivity and reliability of saliva analysis in the diagnosis and monitoring of oral and periodontal diseases and their response to treatment (Denny et al. 2008; Lee et al. 2009; Al-Tarawneh et al. 2011; Al Kawas et al. 2012; Zhang et al. 2012; Zhang et al. 2013a).

In the diagnosis of periodontal diseases, the GCF analysis provides a site specific information, but there are limitations to the use of GCF including: the need for trained personnel, the time consumed in collection and processing the filter paper stripes

used for collection, contamination by gingival bleeding, as well as the limited volumes of GCF obtained, all may limit the usefulness of GCF (Taba et al. 2005; Ng et al. 2007; Buduneli and Kinane 2011; Taylor 2014). In addition to the GCF limitations, the advantages of saliva collection especially the non-invasive methods that makes saliva sampling convenient for both the patients and clinicians/researchers, and the large volumes obtained, favoured saliva sampling over GCF sampling. Furthermore, as a biologically rich fluid, whole saliva mostly contains the same biomarkers found in GCF, and represents a fluid that contains pooled samples from all periodontal sites. Therefore, the analysis of whole saliva provides an overall assessment of the periodontal disease status rather than site limited information provided by GCF analysis (Miller et al. 2006; Ng et al. 2007; Buduneli and Kinane 2011; Taylor 2014).

1.3 Biomarkers of periodontal diseases

Numerous clinical cross-sectional and longitudinal studies, in addition to in vitro and in vivo experimental studies, investigated and identified a wide range of pro- and anti-inflammatory cytokines, chemokines, enzymes, bone metabolic by-products, bacterial products, and various other proteins and inflammatory mediators as biomarkers for periodontal diseases in periodontal cells, GCF and saliva obtained from patients with different periodontal diseases, as well as experimental animals. The identified biomarkers were useful in the diagnosis and differentiation of the disease from healthy status, evaluation of the disease severity, progression and response to treatment by studying the correlations of these biomarkers with the clinical periodontal measures. The following sections present salivary and GCF biomarkers for periodontal diseases.

1.3.1 Periodontal diseases biomarkers in GCF

Various studies utilized GCF as a diagnostic tool for periodontal diseases, thus, numerous inflammatory mediators and biomarkers were identified in the GCF. Inflammatory cytokines are released from the cells of the junctional epithelium, gingival fibroblasts, and the inflammatory cells such as neutrophils, dendritic cells and macrophages. Moreover, during the destructive process of periodontitis, enzymes such matrix metalloproteinases are released by the fibroblasts, neutrophils and osteoclasts (Barros et al. 2016). According to Armitage (2004b), more than 65 GCF components have been evaluated as diagnostic biomarkers for the progression

of periodontitis, and that these biomarkers fall into three categories: host-derived enzymes and their inhibitors, inflammatory mediators and host-response modifiers, and the tissue breakdown products. Whereas, Loos and Tjoa (2005) reported that almost 100 components in the GCF have been evaluated as biomarkers for the diagnosis and classification of periodontal diseases, and that these components can be serum proteins, antibodies, enzymes, cytokines and different proteins and mediators. In their analysis of proteins in human GCF using mass spectrometry, Kido et al. (2012) detected 104 proteins in the GCF of healthy and periodontitis sites, these proteins were serum, cytoskeleton, immunity, inflammation and lipid-related proteins and enzymes, with 63 proteins to be higher in the GCF of periodontitis sites. Other study reported that, up 432 different human proteins and 30 bacterial have been identified in the GCF of both healthy subjects and chronic periodontitis patients. Among these proteins, 120 novel human proteins were identified. Neutrophil defensin-1, carbonic anhydrase-1 and elongation factor-1 gamma were among the proteins identified in the GCF of periodontitis patients (Baliban et al. 2012). Silva-Boghossian et al. (2013), identified 214 proteins in the GCF of periodontitis sites and 154 proteins in the GCF of gingivitis sites obtained from patients with chronic periodontitis.

In their review of host derived periodontal biomarkers, Buduneli and Kinane (2011), reported that cytokines and other inflammatory components are detected in the oral fluids such as GCF. Early studies by Offenbacher et al. (1984); (Offenbacher et al. 1986), reported that high levels of PGE_2 were detected in GCF of patients with chronic and aggressive periodontitis, and that these high PGE_2 levels were associated with the increased attachment loss, severity and aggressiveness of the periodontitis, indicating that PGE_2 is biomarker for tissue destruction in periodontal diseases. Nakashima et al. (1994), measured the PGE_2, alkaline phosphatase (ALP), and osteocalcin (OC) levels in the GCF of healthy, gingivitis and periodontitis patients. OC is a major component of bone matrix produced by the osteoblasts and could be considered as an indicator for alveolar bone degradation. High levels of PGE_2, ALP and OC were detected in the GCF of the periodontitis patients as compared to the gingivitis and healthy subjects, as well as, OC, PGE_2 and ALP were positively correlated with each other. Nakashima et al. (1994) results suggested GCF-PGE_2, ALP and OC as potential markers of periodontitis. PGE_2 has been

detected in higher levels in gingival tissue and GCF proportional to the severity of periodontal disease.

Studies reported that there is a positive correlation between increased GCF levels of IL-1β and periodontitis (Engebretson et al. 2002; Barksby et al. 2007; Buduneli and Kinane 2011; Chaudhari et al. 2011). Significantly higher concentration of IL-1β, IL-6, and β2-microglobulin were found in the GCF of patients with severe periodontitis as compared to healthy controls (Mogi et al. 1999). High levels of IL-1β and neutrophil elastase (NE) in GCF have been associated with increased levels of gingival inflammation in experimental gingivitis studies, which suggested NE as a good marker for gingival inflammation (Gonzales et al. 2001; Herrmann et al. 2001). TNF-α, IL-1β, -6, -8 and -18, were heavily expressed in human gingiva, and high levels have been detected in GCF of periodontitis patients (Preiss and Meyle 1994; Boch et al. 2001; Graves and Cochran 2003; Toker et al. 2008; Pradeep et al. 2009; Fitzsimmons et al. 2010; Teles et al. 2010). High levels of IL-6 and oncostatin M (OSM), were detected in GCF of chronic periodontitis patients, and the total amounts of IL-6 and OSM were positively correlated with the severity of periodontitis (Lin et al. 2005). In their study, Silva et al. (2008) followed the progression of periodontal destruction in 56 moderate to severe chronic periodontitis patients, and they detected significantly higher GCF levels of RANKL, IL-1β, and MMP-13 activity in active sites than the inactive sites. IL-11 and IL-17 levels were investigated in GCF of 40 chronic periodontitis patients and 20 healthy controls. The IL-11/IL-17 ratio was significantly higher in the healthy subjects than the periodontitis patients, whereas shallow pockets in the periodontitis patients demonstrated higher ratios than the deep pockets in the same patients. These results suggested that, periodontal destruction in periodontitis may be attributed to an imbalance in the pro- and anti-inflammatory cytokines (Yetkin Ay et al. 2009). A cross-sectional study carried out on 20 chronic periodontitis patients, 17 generalized aggressive periodontitis patients and 10 gingivitis patients. The study revealed that the GCF IL-1β levels and elastase activity were higher in the deep pockets as compared to the shallow pockets in the periodontitis patients. The results suggested that these two biomarkers might be helpful to indicate the periodontal tissue destruction (Rescala et al. 2010).

Growth factors were among the biomarkers detected in GCF of patients with different periodontal diseases. Growth factors, have various activities which may overlap and

participate in the immune responses (Taylor 2014). High levels of growth factors such as transforming growth factor –alpha and -beta (TGF-α, -β), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were found in GCF of patients with periodontal diseases (Chang et al. 1996; Uematsu et al. 1996; Skaleric et al. 1997; Booth et al. 1998; Mogi et al. 1999; Kakimoto et al. 2002; Ohshima et al. 2002; Ohnishi and Daikuhara 2003).

Different types of matrix metalloproteases and their inhibitors have been detected in the GCF of patients with periodontal diseases (Golub et al. 1997; Kinane et al. 2003; Buduneli and Kinane 2011; Kinney et al. 2014). Higher levels of MMP-8 and MMP-9 were detected in the GCF of chronic periodontitis patients as compared to localized aggressive periodontitis (LAgP) patients and healthy controls. Whilst, significantly elevated levels of MMP-1 and tissue inhibitor of MMP-1 (TIMP-1) were detected in the GCF of the LAgP patients as compared to the chronic periodontitis patients and healthy controls. Moreover, no difference was found in the GCF MMP-3 levels among the three groups (Ingman et al. 1996). In addition to MMP-8, Golub et al. (1997) found high levels of MMP-13 in GCF samples of periodontitis patients. Chen et al. (2000), detected high levels of MMP-8 and neutrophil elastase in the GCF of periodontitis patients, and that the MMP-8 levels reduced significantly following treatment. High levels of GCF MMP-3, and TIMP-1 were found in periodontitis sites as compared to healthy sites in 40 subjects monitored over a 6 month period, which indicated the high risk for periodontal disease progression at the diseased sites (Alpagot et al. 2001). The GCF MMP-8 can be used to differentiate periodontitis from gingivitis and healthy sites, as well as, to monitor the response to treatment in chronic periodontitis patients (Mantyla et al. 2003). Additionally, Kinane et al. (2003) reported that the GCF MMP-8 levels were significantly reduced in chronic periodontitis patients following non-surgical periodontal treatment. The GCF MMP-8 and -9 activities were positively correlated with the periodontal disease severity which was negatively correlated with TIMP-1, -2 levels (Pozo et al. 2005).

High levels of MMP-7, TIMP-1, and extracellular matrix metalloproteinase inducer (EMMPRIN) were detected in GCF of patients with different periodontal diseases, indicating that these proteins are engaged in the progression of periodontal diseases (Emingil et al. 2006b). In another study, Emingil et al. (2006a) demonstrated higher levels and activities of both MMP-25 and -26 in the GCF of chronic and generalised

aggressive periodontitis (GAgP) patients as compared to gingivitis and healthy subjects, and that these MMP-25/-26 levels and activities were associated with the severity of periodontal inflammation, indicating that these novel MMPs may play a role in the progression of periodontal diseases, and suggesting them as diagnostically useful biomarkers. Significantly high GCF levels and activity of MMP-13 were reported in active sites of periodontitis patients, suggesting that MMP-13 could serve as a biomarker for the disease progression (Hernandez et al. 2006). In their study, Beklen et al. (2006) reported that the GCF MMP-8 and -9 levels were positively correlated with the disease activity in chronic periodontitis patients. Though the mean levels were reduced following treatment, persistently high levels of GCF MMP-8 were detected in sites with progressive periodontitis among smokers and non-smoking periodontitis patients, indicating the sites at high risk, as well as, the patients with poor response to non-surgical periodontal treatment, suggesting MMP-8 as a useful biomarker to follow the response to treatment in periodontal diseases (Mantyla et al. 2006).

A statistically significant association was found between the GCF MMP-8 concentrations from shallow gingival crevices and the extent of attachment loss, an association which suggested the GCF MMP-8 as a prognostic biomarker for attachment loss in periodontal diseases (Passoja et al. 2008). High levels and activity of MMP-13 were detected in GCF samples obtained from active periodontitis sites of patients with moderate and advanced periodontitis (Silva et al. 2008). Rai et al. (2008), reported that higher levels of MMP-9 were found in GCF of periodontitis patients in comparison to gingivitis and healthy subjects, as well as, higher levels of GCF MMP-2 were detected in the gingivitis patients in comparison to the periodontitis and healthy subjects, Rai et al. (2008) also found that the GCF MMP-2 and -9 were positively correlated with both the probing depth and gingival bleeding suggesting these 2 GCF MMPs as biomarkers for periodontal diseases and that they may aid in the early detection of periodontitis and gingivitis. The high GCF levels of MMP-9 and -13 found within the active sites of moderate and advanced chronic periodontitis patients, led Hernandez Rios et al. (2009) to suggest these proteases as useful biomarkers for the progression of tissue destruction in periodontitis. By the end of a 6 months monitoring phase, high levels of MMP-8, MMP-9, OPG and IL-1β were detected in GCF samples of patients with progressive periodontitis as compared to

more stable patients, suggesting this panel of biomarkers as a sensitive tool to distinguish patients with risk of progressive periodontitis from those who are more stable or responding positively to treatment (Kinney et al. 2014).

In addition to the MMPs, other inflammatory mediators, cells, proteins and enzymes were also investigated in the GCF of patients with different periodontal diseases. Neutrophil elastase (NE) is one of the most destructive proteolytic enzymes which has the ability to degrade almost all extracellular matrix components as well as plasma proteins, activates pro-MMPs and inactivates TIMP-1. High levels of this elastase was detected in the GCF of patients and subjects with different periodontal conditions (Meyle et al. 1992; Eley and Cox 1996; Sorsa et al. 2006; Geraghty et al. 2007). In a longitudinal study, Eley and Cox (1996) showed that the increased levels and activity of elastase in the GCF of periodontitis patients was predictive for attachment loss. Furthermore, the long term follow up of the patients undergoing supportive periodontal treatment by Eley and Cox (1996), revealed that the GCF elastase was positively correlated with the attachment loss. Elastase and other enzymes were also investigated in the GCF of patients with periodontal diseases. In their study, Cox and Eley (1992) analysed cathepsin B/L, elastase, tryptase, trypsin, and dipeptidyl peptidase (DPP) IV-like activities in GCF samples obtained from 20 chronic periodontitis patients before and after non-surgical treatment. They found that the levels and activities of these enzymes were significantly higher in the pre-treatment status as compared to the post-treatment status, and that the enzymes levels and activities were positively and significantly correlated with the periodontal parameters of the patients. Therefore, Cox and Eley (1992) suggested these GCF proteases as useful markers to reflect the clinical status of periodontal lesions and may be beneficial in monitoring the disease activity. Another study by Eley and Cox (1995), reported that the significantly high levels of DPP II/IV, total activity and concentration found in the GCF obtained from 120 sites with rapid and gradual attachment loss in 48 periodontitis patients may be predictors for periodontal attachment loss.

β-Glucuronidase is a proteolytic enzyme released from neutrophils lysosomes, and its' action is the degradation of proteoglycans and the ground substance (Lamster et al. 2003). Number of studies investigated the β-glucuronidase levels and activity in the GCF of patients with different periodontal diseases especially chronic and

aggressive periodontitis. The studies revealed that the levels and activity of this enzyme were significantly increased in the GCF of periodontal diseases patients, and were positively correlated with the clinical periodontal parameters. Therefore, the results of these studies suggested β-glucuronidase as a useful biomarker for the PMNs activity, loss of attachment, detection of active periodontitis sites and the progression of the disease (Lamster et al. 1988; Lamster et al. 1994; Lamster et al. 1995; Lamster et al. 1996; Albandar et al. 1998a; Layik et al. 2000; Lamster et al. 2003).

The degradation of the collagen matrix in the ECM and ground substance by the action of proteolytic enzymes such as MMPs, will result in the release of collagen fragments or peptides into the circulation, these substances were assayed as diagnostic markers for bone turnover in periodontitis (Giannobile 1997; Giannobile et al. 2003). One of these markers is the C-telopeptide pyridinoline cross-links of type-1 collagen (ICTP), several experimental and clinical studies investigated the levels of this marker in the GCF of patients with periodontitis. High levels of GCF ICTP were detected in periodontitis patients, and these high levels were positively correlated with the clinical parameters of periodontal tissues destruction (Talonpoika and Hamalainen 1994; Giannobile et al. 1995; Golub et al. 1997; Shibutani et al. 1997; Palys et al. 1998).

1.3.2 Salivary biomarkers for periodontal diseases

Saliva contains constituents from the salivary glands, GCF and dental plaque from all periodontal sites, as well as it is readily available, easily collected and abundant when compared to GCF. Hence, the interest in saliva analysis for the diagnosis and monitoring of periodontal diseases is growing and gaining popularity. Numerous studies investigated and identified several biomarkers and inflammatory mediators in saliva of patients with different periodontal diseases (Kaufman and Lamster 2000; Kinane and Chestnutt 2000; Lamster et al. 2003; Kinney et al. 2007; Buduneli and Kinane 2011; Taylor 2014; Korte and Kinney 2016; Taylor and Preshaw 2016). The proper diagnosis, differentiation, prediction and follow up of periodontal diseases in response to treatment, require the consideration of the disease complex pathogenesis and progression. Therefore, Korte and Kinney (2016), suggested that the host-derived biomarkers can be divided according to the three phases of periodontal diseases: inflammation, connective tissue degradation, and bone

turnover. In addition to the periodontal bacteria and their products. Hence, the identified biomarker or mediator may offer an indication about the current status and what stage of the disease pathogenesis the patient is experiencing. However, some of the biomarkers may play roles in both inflammation and tissue degradation, others may be associated with both inflammation and bone resorption (Korte and Kinney 2016).

In respect to the biomarkers of inflammation, numerous studies investigated the relations of several cytokines such as interleukins with the periodontal diseases pathogenesis. In particular, it has been shown that IL-1β demonstrated high levels in saliva of patients with periodontal diseases especially periodontitis (Tobon-Arroyave et al. 2008; Fine et al. 2009; Gursoy et al. 2009; Mirrielees et al. 2010; Ebersole et al. 2013; Taylor 2014; Jaedicke et al. 2016; Korte and Kinney 2016). Evidence derived from several studies, strongly correlated salivary IL-1β with the progression of periodontal diseases suggesting this interleukin as a good biomarker for periodontitis and can be useful to discriminate periodontitis patients from healthy subjects as well as active periodontal sites from healthy or inactive sites (Miller et al. 2006; Tobon-Arroyave et al. 2008; Gursoy et al. 2009; Gursoy et al. 2011; Kaushik et al. 2011; Ebersole et al. 2013; Rathnayake et al. 2013). For instance, several studies revealed that there were significantly positive correlations between the high levels of salivary IL-1β and the clinical periodontal measures of periodontal diseases including: bleeding on probing, gingival and plaque indices, and clinical attachment loss (Miller et al. 2006; Kaushik et al. 2011; Kinney et al. 2011; Sexton et al. 2011; Yoon et al. 2012; Rathnayake et al. 2013; Salminen et al. 2014). Longitudinal studies revealed that the IL-1β levels in saliva of periodontitis patients reduced significantly in response to periodontal treatment (Kaushik et al. 2011; Kinney et al. 2011; Sexton et al. 2011). Moreover, studies also demonstrated that salivary IL-1β levels were positively associated with the extent of alveolar bone loss (Ng et al. 2007; Scannapieco et al. 2007; Fine et al. 2009). Nevertheless, some studies reported that, though IL-1β was detected in saliva of periodontitis patients, but there was no significant difference in its levels as compared to control groups (Christodoulides et al. 2007; Ramseier et al. 2009; Teles et al. 2009). For example,Teles et al. (2009) found that the mean salivary levels of IL-1β were not able to discriminate between periodontitis and healthy status.

Most of the available studies revealed that, there was no statistically significant difference in the salivary IL-6 levels between periodontitis patients and healthy controls and that the salivary IL-6 levels were not associated with the periodontitis clinical measures or the alveolar bone loss (Ng et al. 2007; Scannapieco et al. 2007; Gursoy et al. 2009; Ramseier et al. 2009; Teles et al. 2009; Rathnayake et al. 2013). However, other studies reported high levels of salivary IL-6 in periodontitis patients (Costa et al. 2010; Ebersole et al. 2013; Prakasam and Srinivasan 2014). Other interleukins were also investigated in saliva of patients with periodontal diseases. Three studies found that IL-4, -17A, -17E, IL-17A/E ratio and IL-18 were significantly elevated in saliva of periodontitis patients and were positively correlated with the clinical parameters of periodontitis (Ozcaka et al. 2011b; Awang et al. 2014; Prakasam and Srinivasan 2014). Other studies reported that IL-2, -3, -4, -8, -9, -10, -12, -13, -17, and -33 were detected in saliva of periodontitis patients but their levels were either lower than the control subjects or the difference was not statistically significant, and that they were not associated with the alveolar bone loss and the periodontal measures, on the other hand, some interleukins such as IL-5 were not detected by some studies (Vastardis et al. 2003; Scannapieco et al. 2007; Fine et al. 2009; Ramseier et al. 2009; Teles et al. 2009; Ozcaka et al. 2011b; Sexton et al. 2011; Buduneli et al. 2012; Rathnayake et al. 2013; Prakasam and Srinivasan 2014).

Though some studies reported the detection of TNF-α in saliva of patients with periodontal diseases, the TNF-α levels were either lower than the control subjects or the difference between them was not statistically significant, and no association was found between the salivary TNF-α levels and any periodontitis parameter. Whereas, other studies were not able to detect TNF-α in saliva of periodontitis patients. Subsequently, the studies' results indicated that the salivary TNF-α may not be useful as a biomarker for periodontitis (Gursoy et al. 2009; Ramseier et al. 2009; Teles et al. 2009; Mirrielees et al. 2010; Ebersole et al. 2013; Rathnayake et al. 2013). However, one study reported significantly higher levels of TNF-α in saliva of periodontitis patients in comparison to control subjects (Frodge et al. 2008). Another study which was longitudinal, revealed that the salivary TNF-α levels were significantly reduced in periodontitis patients following periodontal treatment (Sexton et al. 2011).

Though interferon gamma (IFN-γ) was detected in saliva of HIV patients with periodontitis, but their levels were not statistically different in comparison to healthy

controls (Vastardis et al. 2003). It has been reported that the salivary levels of IFN-γ were not associated with the amount of alveolar bone loss (Scannapieco et al. 2007). On the other hand, Ramseier et al. (2009) were not able to detect IFN-γ in saliva of periodontitis and gingivitis patients. Further to IL-1β, -2, -4, -5, -6, -8, -10, and TNF-α, the salivary levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN-γ in periodontitis patients were not statistically different when compared to healthy control subjects (Teles et al. 2009). In their longitudinal study of children who were at risk of LAgP, Fine et al. (2009) also detected GM-CSF and IFN-γ in saliva of children who developed lesions with bone loss but the difference was not statistically significant in comparison to those who remain healthy.

Macrophage inflammatory protein-1α (MIP-1α) was also investigated in saliva of patients with periodontal diseases. The salivary MIP-1α levels were elevated in children who were at risk of LAgP and developed lesions with bone loss in comparison to healthy controls (Fine et al. 2009). The association of MIP-1α with the bone loss in periodontitis was confirmed again in another longitudinal study by Fine et al. (2014) when they detected high levels of MIP-1α in saliva of adolescents who were at risk of aggressive periodontitis and developed lesions with bone loss. Another longitudinal study carried out by Sexton et al. (2011), reported that the salivary MIP-1α levels reflected the severity of periodontitis and they were reduced in response to periodontal treatment, suggesting the salivary MIP-1α as a potential biomarker for periodontitis. Al-Sabbagh et al. (2012), reported high levels of MIP-1α in saliva of periodontitis patients in comparison to healthy controls, and the salivary MIP-1α levels were positively correlated with the periodontitis clinical measures. A longitudinal study investigated the levels and the role of the inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) in chronic periodontitis, it was found that the salivary MCP-1 levels were significantly higher in the periodontitis patients in comparison to the healthy controls, and the levels were positively correlated with the periodontitis clinical measures. Furthermore, the salivary MCP-1 levels were significantly reduced in response to treatment (Gupta et al. 2013).

Though high levels of the growth factors TGF-β, EGF and VEGF were detected in GCF of periodontitis patients, there are limited studies which investigated the same growth factors in saliva of periodontitis patients (Taylor 2014; Jaedicke et al. 2016). VEGF is known to play a role in the angiogenesis of both healthy and diseased

tissues, it has been shown that the VEGF levels were significantly elevated in saliva of periodontitis patients as compared to healthy controls, which suggested that the periodontal disease status influence the salivary VEGF levels (Booth et al. 1998). It has been shown that HGF is secreted by human gingival fibroblasts and this production is in response to, and regulated by the inflammatory cytokines and bacterial products (Sugiyama et al. 1996; Sugiyama et al. 2000; Ohshima et al. 2001). Studies reported that HGF may enhance the production of the MMPs, and stimulates the wound healing through vascularization and keratinocyte proliferation, additionally it has been hypothesized that HGF may drive the apical migration of epithelial cells in periodontitis (Dunsmore et al. 1996; Sugiyama et al. 1996; Matsumoto and Nakamura 1997; Ohshima et al. 2001; Kakimoto et al. 2002; Ohnishi and Daikuhara 2003). Moreover, a recent study reported that HGF play roles as a regulator of inflammation and autoimmunity (Molnarfi et al. 2015). Salivary HGF levels were investigated in four independent studies. Significantly higher levels of salivary HGF were detected in periodontitis patients as compared to healthy control subjects, and the salivary HGF levels were positively correlated with the gingival, plaque, and papillary bleeding indices, suggesting salivary HGF as a potential biomarker for periodontitis (Wilczynska-Borawska et al. 2006). A longitudinal study of 5-year time period, reported a positive association between the salivary HGF levels and the extent of bone loss in periodontitis (Scannapieco et al. 2007). The salivary HGF levels were significantly elevated in patients with moderate and severe periodontitis in comparison to healthy controls (Rudrakshi et al. 2011). Once again, Wilczynska-Borawska et al. (2012), reported that significantly higher levels of salivary HGF were found in periodontitis patients as compared to healthy control subjects, and that the salivary HGF levels were positively correlated with the gingival, plaque, and papillary bleeding indices, which confirmed the results of their previous study (Wilczynska-Borawska et al. 2006).

Several studies investigated the biomarkers of connective tissue destruction in saliva of patients with periodontal diseases. The MMPs are the most important enzymes associated with the destruction of the supporting periodontal tissues. In agreement with the GCF studies, the salivary neutrophil collagenase (MMP-8) was the target of several studies. Data derived from studies reported significantly elevated levels and activity of MMP-8 in saliva of periodontitis patients, as well as positive correlations of

the salivary MMP-8 with increased bleeding on probing, clinical attachment loss and periodontal pocket depth (Iijima et al. 1983; Gangbar et al. 1990; Hayakawa et al. 1994; Makela et al. 1994; Ingman et al. 1996; Matsuki et al. 1996; Miller et al. 2006; Herr et al. 2007a; Rai et al. 2008; Ramseier et al. 2009; Costa et al. 2010; Gursoy et al. 2010; Mirrielees et al. 2010; Kinney et al. 2011; Ebersole et al. 2013; Rathnayake et al. 2013; Miricescu et al. 2014). The salivary MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) and elastase were also investigated in relation to periodontal diseases, studies reported that their levels were significantly higher in periodontitis patients as compared to healthy subjects (Makela et al. 1994; Pederson et al. 1995; Shetty and Pattabiraman 1998; Ramseier et al. 2009; Isaza-Guzman et al. 2011; Kinney et al. 2011). However, some studies found that there were no differences in the salivary MMP-9 and elastase levels between periodontitis patients and healthy controls (Ingman et al. 1996; Gursoy et al. 2009). In respect to MMP-1 (fibroblast or interstitial collagenase) and MMP-3 (stromelysin-1), and MMP-14 (a membrane type MMP), no evidence has been reported about the association of these MMPs with periodontitis (Ingman et al. 1996; Gursoy et al. 2010; Taylor 2014).

A study of the collagenase activity in whole saliva of both healthy subjects and periodontitis patients revealed that, the total activity in saliva of the healthy controls was in the form of the inactive pro-collagenase, whereas the activity in the whole saliva of periodontitis patients was mainly in the form of the active collagenase (Hayakawa et al. 1994). Moreover, the TIMP-1 levels were higher in the healthy controls as compared to the periodontitis patients, which indicates the higher collagenase activity and low inhibition activity in periodontitis (Hayakawa et al. 1994). Two other studies also reported that the TIMP-1 levels were significantly higher in saliva of healthy subjects in comparison to periodontitis patients (Gursoy et al. 2010; Isaza-Guzman et al. 2011). Nevertheless, one study reported that there were no statistically significant differences in the levels of both TIMP-1 and MMP-1 in saliva of chronic and LAgP periodontitis patients in comparison to healthy controls (Ingman et al. 1996).

In comparison to GCF, there are limited longitudinal studies that measured the levels of MMPs in saliva of periodontitis patients following the natural progression of the disease and in response to treatment. In their longitudinal study carried out on 66 chronic periodontitis patients who received 2 modes of treatment, Gorska and Nedzi-

Gora (2006), reported high levels of salivary MMP-8, MMP-9, TIMP-1, as well as MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios in the patients prior to treatment. After treatment, only the salivary MMP-9/TIMP-1 ratio was significantly reduced in the 33 patients who received the periostat treatment (scaling and root planning + 20 mg doxycycline twice daily) as compared to the pre-treatment status. Whereas the other 33 patients who received the conventional treatment (scaling and root planning only) demonstrated significantly reduced levels of both the salivary MMP-8 and salivary MMP-8/TIMP-1 ratio as compared to the pre-treatment levels (Gorska and Nedzi-Gora 2006). The salivary MMP-9 levels were also reduced in the patients who received the conventional treatment more than those who received the periostat treatment; however, the difference was not significant as compared to the pre-treatment levels. In contrast to the MMPs, the salivary TIMP-1 levels were significantly elevated following the periostat treatment as compared to the pre-treatment levels, but not after the conventional treatment (Gorska and Nedzi-Gora 2006).

A cohort study monitored periodontitis in 219 subjects over a 4-year period, during which time they didn't receive any dental treatment (Kibayashi et al. 2007). The study used a multiple logistic model to follow the periodontitis progression in relation to smoking, the model involved measuring the changes in periodontal pocket probing depth as compared to levels of salivary biomarkers and other lifestyle factors. This logistic model revealed that among the lifestyle factors the smoking habit exerted the greatest influence on periodontitis risk, and the disease progression was related to smoking habit but not to the levels of any of the 9 proteins biomarkers combination (IL-1β, MMP-8, MMP-9, lactoferrin, IgA, albumin, aspartate aminotransferase AST, lactate dehydrogenase LDH, and alkaline phosphatase ALP) (Kibayashi et al. 2007). The levels of salivary lactoferrin, AST, and LDH were significantly reduced in smokers, a finding concordant with the well-known immunosuppressive effects of smoking on the host-defence system, which may promote periodontitis progression (Nishida et al. 2006; Kibayashi et al. 2007).

A case-controlled longitudinal study carried out on 68 adult patients with chronic periodontitis, in which 33 patients received oral hygiene instructions (OHI) alone and 35 treated with scaling and root planning (SRP) combined with OHI (Sexton et al. 2011). The salivary levels of MMP-8 and OPG (along with the previously mentioned

IL-1β, IL-8, MIP-1α, and TNF-α) were measured before treatment and after treatment at weeks 16 and 28. The periodontal health was improved in both treatment groups, and the salivary levels of MMP-8 and OPG (as well as the other biomarkers) were significantly reduced following treatment in the SRP treated patients as compared to the OHI group. The study revealed that the salivary levels of all the biomarkers especially MMP-8 (excluding IL-8) reflected the periodontitis severity and response to treatment, suggesting them as useful biomarkers for monitoring periodontitis status (Sexton et al. 2011).

Another longitudinal study investigated the role of a panel of potential salivary biomarkers and periodontal pathogens in the progression of periodontitis in a cohort of 100 participants during 1 year (Kinney et al. 2011). First, there was a 6 month-monitoring phase, during which the participants received no treatment. As compared to baseline, during the monitoring phase neither the periodontal status nor the salivary biomarkers levels in the participants were significantly changed. By the end of the monitoring phase, the participants received appropriate treatment, followed by a 6-month “disease-recovery” phase during which the disease recovery and progression were monitored. By the end of the 12 months, the study revealed that the levels of salivary MMP-8, MMP-9, OPG, as well as IL-1β were significantly reduced following treatment in participants with moderate to severe periodontitis (Kinney et al. 2011). Two studies reported the significant reduction of active collagenase in saliva of both localized juvenile (aggressive) periodontitis and adult (chronic) periodontitis in response to surgical and non- surgical periodontal treatment (Gangbar et al. 1990; Uitto et al. 1990).

In regard to the ability of salivary MMPs to differentiate periodontitis patients from gingivitis and healthy subjects, to determine the severity and extent of periodontitis, and to evaluate the response to periodontal treatment, it looks like the salivary MMP-8 to be the best among the other MMPs, as well as a better biomarker for periodontitis than other mediators such as IL-1β (Rathnayake et al. 2013). A previous study reported that the combined elevated salivary levels of MMP-8 and IL-1β were positively correlated with the clinical periodontitis measures and increased the risk for experiencing periodontal disease 45-fold (Miller et al. 2006). As compared to IL-1β, MIP-1α, OPG and TNF-α, the receiver operating characteristic curve (ROC) analysis revealed that the salivary MMP-8 had the highest area under the curve value (0.7,

p=0.01) and was the best to discriminate patients who responded to treatment from those who didn't (Sexton et al. 2011). Using the ROC analysis, it was found that a combination of salivary biomarkers and periodontal pathogens augmented the prediction of disease category. The combination of salivary MMP-8, -9 and OPG analysis along with the anaerobic periodontal bacteria *P. gingivalis* and *T. denticola* analysis, provided more precise predictions of periodontal disease category, and the combination of the elevated salivary MMP-8 and *T. denticola* biofilm levels was the best in predicting periodontal disease severity (Ramseier et al. 2009). The ROC analyses of the combination (IL-1β, IL-6 and MMP-8) demonstrated high areas under the curves (0.963-0.984) indicating good discrimination of periodontitis from health status, these results indicated that the biomarker panel comprising IL-1β, MMP-8 and IL-6 have a particular diagnostic potential (Ebersole et al. 2013). A recent study reported that salivary MMP-8 can be used alone or together with IL-1β and *P. gingivalis* to calculate the cumulative risk score of periodontitis at the subject level as an efficient diagnostic tool, which can be useful in large-scale public health surveys when a full periodontal examination is inapplicable (Sorsa et al. 2016).

During the active phase of periodontitis which is associated with death of cells, a number of enzymes, proteins and other molecules will be released into the surrounding tissues making these molecules viable markers of disease activity, one of these enzymes is the soluble cytoplasmic enzyme aspartate aminotransferase (AST) (Page 1992). A number of studies investigated the relation between the elevated levels of salivary AST and periodontitis, results of such studies linked the progression of periodontitis as defined by gingival bleeding, pocket depth, and suppuration with the elevated levels of salivary AST (Todorovic et al. 2006; Totan et al. 2006). Other studies investigated the effects of periodontal treatment on the salivary AST levels, the studies reported that the salivary AST levels decreased significantly in response to non-surgical periodontal treatment (Nomura et al. 2006; Miller et al. 2010; Nomura et al. 2012). In addition to AST, other host derived enzymes related to cellular damage and inflammation such as alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and alanine transferase (ALT), have been studied as salivary biomarkers for the detection and progression of periodontitis. Studies reported evidence for the association of elevated salivary levels of these enzymes with the progression of periodontitis and its clinical measures such as

periodontal pocket depth, and gingival bleeding (Cesco Rde et al. 2003; Nomura et al. 2006; Totan et al. 2006; Zappacosta et al. 2007; Gursoy et al. 2008; Kugahara et al. 2008; Miller et al. 2010; Nomura et al. 2012). However, one study reported no association between the salivary ALP, AST, and ALT with periodontitis (Nomura et al. 2006), another study didn't find a relation between salivary LDH and periodontitis (Gursoy et al. 2009).

Biomarkers of alveolar bone turnover/resorption such as the cytokines RANKL, OPG, osteocalcin, and calprotectin have been investigated in saliva of patients with periodontal diseases (Giannobile et al. 2003; Miller et al. 2006; Buduneli and Kinane 2011; Korte and Kinney 2016). A number of cross-sectional studies reported significantly elevated levels of RANKL in saliva of periodontitis patients as compared to healthy controls (Buduneli et al. 2008; Tobon-Arroyave et al. 2012; Tabari et al. 2013). However, one study was not able to find significant differences in the salivary RANKL levels between periodontitis patients and healthy controls (Frodge et al. 2008). Other studies investigated OPG which plays a role in preventing bone resorption and acts as a neutralizing receptor for RANKL (Cappellen et al. 2002). The ratio of RANKL/OPG plays a crucial role in the bone resorption and reconstruction (Cappellen et al. 2002; Jin et al. 2007). Significantly lower levels of OPG have been found in saliva of periodontitis patients as compared to healthy controls (Ramseier et al. 2009; Tobon-Arroyave et al. 2012). Other studies didn't find significant differences in the salivary OPG levels between periodontitis patients and healthy controls (Miller et al. 2006; Frodge et al. 2008; Costa et al. 2010; Kinney et al. 2011; Tabari et al. 2013). Few longitudinal studies demonstrated that the salivary OPG levels declined significantly in response to periodontal treatment (Kinney et al. 2011; Sexton et al. 2011).

In regard to the other bone regulating cytokines and molecules such as the neutrophil protein calprotectin, it was found that the salivary levels of this marker to be increased in periodontitis patients (Ramseier et al. 2009). Whereas, Kinney et al. (2011), found that both the healthy controls and the periodontitis patients demonstrated significantly elevated salivary calprotectin levels by the end of their 12 month-period longitudinal study as compared to baseline. Studies reported inconsistent evidence regarding the association of both salivary osteonectin and osteocalcin with periodontitis (Bullon et al. 2005; Bullon et al. 2007; Burton 2007;

Ozcaka et al. 2011a; Miricescu et al. 2014). Moreover, a 5-year period longitudinal study demonstrated a negative association between the salivary osteonectin levels and the extent of alveolar bone loss (Scannapieco et al. 2007).

Peptides represent other markers for bone turnover, they are released as a result of alveolar bone and collagen destruction. Among these peptides is the ICTP, which is a specific marker for alveolar bone deterioration and considered as a potentially useful indicator to discriminate periodontitis from gingivitis patients (Taylor 2014; Korte and Kinney 2016). Some studies reported elevated salivary levels of ICTP in periodontitis patients as compared to healthy controls and that these elevated ICTP levels were positively correlated with the clinical measures of periodontitis (Gursoy et al. 2010; Ozcaka et al. 2011a; Gursoy et al. 2013). However, other studies were not able to detect ICTP in saliva of periodontitis patients (Ng et al. 2007; Frodge et al. 2008; Ramseier et al. 2009; Al-Sabbagh et al. 2012).

1.4 Proteomics and biomarkers identification

1.4.1 Proteomics in the diagnosis of periodontal diseases

The majority of the published literature on salivary and GCF biomarkers for periodontal diseases indicate that these biomarkers were selected for investigation on the basis of their previously known roles in immunity and inflammation, and they were identified by techniques such as ELISA, western blot, polymerase chain reaction techniques (PCR), immunohistochemistry, microbiological techniques and others. Despite the fact that they are well-known, verified, reliable, and improvements are continually introduced to these techniques, they always need candidate biomarkers and they can detect a limited number of proteins or cytokines in saliva and GCF samples. Hence, there is a need for more open and unbiased approaches to identify biomarkers for diseases with complex pathogenesis such as periodontal diseases (Haigh et al. 2010; Taylor 2014). There is a growing interest to use alternative techniques such as genomics, proteomics and other advanced approaches that are able to identify changes in genes, and proteins. These advanced techniques do not require candidate proteins and can concurrently identify wide range of proteins in one sample (Haigh et al. 2010). Therefore, proteomics offers the potential to identify numerous disease associated proteins (Xie et al. 2011; Zhang et al. 2013a; Taylor 2014). Proteomics can be defined as “the study of protein

properties on a large scale to achieve more extensive vision about diseases processes, cellular processes and networks at the protein level" (Blackstock and Weir 1999), or as "the large-scale study of proteins, especially their structures and functions" (Zhang et al. 2010b). The term proteome is an admixture of protein and genome, the concept of proteome was first coined by the PhD student Marc Wilkins in 1994 (Wilkins et al. 1996). Whereas, the term proteomics was first introduced in 1997 to make an analogy with genomics which studies the genome (James 1997).

Diverse proteomic techniques have been used to analyse the protein content of saliva both qualitatively and quantitatively (Xiao and Wong 2010). Denny et al. (2008), reported that the identified salivary proteome comprises the sum of 1166 proteins. However, substantial improvements and modifications have been and are always introduced to the proteomic analysis of saliva such as using a combination of advanced techniques for protein separation, as well as advances in the spectrometry technologies using liquid chromatography-mass spectrometry (LC-MS) quantitative protocols (Xiao and Wong 2010; Xie et al. 2011), therefore, it is expected to identify more salivary proteins in the future. In the last 2 decades, various studies used proteomics to identify and investigate salivary and GCF protein biomarkers for periodontal diseases (Haigh et al. 2010; Taylor 2014). Salivary proteomic studies might reveal high quality diagnostic and prognostic protein biomarker signatures, such biomarkers may help in the diagnosis, personalizing treatment paths and monitoring patients with high susceptibility for periodontitis in their young adulthood before the clinical onset of tissue destruction associated with periodontitis (Giannobile et al. 2009; Kornman and Duff 2012; Salazar et al. 2013).

One of the earliest studies that used proteomics in the diagnosis of periodontal diseases identified two of the S100 family of calcium binding proteins in GCF and saliva of periodontitis patients, the S100 proteins are known to have regulatory roles in inflammation. Using two-dimensional-gel electrophoresis (2-D PAGE) with peptide mass fingerprinting and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), Kojima et al. (2000) identified the S100A8 (MRP8) and S100A9 (MRP14) proteins in GCF and saliva of periodontitis patients, the results were then confirmed by western blot. The two proteins were highly expressed in both the GCF and saliva of periodontitis patients as compared to healthy and edentulous controls, and they were more abundant in GCF. The study results suggested these

two S100 proteins in GCF as biomarkers for periodontitis and introduced proteomics as a new diagnostic approach for periodontal diseases (Kojima et al. 2000). Another proteomic study analysed GCF samples obtained from periodontitis patients and healthy controls. Using 1-D PAGE and LC-MS/MS, the study detected the protein azurocidin which was highly expressed in the GCF of periodontitis patients in comparison to healthy controls. Azurocidin is one of the antibacterial proteins produced by neutrophils, it is also known to have chemotactic effects, and it was found that azurocidin inhibits the differentiation of bone marrow-derived macrophages to osteoclasts suggesting a protective role against alveolar bone loss during the early stages of periodontitis. The increased expression of azurocidin during periodontitis was verified by ELISA assay of the periodontitis patients GCF samples compared to those of the healthy individuals. The study results suggested azurocidin as a potential biomarker for the early detection of inflammatory periodontal destruction in periodontitis (Choi et al. 2011).

The 2-D PAGE and LC techniques were used to identify changes in α-amylase, Ig heavy chain, and albumin along with cystatin levels in saliva of patients with gingivitis and chronic periodontitis. These approaches provided information about the salivary proteome profile during gingival and periodontal inflammation, and considered as contribution to the improvement in the salivary diagnostics of periodontal diseases (Goncalves Lda et al. 2010; Goncalves Lda et al. 2011). In their study, Salazar et al. (2013) used highly sensitive LC-MS approaches to identify 20 salivary human proteins. Salivary MMP-8, MMP-9, α_2-macroglobulin, S100-P protein and complement C3 were among the identified proteins, they demonstrated more than 1.5-fold difference in their abundance and they were higher in periodontitis patients as compared to healthy controls. This finding confirmed previous studies which used proteomics and approaches other than proteomics to identify the same proteins as potential salivary biomarkers for periodontitis (Kojima et al. 2000; Zhang et al. 2009; Haigh et al. 2010; Choi et al. 2011; Heo et al. 2011). Using gene ontology analysis, and Ingenuity pathway analysis, Salazar et al. (2013) demonstrated that the identified salivary proteins were mostly related to the acute phase signalling pathway and regulation of inflammatory response in periodontitis. With an exception for the S100-P protein which demonstrated the highest abundance and strongest difference (fold change 2.4), Salazar et al. (2013) found that only small differences in the levels of

the identified salivary proteins, were enough to differentiate periodontitis patients from healthy subjects, a finding that was in agreement with other studies which used 2-D PAGE and MS techniques (Wu et al. 2009; Haigh et al. 2010; Chan et al. 2012; Range et al. 2012).

However, despite the fact that various proteomic techniques are able to detect wide range of proteins and biomarkers in saliva and GCF samples, issues such as sensitivity, recovery and reproducibility have been reported and thought to be limitations to obtain reliable data. To overcome such challenges, new protocols and advanced alternative technologies have been used and are always improving. It is believed that these new protocols and technologies will optimize the biomarkers detection and the generated data (Vitorino et al. 2012; Juncker et al. 2014).

1.4.2 Advances in saliva proteomics

The salivary proteomic studies generated a huge amount of data, and dealing with this data was indeed a challenge for most of the researchers as they encountered difficulties such as the inability to interface and cross-reference data obtained from saliva proteomics with that derived from other studies including transcriptomics, genomics and metabolic studies (Ai et al. 2012). Bioinformatics is one of the key advances in the field of proteomics that may play a significant role in the investigation, exploration, and utilization of data obtained from proteomics and related studies, which will eventually help in the identification of novel salivary biomarkers (Ai et al. 2012; Rosa et al. 2012; Taylor 2014). Furthermore, the emergence and development of what is known as salivomics knowledge database (SKB), enabled the researchers to overcome challenges in data exchange and interpretation (Hu et al. 2006; Ng et al. 2006; Huang and Zhu 2009; Takeda et al. 2009; Ai et al. 2012; Wong 2012). SKB can be defined as “a data management system and web resource constructed to support saliva diagnostics research” (Ai et al. 2012). Which means that SKB is enriched with data derived from saliva proteomics, transcriptomics, metabolomics and other approaches. Therefore, bioinformatics and SKB provide information about salivary proteins which may encourage the invention and development of individualized diagnostic approaches which could be used in the dental clinics as well as domestically (Ai et al. 2012; Wong 2012).

New systems such as microfluidics, micro-electromechanical and nano-electro-fluidic systems (MEMS and NEMS) have been introduced in the last decade and are under continuous development, these systems raised the sensitivity and specificity of salivary diagnostics and offer the chance for pre-symptomatic diagnosis (Wong 2006). These advanced systems have been introduced in various biosensors, the MEMS-NEMS based biosensors are supplied with multiple probes for various proteins and nucleic acid targets which enable simultaneous and rapid detection of multiple salivary components (Wong 2006). One of the major advantages of these sensors is that they enable the use of minute amount of saliva sample with an easy and rapid measurement of salivary components (Wong 2006; Herr et al. 2007a). These advanced technologies encouraged the invention of miniaturized “lab-on-a-chip” chairside and handheld devices suitable for both clinical and domestic use such as point-of-care (POC) devices (Wong 2006; Herr et al. 2007a; Fuentes et al. 2014; Taylor 2014). The invention of POC devices is one the novel advances in saliva diagnostics, these devices hold the promise for rapid and simultaneous measurement of multiple salivary biomarkers with the ability for data storage, exchange and transmission. Hence, POC devices enable both the clinicians and the patients to assess the status and progression of periodontal diseases as well as the efficacy and response to treatment (Wong 2006; Christodoulides et al. 2007; Herr et al. 2007b; Taylor 2014). In addition to their clinical and domestic use, POC devices can be used in epidemiological surveys especially in remote and impoverished areas where laboratories, sample storage and transport facilities may not be available or accessible (Taylor 2014).

However, any advanced technology or device such as POC devices must be commercially attractive (Urdea et al. 2011). Therefore, these developed technologies and devices must be ultrasensitive and ultra-specific in detecting and measuring multiple diseases biomarkers, rapid, high throughput, automated, portable, miniaturized, easy to use especially by patients, and most importantly with reasonable cost (Herr et al. 2007a; Taylor 2014).

1.5 Aims and objectives

1.5.1 Aims of the study

1. To identify novel salivary biomarkers that may predict the onset of periodontal disease.
2. To investigate salivary biomarkers that can be used to indicate the severity of chronic periodontitis.
3. To characterize novel salivary biomarkers that may predict the clinical course of periodontitis in response to non-surgical treatment.
4. To compare biomarkers in saliva and gingival crevicular fluid of periodontitis patients

1.5.2 Objectives

1. Identify novel salivary biomarkers by proteomic analysis of saliva from periodontitis patients using antibody arrays, along with the interrogation of the existing data from previous transcriptomic and proteomic studies.
2. Characterization of these novel biomarkers in saliva of periodontitis and gingivitis patients using ELISA.
3. Correlate the levels of the identified salivary biomarkers with the clinical parameters of gingivitis and periodontitis utilizing study databases.
4. Measure the biological activity of one of the identified biomarkers in saliva using fluorescent peptide digestion assay.
5. Assay biomarkers in GCF samples from periodontitis and gingivitis patients.
6. In vitro study of the identified biomarkers production by human gingival fibroblasts.

- **I. Gingival diseases**
  - **A. Dental plaque induced gingival diseases**
    - **1. Gingivitis associated with dental plaque only**
    - **2. Gingival diseases modified by systemic factors**
      - **a. Associated with the endocrine system**
      - **b. Associated with blood dyscrasias**
    - **3. Gingival diseases modified by medications**
    - **4. Gingival diseases modified by malnutrition**
  - **B. Non-plaque induced gingival lesions**
    - **1. Gingival diseases of specific bacterial origin**
    - **2. Gingival diseases of viral origin**
    - **3. Gingival diseases of fungal origin**
    - **4. Gingival lesions of genetic origin**
    - **5. Gingival manifestations of systemic conditions**
      - **a. Mucocutaneus disorders**
      - **b. Allergic reactions**
    - **6. Traumatic lesions**
      - **a. Chemical injury**
      - **b. Physical injury**
      - **c. Thermal injury**
    - **7. Foreign body reactions**
    - **8. Not otherwise specified (NOS)**
- **II. Chronic periodontitis**
  - **A. Localized**
  - **B. Generalized**
- **III. Aggressive periodontitis**
  - **A. Localized**

	B. Generalized
IV.	**Periodontitis as a manifestation of systemic diseases**
	A. Associated with haematological disorders
	B. Associated with genetic disorders
	C. NOS
V.	**Necrotizing periodontal diseases**
	A. Necrotizing ulcerative gingivitis (NUG)
	B. Necrotizing ulcerative periodontitis (NUP)
VI.	**Abscesses of the periodontium**
	A. Gingival abscess
	B. Periodontal abscess
	C. Pericoronal abscess
VII.	**Periodontitis associated with endodontic lesions**
VIII.	**Developmental or acquired deformities and conditions**
	A. Localized tooth-related factors that modify or predispose to plaque-induced gingival diseases or periodontitis
	B. Mucogingival deformities and conditions around teeth
	C. Mucogingival deformities and conditions on edentulous ridges
	D. Occlusal trauma

Table 1.1: Classification of periodontal diseases.

Demonstrating the classification of periodontal diseases and conditions by the International Workshop for the Classification of Periodontal Diseases and Conditions (1999) and the American academy of periodontology. As a general guide, the extent of the disease can be characterized as localized when ≤ 30% of sites involved and generalized when >30% of sites involved. The severity of periodontitis can be characterized on the basis of the CAL into: slight or mild = 1 or 2 mm CAL, moderate = 3 or 4 mm CAL, and severe = ≥5 mm CAL, (Armitage 1999; Highfield 2009).

Case definition	5th European Workshop in Periodontology criteria	AAP criteria
Incipient or moderate periodontitis	Presence of proximal attachment loss of ≥ 3 mm in ≥ 2 non-adjacent teeth.	Presence of ≥2 interproximal sites with CAL of ≥4 mm (not on same tooth) or ≥2 interproximal sites with PD ≥5 mm (not on same tooth).
Substantial or severe periodontitis	Presence of proximal attachment loss of ≥5 mm in ≥30% of teeth present.	Presence of ≥2 interproximal sites with CAL of ≥6 mm (not on same tooth) and ≥1 interproximal site with PD ≥5 mm.

Table 1.2: Periodontitis case definition.

Demonstrating the periodontitis case definition by 2 studies. The first study presents the periodontitis case definition by the 5th European Workshop in Periodontology (Tonetti and Claffey 2005), measuring the CAL at the proximal sites of non-adjacent teeth**.** The second study (Page and Eke 2007), presents the periodontitis case definition by the AAP, measuring the CAL and the PD at the interproximal sites of more than 1 tooth.

Case	Definition
No periodontitis	No evidence of mild, moderate, or severe periodontitis
Mild periodontitis	≥2 interproximal sites with CAL ≥3 mm, and ≥2 interproximal sites with PD ≥4 mm (not on same tooth) or one site with PD ≥5 mm
Moderate periodontitis	≥2 interproximal sites with CAL ≥4 mm (not on same tooth), or ≥2 interproximal sites with PD ≥5 mm (not on same tooth)
Severe periodontitis	≥2 interproximal sites with CAL ≥6 mm (not on same tooth) and ≥1 interproximal site with PD ≥5 mm

Table 1.3: Update on the periodontitis case definition.

Demonstrating the update on the periodontitis case definition by the CDC-AAP. The criteria for the moderate and severe periodontitis remain same as the previous case definitions, this updated case definition also measures the CAL and the PD at the interproximal sites of more than 1 tooth for the definition of mild periodontitis, and the third molars were excluded (Eke et al. 2012).

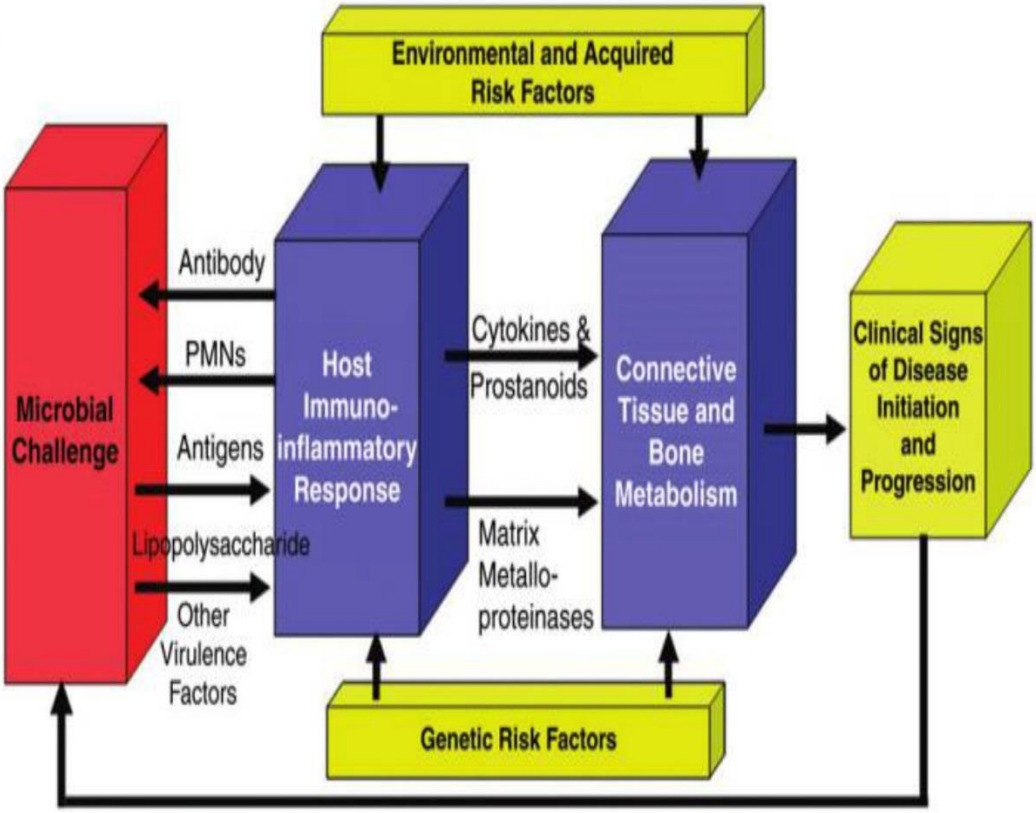

Figure 1.1: The pathogenesis of periodontal diseases.

As multi-factorial diseases, many factors contribute to the pathogenesis of periodontal diseases. The dental biofilm bacterial antigens and products such as LPS and other virulence factors represent the microbial challenge that stimulates and initiates the host inflammatory response. The PMNs which is the first line of defence as well as the macrophages and dendritic cells try to remove this bacterial challenge, these innate immunity cells carry receptors that recognize the bacterial antigens (adaptive immune response) present them to the immune system and release cytokines which stimulates the T cells (cell mediated response) and the B cells (humoral response to produce antibodies that attack bacteria). Furthermore, the bacterial challenge stimulates the inflammatory and local periodontal cells produce pro-inflammatory mediators such as cytokines and prostanoids which regulate the inflammatory process, attract more inflammatory cells, stimulate the production of enzymes such as matrix metalloproteinases and activate the osteoclastic activity which all lead to the destruction of both the periodontal connective tissue and alveolar bone. These responses are presented clinically by bleeding, periodontal pockets, loss of attachment, and finally loss of teeth. Moreover, the sequence of these events are affected by risk factors which may modify or exaggerate the host immune response and periodontal disease pathogenesis, these factors may be local environmental, systemic diseases, or genetic factors (Page and Kornman 1997; Kornman 2008).

Chapter 2 Materials and Methods

2.1 Clinical studies

Saliva and gingival crevicular fluid (GCF) samples assayed in this research were obtained from three independent clinical studies carried out as part of previous projects by the periodontology research group at the School of Dental Sciences/ Newcastle University. Details of these studies are described below.

2.1.1 Ethical approval

Each clinical study included in this research was ethically approved. Clinical study A was ethically approved by the Newcastle and North Tyneside 1 Research Ethics Committee (REC) reference (12/NE/0396). Clinical study B was ethically approved by the Newcastle and North Tyneside 1 REC reference (09/H0905/49). Clinical study C was ethically approved by the Sunderland Local REC reference (06/Q0904/8). The application to the ethics committees enclosed a protocol for each study, which highlighted the possible ethical concerns related to the study. The saliva and GCF samples were collected purely for research purposes and would otherwise not be collected. The purpose and reason for collecting samples was made clear to the prospective participants in the information sheet. The periodontal examination and treatment provided as part of the study, constituted routine clinical care. Furthermore, all participants in the study were given information and instructions on how to better maintain their oral health. All data recorded and samples collected were stored securely and anonymously, using a coding system. The information that was generated as part of this research study did not have an impact on the patients' clinical care and treatment other than that relating to any required periodontal clinical management.

2.1.2 Consent

At the first visit of each clinical study, written informed consent was obtained from all patients and healthy control volunteers. Here, the clinician confirmed that the patient had understood the written information sheet they had received about the study. Subsequently, the background and aims of the study were verbally confirmed by the clinician, moreover, all potential benefits and risks that the participants may

encounter by their involvement in the study were explained by the clinician. All participants were offered the chance to ask any question related to the study, as well as, the option to refuse the participation or withdraw from the study at any time. Participants who verbally accepted to participate in the study were asked to sign two copies of the consent form, one of which was kept in the patients'/volunteer case report form and the other copy was given to the patient.

2.1.3 Study groups

The studies were carried out and participants recruited at the Dental Hospital, School of Dental Sciences/Newcastle University (Figure 2.1, Figure 2.2 & Figure 2.3). All participants met the criteria of being adult males or females of 18-65 years of age, had a minimum of 20 natural teeth (excluding 3rd molars), were non-smokers and were not pregnant or nursing females. Subjects were excluded if they had crown, bridge, or rampant caries. Subjects with dentures (except the edentulous group in study B), who had a history of oral or systemic disease (such as xerostomia, Sjögren's syndrome, mucocutaneous and vesicobullous disorders, diabetes, systemic infectious disease), patients on treatment that my interfere with study (such as head and neck radiotherapy, systemic corticosteroids, long term use of phenytoin, cyclosporine, coumarin, warfarin, and heparin), who had extensive dental/orthodontic treatment, who had dental implants, cardiac pacemakers or automatic implanted cardiac defibrillators were also excluded. The edentulous group included in study B, were edentulous for more than 1 year with healthy oral tissues.

Clinical study A

This study was carried out on patients with chronic periodontitis and healthy control volunteers. During the visit 1 (the "baseline" assessment, one month before start of the study proper) the consent form, medical/dental history, and saliva samples were obtained for all participants. Oral/dental examination, and periodontal screening were carried out at this visit. At visit 2 (month 0), medical/dental history updated, saliva sampling, and periodontal assessment, were carried out for all participants. After sampling and periodontal assessment, non-surgical treatment was offered for periodontitis patients, whereas, healthy subjects had scaling and polishing and they then exited the study. Visits 3 (month 1), 4 (month 2), 5 (month 3), 6 (month 6) for all periodontitis patients, involved the previous steps with non-surgical treatment as maintenance. The study was ended at visit 6. Saliva samples involved in the present

research, were obtained at visits 1 (Baseline), 2, and 6. Healthy saliva samples comprised 2 sets. The first set comprised 34 saliva samples obtained at visit 1 (Baseline): some of these samples were used in proteome profiler arrays (PPA) assays and all were analysed for uPAR levels using ELISA. The second set comprised 40 saliva samples obtained at visit 2 of the study and were analysed for: uPAR, uPA, and VDBP levels using ELISA, as well as, uPA activity using activity assay. Saliva samples from periodontitis patients included 3 sets. The first set comprised 30 saliva samples from patients with untreated chronic periodontitis obtained at visit 1 (Baseline): some were used in the PPA assays and all were analysed for uPAR levels using ELISA. The second set comprised 45 pre-treatment saliva samples from 45 periodontitis patients obtained at visit 2 of the study and were analysed for: uPAR, uPA, and VDBP levels using ELISA, as well as, uPA activity using activity assay. The third set comprised 45 post-treatment saliva samples obtained from the same 45 periodontitis patients at visit 6 of the study which were analysed for uPAR, uPA, and VDBP levels using ELISA. (Figure 2.1).

Clinical study B

This study involved edentulous subjects, healthy volunteers, gingivitis patients, and periodontitis patients. The study started at visit 1 (day 0) for all participants. In this visit the informed consent form, the medical/dental history, and saliva samples were obtained. An oral/dental examination and periodontal screening were also carried out at this visit. At visit 2 (5-30 days from day 0) the same procedures as visit 1 were repeated and it was the last visit for the edentulous, healthy, and gingivitis participants. Visit 2 was followed by 1-2 non-surgical treatment visits (within 2 weeks of visit 2), and follow up/recall visits (3, 6, and 9 weeks after treatment), for the periodontitis patients. Finally, visit 3 (12±2 weeks after treatment) at which same steps as visit 1 and 2 were repeated again, and the study was completed. Five groups of saliva samples obtained at visit 2 and two groups obtained at visit 3 of study B, were assayed for uPA levels and uPA activity. The groups included: 26 samples from edentulous subjects, 29 samples from dentulous healthy subjects, and 25 samples from gingivitis patients, all obtained at visit 2 of the study. Saliva samples from mild/moderate periodontitis patients comprised 31 pre-treatment samples obtained at visit 2, and 31 post-treatment samples obtained at visit 3. Lastly, saliva

samples from advanced periodontitis patients comprised 27 pre-treatment samples obtained at visit 2, and 27 post-treatment samples obtained at visit 3. (Figure 2.2).

Clinical study C

The study involved investigations carried out on 2 groups of participants. The first group comprised periodontally healthy volunteers, gingivitis patients, and periodontitis patients all with type-2 diabetes mellitus. The second group comprised periodontally healthy volunteers, gingivitis patients, and periodontitis patients all were systemically healthy. The clinical protocol of the study included 7 visits. All participants were assessed at a pre-treatment screening visit (visit 1), in which the informed consent form, medical/dental history, and samples (saliva, GCF, and serum) were obtained. Furthermore, oral, dental, and periodontal examinations were carried out at this visit. At the end of pre-treatment visit, the healthy and gingivitis participants in both groups were not seen again. Two months after the pre-treatment screening (visit 1), non-surgical periodontal treatment was offered for the periodontitis patients at visit 2 which was the start of the study proper (month 0 time point). Visit 3 (week 3), and visit 4 (week 6), both involved only oral examination and prophylaxis. At visit 5 (month 3), and visit 6 (month 6) the same steps as visit 1 were repeated with prophylaxis and additional treatment if required. The same steps as visits 5 and 6 were followed at visit 7 (month 12), which was the end of the study. From clinical study C, only GCF samples obtained from the systemically healthy group (not from the type-2 diabetes group) were assayed for uPA levels in the present research. The samples were divided into 2 sets. The first set of samples comprised 3 groups: 7 healthy GCF samples, 13 gingivitis GCF samples, and 9 pre-treatment periodontitis GCF samples, all obtained at the pre-treatment screening (visit 1) of the study. The second set comprised 2 groups of post-treatment GCF samples obtained from the same periodontitis patients, including: 9 post-treatment samples at visit 5, and 9 post-treatment samples at visit 6 of the study. (Figure 2.3).

2.1.4 Clinical assessment

At the pre-treatment or first visit of each study, all patients and subjects received a full-mouth periodontal screening, which included recording periodontal disease indices at 6 sites per tooth. When clinically indicated, radiographs were taken for participants and, thereafter, clinical and radiographic data were used to confirm the periodontal diagnosis based on the diagnostic criteria (Table 2.1). Following the

confirmation of diagnosis at this visit, patients were screened again on treatment and post-treatment visits.

Periodontal disease indices

A number of periodontal indices were used in the three clinical studies for the clinical assessment of participants at the first visit and to monitor the disease progression and response to treatment in the following visits. These periodontal indices used were: modified gingival index (MGI), probing or pocket depths (PD), bleeding on probing (%BOP), and clinical attachment loss (CAL). The University of North Carolina (UNC) 15 probe (Dentsply, Addlestone, UK) was used for the indices measurements.

Modified gingival index: The modified gingival index by (Lobene et al. 1986), was used for the clinical assessment of gingival inflammation as follows:

0 Absence of inflammation

1 Presence of mild inflammation: slight change in colour, little change in texture of any portion but not the entire gingival margin or papillary gingival unit.

2 Mild inflammation: criteria as above but involving the entire marginal or papillary gingival unit.

3 Moderate inflammation: glazing, redness, oedema and/or hypertrophy of the marginal or papillary gingival unit.

4 Severe inflammation: marked redness, oedema and/or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

In both clinical studies A and B, the above scores were recorded by visual examination for 6 sites per tooth, the mean of the scores for each tooth was calculated, and then the mean of the mean scores was calculated for all teeth examined. In clinical study C, the scores were also recorded by visual examination for 6 sites per tooth, the mean of the scores for each tooth was calculated, and then the mean of the mean scores was calculated for the four target teeth from which GCF samples were obtained.

Probing depth: Using the UNC periodontal probe, probing depths or periodontal pockets for the three clinical studies were measured at 6 sites per tooth for all standing teeth except 3rd molars. The periodontal pocket represents the distance from the gingival margin to the base of the pocket measured by millimetres (mm). The probe was inserted into the gingival sulcus and advanced apically, along the long axis of the tooth, until resistance of the tissue was felt at the base of the gingival sulcus or pocket. The PD measurements were recorded by direct visualisation of the markings on the probe. The PD final score was calculated in a similar manner to MGI.

Bleeding on probing: Following the measurement of periodontal pockets in one aspect of a quadrant (such as buccal aspect of lower left quadrant), the probing depth sites (periodontal pockets) were re-examined to determine whether post-probing bleeding occurred. In a similar manner to probing depth, bleeding status was recorded at 6 sites per tooth depending on the presence (score 1) or absence of bleeding (score 0) from the base of the pocket following probing. The final score was presented as percentage of the bleeding sites for all teeth examined (%BOP), for example if there were 75 bleeding sites for 20 teeth then the final score will be as following:

6 sites $\times$ 20 teeth=180, 75/180= 0.417 $\times$ 100= 41.7 %BOP

Clinical attachment loss (CAL): This is the sum of probing depth and recession, hence, it represents the distance from the cemento-enamel-junction (CEJ) to the base of the periodontal pocket measured in mm. Again, CAL was measured at 6 sites per tooth for all teeth present, excluding 3rd molars. Then the total score for each patient or subject was used in the study.

Before calculating the CAL, recession was measured using the UNC probe. Recession represents the distance from the CEJ to the gingival margin measured by mm. It was recorded during PD measurement whilst the probe was inserted into the gingival sulcus. When the CEJ was located above the gingival margin, recession was recorded by direct visualisation of the probe markings. When the CEJ was located below the gingival margin (such as in case of false pocketing), the position of the CEJ was estimated in relation to the gingival margin and a negative recording was

made. Recession was recorded at 6 sites per tooth for all standing teeth excluding the 3rd molars.

2.1.5 Saliva and GCF sampling

Saliva samples

In clinical study A, whole unstimulated saliva samples were obtained from study participants at least one hour after their last food or drink intake and, at least one hour after their last oral hygiene measure (tooth brushing, flossing, and/or mouth rinse). The participants were seated in the dental chair, avoiding any noise or distraction. Neither stimulation nor examination of the oral tissues and mouth were carried out during sample collection. A pre-labelled, sterile, 50 ml polypropylene tube was given to each participant and the participant was instructed to simply drool saliva into the tube until approximately 5-10 ml of saliva were collected. The estimated collection time was 5-10 minutes, time and date of collection were recorded. The samples were placed on ice and taken to the lab for processing. Saliva samples were centrifuged (at 1500g, 15 minutes, 4°C), the supernatant was pipetted into aliquots (to avoid multiple freeze-thaw cycles), snap frozen in liquid nitrogen and then stored at -80°C till use in different assays.

In clinical study B: The same steps of saliva sampling as in study A were followed in regard to participants seating, time of collection, processing and storage of samples. The only difference was that the samples were stimulated, each participant was instructed to place a sterile marble into the mouth and asked to roll it gently around the moth to stimulate the oral musculature, promote mixing of GCF and saliva, and stimulate salivary flow.

GCF samples

GCF samples were collected using Periopaper strips (Oraflow Inc., New York) and the volume quantified using a calibrated Periotron 6000 (Preshaw et al. 1996; Wassall and Preshaw 2016). As recommended by the manufacturer, the Periotron was allowed to ‘warm up’ before use, and then zeroed with a blank (dry) Periopaper. The reading dial was adjusted until the digital display indicated zero. To minimise contamination by blood, GCF samples were collected prior to periodontal pockets probing. At the first visit (pre-treatment screening visit), 4 samples were collected from each participant, from the mesio-buccal aspects of the four 1st molars. If the 1st

molar was absent in a quadrant, the 2nd molar was used, then the 2nd premolar, then the 1st premolar, then the canine or incisor teeth (the sampled teeth were designated target teeth). To collect a sample, first the site was isolated with cotton rolls and a saliva ejector, then dried by a gentle stream of air. If present, supra-gingival plaque was carefully removed with a curette prior to sampling. A Periopaper was placed carefully into the gingival sulcus till mild resistance was felt and was held in place for 30 seconds. After collection of GCF, Periopapers were transferred immediately to the Periotron jaws to minimise evaporation. Periopapers were carefully positioned in a standardised position between the jaws, so that the black line on the paper was at the outer rim of the jaw plate. The GCF volume (in Periotron units) was recorded when 'mode II' illuminated on the Periotron display. The Periopaper was then placed into a sterile plastic 0.5 ml micro-tube (Sarstedt, Leicester, UK) containing 150µl autoclaved and filtered PBS (Dulbecco's phosphate buffered saline). The GCF samples were kept on ice at the chair side and transferred, within 20 minutes of sampling, to the laboratory and frozen at -80°C (Cutler et al. 1999) till subsequent elution & analysis. For longitudinal study of periodontitis patients, the same steps were followed to collect GCF samples from the same 4 sites at months 3, 6 and 12. GCF samples from the Periopaper required elution, which was executed by thawing the 4 sites samples on ice for 15 minutes and then 50µl of 1% bovine serum albumin (BSA) was added. GCF samples were centrifuged (using Sigma 3K10 centrifuge) at 300 rpm for 60 minutes at 4 °C and then at 12000 rpm for 2 minutes at 4 °C. The Periopapers were then removed with college tweezers, with the ends of the tweezers being rinsed with PBS between samples. The final 4 sites GCF samples for each participant were stored at -80°C till use in different assays. In the present research due to the minimum volumes left from the previous projects in the periodontology group, the 4 sites GCF samples were pooled into one sample for each participant.

2.2 Laboratory investigations

2.2.1 Materials, reagents, and equipment

The plastic-ware used in all assays was supplied by Greiner Bio One (Stonehouse, UK). All ELISA kits (Quantikine and DuoSet), PPA kits, and reagents were purchased from R & D Systems (Abingdon, UK). uPA activity fluorometric assay kits, in addition to cell culture reagents and media, were purchased from Sigma-Aldrich (Dorset, UK). For PPA assays, 3D rocking platform shaker STR (from Stuart Scientific,

Staffordshire, UK) was used in incubations and washing steps. An autoradiography film cassette, and highly sensitive autoradiography film (from Amersham Hyperfilm ECL, Buckinghamshire, UK), was used to take radiographs of the array membranes. Image analysis software (Gene Tools software Syngene, Cambridge, UK), was used for the analysis of the pixel intensity (PI) of the radiographic images.

In ELISA assays, phosphate buffered saline (PBS), reagent diluent (RD), and calibrator diluent (CD) (R & D systems) were used for the dilution of the standards and antibodies. Wash buffer (0.05%Tween in PBS) (R & D systems) was diluted by 1:25 in distilled water, and an auto-washer from BioTek Instruments Ltd (Swindon, UK) were used to wash the plates as required. A horizontal orbital microplate shaker (from IKA-Labortechnik, Staufen im Breisgau, Germany) set at 500±50 rpm, was used during the incubations of Quantikine ELISA plates. Optical density for each assay was determined using Synergy HT microplate reader from BioTek Ltd. The plate reader was set to 450 nm with wavelength correction set to 540 or 570 nm. Standard curves were generated and results were calculated using Software Gen5 1.11 from BioTek Ltd. For uPA activity assay, same plate reader was used to read the fluorescence of the activity assay. 96 well flat-bottom black plates from Greiner Bio One were used for the uPA activity assay.

The recombinant proteins used in cell culture included: human recombinant IL-1β (R&D Systems) which was prepared at 25 µg/ml in 0.1% w/v endotoxin-free bovine serum albumin (BSA) in Dulbecco's phosphate buffered saline (PBS). IL-1β stock aliquots stored at -80 °C, further dilutions prepared as needed using serum-free medium (SFM) "SFM was Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DMEM) from Sigma-Aldrich, added to it L-glutamine (2 mM), penicillin (200 U/ml), and streptomycin (200 µg/ml), without foetal bovine serum (FBS)". Lipopolysaccharides (LPS) used for the stimulation of human gingival fibroblasts (hGFs) were: *Escherichia coli* (*E. coli*) LPS strain 0111:B4 (Invivogen, Nottingham, UK) which was prepared as a 5 mg/ml stock solution in sterile filtered water, and *Porphyromonas gingivalis* (*P.gingivalis*) ultrapure TLR4 agonist LPS (Invivogen) prepared as a 1 mg/ml stock solution in sterile filtered water. LPS stocks were stored as 20 µl aliquots at -20 °C, further dilutions were prepared with sterile filtered water and SFM as required.

2.2.2 Cell culture

A Class II hood (laminar flow unit-BioAir, Biological Instrumentation Services, Manchester, UK), was used for all cell culture procedures. Sterile CELLSTAR (Greiner Bio One) cell culture plastic-ware including: T75 and/orT25 flasks were used for cell growth, and 6-multi-well plates were used for cells stimulation. All reagents used were sterile and endotoxin-free. Cells were incubated in a 5% CO2 atmosphere at 37 °C unless otherwise indicated. DMEM+ medium (SFM supplemented with 10% v/v FBS) was used for cell growth. For cells stimulation the SFM medium was used. A Ca2+/Mg2+ free Dulbecco's phosphate buffer saline (PBS) was used for wash of cells. Trypsin ethylene diamine tetra acetic acid (EDTA) solution (supplied as a bottle of 500 ml of 1x solution of 0.5g porcrine trypsin and 0.2g EDTA. 4Na per litre of Hanks' balanced salt solution with phenol red, from Sigma-Aldrich) was used to detach cells from the bottom of growth flasks and/or multi-well plates. Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) 99.5% pure "cryo-protectant agent for the preservation of cells" was used in cell freezing media to protect cells from ice crystal induced injury.

The cells used in the cell culture experiments were primary hGFs cells previously prepared by Dr Rachel Williams from gingival tissue obtained from separate patients undergoing canine tooth exposure surgery at Newcastle Dental Hospital (Williams et al. 2016), this was approved by the National Research Ethics Service Committee North East (Newcastle and North Tyneside 2, REF: 07/Q1003/41). The cells were stored in cryo-vials in liquid nitrogen till use.

2.3 Assays methods

2.3.1 Proteome profiler arrays

The PPA works on the principle of two-site sandwich immunoassay, in which samples are incubated with membranes in an overnight procedure, and any captured analyte is detected by a cocktail of biotinylated antibodies specific for each array (Figure 2.4). A chemiluminescent substrate mix is used to demonstrate the positive spots on the membranes, to view these positive spots multiple radiographs are needed. Human proteome profiler protease array kits (ARY021, R & D systems) and human proteome profiler cytokine array kits (ARY022, R & D systems), were used to identify candidate biomarkers in whole unstimulated saliva of periodontitis patients.

The protease array membranes contain 34 capture antibodies in duplicate for a range of human proteases, whereas, the cytokines array membranes contain 102 capture antibodies in duplicate for human cytokines. Each PPA membrane has three pairs of positive reference spots on three corners, and a pair of negative reference spots next to the last protease on the protease detection membrane and on the lower right hand corner of the cytokine detection membrane (Figure 2.5). The reference spots are used for three reasons: firstly, as a positive control for the performance of the array as the positive reference spots will have signals while the negative reference spots will be blank, secondly, to help in the alignment of the overlay template, and finally to be used as a reference for the calculation of the relative density of the proteins spots.

The human protease PPA assay

Aliquots of saliva samples previously obtained from healthy subjects and chronic periodontitis patients from the baseline visit of study A, stored at -80°C, were analysed in the protease PPA assays. Each sample aliquot used once only to avoid repeated freeze-thaw cycles. All samples and reagents were prepared at room temperature (RT). Using flat-tip tweezers each protease PPA membrane with the identification number faced upward, was placed in a separate well of a 4-well multi-dish (supplied with each kit, R & D systems) in which 2 ml of array buffer 6 were pipetted per well. Array buffer 6 served as a block buffer. The membranes were incubated in the blocking buffer for 1 hour on the platform shaker at RT. While the membranes were blocking, the samples were prepared and adjusted with array buffers to a volume of 1.5 ml; thus,

0.5 ml of array buffer 4 + 300 µl of the sample + 700 µl of array buffer 6 were combined. According to the manufacturer, the sample volume should not be less than 100µl, adjusted to 1.5 ml with the 2 array buffers. Then, 15 µl of the specific biotinylated detection antibody cocktail (DAC) were added up to each prepared sample, mixed and incubated for 1 hour at RT.

By the end of blocking, the blocking buffer was aspirated, then the prepared sample/antibody mixtures were added, and incubated overnight on the platform shaker at 2-8°C in a refrigerator. According to the manufacturer's recommendations, overnight incubation is necessary for optimal sensitivity of the assay. After the

overnight incubation, the membranes were carefully removed and each membrane placed into an individual plastic container (such as sterile Petri dish) containing 20 ml of 1X wash buffer. Each membrane was washed at RT for 10 minutes on the platform shaker, for a total of 3 washes. After washing, the membranes were carefully removed from the washing containers, allowed to drain by blotting onto dry paper towels, then the membranes were placed into the multi-well dish containing 2 ml of streptavidin-HRP/well (diluted by 1:2000 in array buffer 6 immediately before use), protected from light by covering the multi-well dish with aluminium foil, and incubated for 30 minutes at RT on the shaker. The membranes were washed again, and allowed to drain carefully on paper towels. Each membrane was placed on a plastic sheet with the identification number facing up. 1 ml of the chemiluminescent reagent mix (CRM, prepared by mixing equal volumes of reagents 1 and 2 immediately before use), was added on each membrane ensuring complete coverage, and carefully covered by a top plastic sheet protector. Any air bubbles generated were gently smoothed out to ensure an even spread of the CRM on all corners of each membrane. The covered membranes were incubated for 1 minute protected from light with aluminium foil. Using paper towels, any excess of CRM was squeezed out. The top plastic sheet was removed then the remaining liquid was carefully wiped off using absorbent paper. Thereafter, radiographs were taken for the protease PPA membranes.

The human cytokines PPA assay

Aliquots of saliva samples previously obtained from healthy subjects and chronic periodontitis patients from the baseline visit of study A, stored at -80°C, were analysed in the cytokine PPA assays. Each sample aliquot used once only to avoid repeated freeze-thaw cycles. All samples and reagents were prepared at RT. Using flat-tip tweezers each cytokine PPA membrane with the identification number faced upward, was placed in a separate well of the 4-well multi-dish in which 2 ml of array buffer 6 were pipetted per well. Array buffer 6 served as a block buffer. The membranes were incubated in the blocking buffer for 1 hour on the platform shaker at RT. While the membranes were blocking, the samples were prepared and adjusted with array buffer 6 to a volume of 1.5 ml; thus,

200 µl of the sample + 1300 µl of array buffer 6 were combined.

The blocking buffer was aspirated, then the prepared samples were added to each well, and incubated overnight on the platform shaker at 2-8°C in refrigerator. According to the manufacturer's recommendations, overnight incubation is necessary for optimal sensitivity of the assay. After the overnight protection, the membranes were carefully removed and each membrane placed into an individual plastic container containing 20 ml of 1X wash buffer. Each membrane was washed at RT for 10 minutes on the platform shaker, for a total of 3 washes. After washing, the membranes were carefully removed from the washing containers, allowed to drain by blotting onto dry paper towels, and placed into the multi-well dish containing 1.5 ml of DAC/array buffers mixture per well which was prepared immediately before use as following:

First, for 4 wells, 6 ml of array buffer 4/6 mixture was prepared in 1:2 ratio
2 ml of array buffer 4 + 4 ml of array buffer 6= 6 ml array buffer 4/6 mixture

Then, 30 µl of DAC needed for each 1.5 ml of the array buffer mixture, therefore 120 µl of DAC were added up to the 6 ml of array buffer mixture.

The membranes were incubated for 1 hour at RT on the platform shaker. Then, the membranes were washed as described before. After washing, and blotting onto paper towels to drain, the cytokine membranes were placed into the multi-well dish containing 2 ml of streptavidin-HRP/well (diluted by 1:2000 in array buffer 6 immediately before use), protected from light and incubated for 30 minutes at RT on the shaker. The membranes were washed again, allowed to drain carefully on paper towels. Each membrane was placed on a plastic sheet with the identification number facing up. 1 ml of the CRM mix (prepared by mixing equal volumes of reagents 1 and 2 immediately before use), was added on each membrane ensuring complete coverage, and carefully covered by a top plastic sheet protector. Any air bubbles generated were gently smoothed out to ensure an even spread of the CRM on all corners of each membrane. The covered membranes were incubated for 1 minute protected from light. Using paper towels, any excess of CRM was squeezed out. The top plastic sheet was removed then the remaining liquid was carefully wiped off using absorbent paper. Thereafter, radiographs were taken for the cytokine PPAs membranes, except the first cytokine PPA assay in which images were captured for the membranes.

PPA assay imaging

In the first cytokine PPA experiment, after incubation with the CRM, images for both the healthy control sample and the periodontitis sample membranes were captured using G:BOX Chemi XL viewer (Syngene, UK), at the Institute for Cell and Molecular Biosciences, Newcastle University. The pixel intensity (PI) of the resultant spots were quantified by the image analysis software.

Radiography of PPAs

In the majority of experiments, PPA assay membranes were analysed using radiography. Thus, after incubation with the CRM, the protease and cytokine membranes were wrapped carefully with plastic protective sheets with the identification numbers facing up, placed in autoradiography film cassette, to be exposed to a highly sensitive autoradiography film for 1-10 minutes (the radiography procedure was carried out in a dark X-ray room at the Institute for Cell and Molecular Biosciences, Newcastle University). It was found that 4-5 minutes exposure time was the best time to develop clear signals on the radiographs. These signals were identified by placing transparency overlay template on the radiograph, aligning it with pairs of positive reference spots on three corners of each array. Finally, the radiographs were scanned, and the PI of each spot on the resultant images was quantified using the image analysis software (section 2.2.1).

Quantification of hybridisation on PPA membranes

The PI values of the resultant spots on the images were exported to an excel spreadsheet file for calculation. The mean PI of each doubled spot was subtracted from the mean PI of the duplicate negative control spot of each array membrane (background correction). The resultant PI values from the protease and cytokine PPAs from each sample and control array membranes, were compared to each other to determine the relative changes in the analytes, this was achieved by the means of PI values fold change, p-value of the logarithm-fold change, and bar graphs demonstrating the high and low protein expression.

2.3.2 Enzyme linked immunosorbent assay (ELISA)

Following the identification of the candidate biomarkers and to confirm the results of PPAs, human urokinase receptor (uPAR) (DuoSet kits DY807, R & D systems), human urokinase (uPA) (Quantikine kits DUPA00, R & D systems) and human

vitamin D binding protein (VDBP) (Quantikine kits DVDBP0, R & D systems) were used to measure the levels of these mediators in saliva and GCF samples obtained from the three clinical studies. For assays of cell culture supernatants, the following assays were used: uPAR DuoSet, human uPA DuoSet (DY1310, R & D systems), and human total matrix metalloproteinase 1 (MMP-1) DuoSet (DY901, R & D systems). All assays were performed at room temperature. For each assay, new aliquots of saliva or GCF samples were used to avoid repeated freeze-thaw cycles. Along with the standards, saliva and GCF samples were diluted as appropriate using reagent diluent (RD) for DuoSet ELISAs, or in calibrator diluent (CD) for Quantikine ELISAs.

DuoSet ELISA assays were carried out according to the manufacturer's protocol on benchtop. Briefly, plates were coated with 100 µl/well of capture antibody (Cab) diluted in PBS (concentrations were: 4 µg/ml for uPAR, 1 µg/ml for uPA, and 2 µg/ml for MMP-1), incubated overnight, washed next day with 400 µl/well of wash buffer (section 2.2.1) for a total of 3 washes using auto-washer (BioTek) to remove any unbound antibodies. The plates were blocked with 300 µl/well of RD for 1 hour. After washing, 100 µl/well of standards and samples were dispensed into the plates and incubated for 2 hours (samples and standards were diluted in RD as required, standards dilution series were: 2000-31.3 pg/ml for uPAR, 4000-62.5 pg/ml for uPA, and 10000-156.3 pg/ml for MMP-1). During incubation, any uPAR or uPA or MMP-1 in the samples is bound by the Cab. The plates were washed again to remove any unbound proteins, and then 100 µl of biotinylated detection antibody (Dab) diluted in RD (concentrations were: 200 ng/ml for uPAR, 400 ng/ml for uPA, and 100 ng/ml for MMP-1) were added per well, and incubated for 2 hours. Following another washing step to remove any unbound antibodies, 100 µl/well of streptavidin-horseradish peroxidase (HRP) solution (1:200 in RD, prepared immediately before use) were added to the plates, which were protected from light using aluminium foil and incubated for 20-30 minutes. During incubation, streptavidin-HRP bound to the biotinylated Dab. Any unbound streptavidin-HRP was removed by a further washing step, then 100 µl of substrate solution were pipetted per well (prepared immediately before use by mixing colour reagents A and B in a 1:1 ratio), protected from light and incubated for 20-30 minutes depending on the analyte. To stop the reaction, 50 µl/well of stop solution (2N Sulphuric acid, R & D systems) was added to the plates,

the colour of the substrate solution in the wells changed immediately from blue to yellow, when the colour change was not uniform or delayed or appeared green it was hastened by gentle tapping of the plates to ensure thorough mixing of the acid stop and substrate solutions in the wells (R & D systems). Once the colour changed, the plates were immediately read by the microplate reader to generate the standard curve (Figure 2.6, Figure 2.7, & Figure 2.8) and calculate the desired biomarkers concentrations in the samples (section 2.2.1).

Quantikine ELISA assays for both uPA and VDBP were also carried out according to the manufacturer's protocol. Briefly, the Quantikine assay plates are supplied with pre-coated antibodies to which 100 µl/well of assay diluent were added, followed by 50 µl of standards or samples (diluted as required in CD) and incubated on the microplate shaker (section 2.2.1) for 1 hour, during this time any uPA or VDBP present is bound by the specific immobilized antibody. The standard concentration ranges were: 2000-31.25 pg/ml for uPA, and 250-15.6 ng/ml for VDBP. The plates were washed with 400 µl/well of wash buffer (section 2.2.1) for a total of 4 washes using auto-washer (BioTek) to remove any unbound samples, then 200 µl/well of conjugate (HRP-linked antibody specific for each biomarker), were added to the plates and incubated for 2 hours on the shaker. After washing the plates to remove any unbound antibody, 200 µl of substrate solution were added per well (prepared immediately before use by mixing colour reagents A and B in a 1:1 ratio), plates incubated for 30 minutes on the benchtop protected from light with aluminium foil. The colour development was stopped by adding 50 µl per well of stop solution (2N Sulphuric acid, R & D systems), the colour of the substrate solution in the wells changed immediately from blue to yellow, when the colour change was not uniform or delayed or appeared green it was hastened by gentle tapping of the plates to ensure thorough mixing of the acid stop and substrate solutions in the wells (R & D systems). The intensity of the resultant colour was measured immediately by microplate reader to generate the standard curve (Figure 2.9 & Figure 2.10) and calculate the desired biomarkers concentrations in the samples (section 2.2.1).

ELISA validation assays

The aim of these assays is to validate an ELISA kit for samples other than specified by the manufacturer in order to secure the most accurate, reliable, and reproducible data (Jaedicke et al. 2012). Before carrying out an ELISA assay on the invaluable

saliva samples obtained from patients, it is necessary to perform these validation or quality control assays to certify the use of the assay with saliva samples. Even if the ELISA kit was verified by the manufacturer for the use on saliva, there is still a need to perform at least one of the validation assays before risking the study samples, such as testing volunteer samples to set a dilution factor at which the desired protein falls within the standard curve range and is possible to be detected by the assay. ELISA validation assays include: determination of optimal dilution, recovery and linearity measurements, intra- and inter-assay variations, and assay sensitivity. Validation assays were carried out on the uPA DuoSet, uPAR DuoSet, uPA Quantikine, and VDBP Quantikine kits.

Determination of optimal dilution: The concentrations of the desired mediators to be expected in saliva samples were completely unknown (i.e. not measured before), so samples were assayed in different dilutions (neat, 1:10, 1:100, and 1:500) in order to determine the optimal dilution factor. This gave a first indication if the assay will work with different samples, as well as important information relating to the most appropriate sample dilution for the rest of the validation assays.

Recovery and linearity measurements: Recovery works on the principle that whatever known amount added into the assay is what should be measured (i.e. recovered) in return as the result of the assay. The design of recovery assay was to test the recovery of a spiked saliva sample and a spiked control. The control was the reagent diluent in DuoSet ELISA and the calibrator diluent in Quantikine ELISA.

Linearity follows the same principle; however, uses different dilutions to assess recovery. The design of linearity assay was to test spiked, and unspiked samples along with spiked control, in four different dilutions 1:2, 1:4, 1:8 and 1:16 for each.

In both recovery and linearity, the control and sample were spiked with the same amount of the provided ELISA standard selected from or near to the middle range of the standard curve. Linearity was useful to investigate whether or not dilutions of samples yielded readings in the assay which were parallel to the standard curve. Linearity was also used to assess whether saliva samples have to be diluted differently because some might have high and some might have low concentrations of the protein of interest, that the dilution itself does not affect recovery in the assay.

A recovery between 80 and 120% is generally acceptable, which is an indication that the assay was suitable for the tested sample and will work properly with the study samples (R & D systems), (Jaedicke et al. 2012). All dilution factors should be considered when conducting the calculations.

The following formulas were used to calculate the recovery % for recovery assay:
For spiked sample: [(assay result for spiked sample - assay result for neat sample)/ (amount spiked)] ×100 neat sample=unspiked sample

For spiked control: (assay result for spiked control/ amount spiked) ×100

The following formulas were used to calculate the recovery % for the linearity assay:
For 1:2 spiked sample: (assay result for spiked sample/ assay result for 1:2 spiked sample) ×100
So on for 1:4, 1:8, and 1:16 dilutions.

For 1:2 unspiked sample: (assay result for unspiked sample/ assay result for 1:2 unspiked sample) ×100
So on for 1:4, 1:8, and 1:16 dilutions.
For 1:2 spiked control: (assay result for spiked control/ assay result for 1:2 spiked control) ×100
And so on for 1:4, 1:8, and 1:16 dilutions.

Intra-and Inter-assay variation:

Intra- and inter-assay variation measurements are often quoted in papers and give an indication of how reproducible data is within one assay and between assays performed on different days, respectively. Intra-assay variation employs the analysis of three different samples, assayed in triplicate on the same plate. Whereas, inter-assay variation employs the analysis of three different samples in three different assays (preferably on three separate days, if this is not possible, at least with three separate preparations of antibodies, samples and standards).

The intra- and inter-assay coefficient of variation (CV), calculated by the formula:
(standard deviation of assay results/ mean of assay results) ×100
CV value less than 10-15 is generally acceptable (Hanneman et al. 2011).

Assay sensitivity:

Values of for assay sensitivity are usually included in the ELISA kits datasheet and indicates the smallest concentration of the protein of interest the assay can detect in the type of sample used (e.g. saliva). Twenty replicates of the ELISA reagent diluent were assayed for the sensitivity step.

Assay sensitivity is defined as “the mean of assay results for the 20 zero standard replicates + 2 standard deviations of the mean” (Jaedicke et al. 2012). The resultant value represents an optical density value to be used in the standard curve equation to calculate the minimum concentration of the desired protein that can be detected by the ELISA.

2.3.3 Cell culture experiments

Cell culture experiments were performed to investigate the expression of both uPAR and uPA in the supernatants of hGFs stimulated with IL-1β, *E. coli* LPS, and *P. gingivalis* LPS. uPA biological activity was also measured in response to the mentioned stimulants. All cell culture experiments were carried out independently on cells obtained from each of 8 donors in total, and the cells were always used in passage 5-10. One preliminary IL-1β stimulation experiment was carried out on cells obtained from one donor as one replicate (T25 flask) for each of the stimulating concentrations and control. Three independent IL-1β stimulation experiments were carried out on cells obtained from 3 donors in a total of 10 replicates (wells) for each of the stimulating concentrations and control. Two independent *E. coli* LPS stimulation experiments were carried out on cells obtained from 2 donors in a total of 6 replicates (wells) for each of the stimulating concentration and control. Two independent *P. gingivalis* LPS stimulation experiments were carried out on cells obtained from 2 donors in a total of 6 replicates (wells) for each of the stimulating concentration and control. The donor variability of hGFs with respect to response to pro-inflammatory stimuli has been investigated in detail in our laboratory previously (Williams et al. 2016) and has been found to be minimal. All cell culture procedures and stimulating experiments were carried out as previously described by Williams (2015) and Williams et al. (2016). The concentrations used in the stimulating experiments were selected near to those used by the same two studies or in other dilutions to investigate the stimulatory effects of different concentrations, including

100 ng/ml of LPS instead of 10 ng/ml, and 5 ng/ml along with 0.05 ng/ml concentrations for IL-1β.

Primary hGF culture procedure

Cell culture procedures were carried out aseptically in a class II hood (section 2.2.1) in the cell culture lab within the oral biology lab, at level 7/school of dental sciences, Newcastle University. All cells were incubated in a 5% CO_2 atmosphere at 37 °C. The hGF cells were taken out of liquid nitrogen, thawed in a water bath at 37°C, then slowly pipetted into T75 flask containing 15 ml DMEM+ medium (section 2.2.1). The hGFs were incubated at 37°C to grow to confluence as a monolayer adherent to the bottom of tissue culture flask with DMEM+. During growth, hGFs exhibited the characteristic spindle-shaped morphology (Figure 2.11). While on confluent growth, the cells appear aligned in the same orientation (Figure 2.12). The hGFs growth was monitored daily by visual inspection using a light microscope (DM IL, Leica Microsystems, Milton Keynes, UK) and the medium was changed every two days until the cells were ≥80% confluent (this may take 7-10 days depending on the cells growth). To passage the cells, 10 ml of PBS was used to wash the cells in the flasks (to washout any unattached or floating cells), then the cells were covered with 3-5 ml of 1x trypsin-EDTA solution (section 2.2.1) and incubated at 37°C for 3 minutes until detached. 5 ml of DMEM+ were added to the cell suspension to neutralize the trypsin. The cells were pelleted by centrifugation (168g, 5 minutes, 20°C) using CR3i multifunction centrifuge (Thermo Fisher Scientific, Loughborough, UK) and re-suspended with 1 ml of DMEM+. At this stage, the cells were counted and sub-cultured using a new flask or seeded into multi well plates for stimulation experiments, or cryopreserved for future use. The hGFs could be used up to passage 13 as they were found to stop dividing at this passage (Williams 2015).

Counting and viability assessment of cells

The hGFs cells were counted on haemocytometer under a light microscope using trypan blue (0.4%) stain. Equal volumes of cell suspension and trypan blue (10 µl of each) were mixed in an Eppendorf tube and incubated at room temperature for 30 seconds, then 10 µl of the mixture was loaded onto a haemocytometer. Trypan blue was used to count the cells and assess their viability, cells that uptake the stain were considered dead. The Bright-Line Haemocytometer (Hausser Scientific, VWR,

Lutterworth, UK) used has a Neubauer ruling pattern and therefore the number of cells/ml was determined using the formula:

Cells/ml = number of cells counted in 1 mm^2 (4 squares at the corners of the Neubauer pattern) x 2 (dilution factor in trypan blue) x 10^4

Morphology and adherence of the cells to culture surfaces were considered. Hence, when the cells' viability was <90% or they were poorly adherent, they were excluded from stimulation experiments.

Cryopreservation of hGFs

Following centrifugation, cell pellets were re-suspended with 1ml of DMEM+ containing 10% v/v DMSO to give a cell density of ≥ $1x10^6$ cells/ml. Each 1 ml aliquot was dispensed into a cryovial which was then placed in a freezing unit (containing propan-2-ol) in a -80°C freezer overnight. The cryovials were transferred to liquid nitrogen for long-term storage. To revive the hGF cells from cryo-preservation, they were thawed by warming to 37°C. Thereafter, the hGFs cells were slowly seeded into a T75 cell culture flask containing 15 ml $DMEM^+$ medium. The flask was incubated overnight at 37°C, next day and after checking the cells were adherent to the bottom of the flask, the medium was changed to remove any residual cryopreservant or non-viable cells and DMSO. The cells were then sub-cultured to be used in an experiment.

In vitro IL-1β stimulation of hGFs

Depending on the experiment and plastic-ware format to be used, hGF cells were slowly seeded at two densities in DMEM+ (2-$4x10^5$ cells/flask in T25 flask, and 1-$2x10^5$ cells/well in 6-multi-well plate). The hGFs, whether in 6-multi-well plate or T25 flask, were incubated at 37°C until 80-90% confluent and at this point the cells were serum-starved. To serum-starve the cells, the DMEM+ medium was carefully pipetted out avoid touching the bottom of the well or flask then, replaced with SFM (2 ml per well or 5 ml per flask) for 24 hours at 37°C. Then, the SFM was carefully pipetted out avoid touching the bottom of the well or flask then, the cells were stimulated for 24 hours at 37°C with IL-1β (2 ml per well or 5 ml per flask of SFM containing two concentrations of IL-1β). hGFs used in IL-1β stimulation experiments were between passages 5 and 10.

A preliminary stimulating experiment was carried out using three T25 cell culture flasks, in which hGFs were stimulated with 5 ml/flask of two concentrations of IL-1β in SFM (5 ng/ml and 0.05 ng/ml, one flask/concentration) and the third flask with control (0 ng/ml of SFM), all incubated at 37°C for 24 hours. The aim of this experiment was to check if the IL-1β will stimulate the hGFs to produce MMP-1 (fibroblast collagenase), which is a measure of pro-inflammatory response by such cells (Eren et al. 2015; Williams et al. 2016). Following the preliminary experiment, a number of further stimulating experiments were carried out using 6-multi-well plates, in which hGFs were stimulated with 2 ml/well of two concentrations of IL-1β in SFM (5 ng/ml, 0.05 ng/ml) and control (0 ng/ml of SFM), all incubated at 37°C for 24 hours. The aim of these experiments, was to investigate if the hGFs are able to produce the desired candidate biomarkers (uPA and uPAR) when stimulated with IL-1β, by assaying the cell culture supernatants using DuoSet ELISAs, as well as, assaying uPA activity in the supernatants by uPA activity assay. Following the 24 hours stimulation with IL-1β, the hGFs supernatants were pipetted out from each well, placed into cryovials, and stored at -80°C till use.

In vitro LPS stimulation of hGFs

In a similar manner to IL-1β stimulating experiments, hGFs cells were slowly seeded into DMEM+ containing 6-multi-well plates at the density of 1-2x10^5 cells/well, incubated at 37°C to confluency of 80-90%, serum-starved with SFM at 37°C for 24 hours, and stimulated for 24 hours at 37°C with bacterial lipopolysaccharide (LPS). In cell culture stimulating experiments, *P. gingivalis and E. coli* LPS were found to stimulate the production of pro-inflammatory cytokines (such as IL-1β, and IL-6), chemokines and MMPs in gingival fibroblasts and other cells (Kraus et al. 2012; Kuo et al. 2012; Williams 2015). hGFs used in LPS stimulation experiments were between passages 4 and 9.

The stimulating experiments were carried out on the hGFs seeded into the 6-multi-well-plates, using *E. coli* and *P. gingivalis* LPS in a concentration of 100 ng/ml and control (0 ng/ml of SFM), all incubated at 37°C for 24 hours. Same as the IL-1β experiments, the hGFs supernatants were assayed first to check if the *E. coli* and *P. gingivalis* LPS were able to stimulate the cells to produce MMP-1 as a measure of pro-inflammatory response. The aim of the stimulation experiments was to investigate if the hGFs cells will secrete the candidate biomarkers (uPA and uPAR) in response to the LPS, by assaying the cell culture supernatants using DuoSet ELISA

kits, as well as, assaying uPA activity in the supernatants by uPA activity assay. Following the 24 hours stimulation with LPS, the cell culture supernatants were pipetted out from each well, placed into cryovials, and then stored at -80°C till use.

2.3.4 uPA activity fluorometric assay

The uPA activity fluorometric assay kit measures urokinase activity ranging from 0.01-0.5 IU/well in a variety of samples. uPA activity in saliva and cell culture supernatants, was determined using the enzymatic cleavage of an AMC (amido-methyl-coumarin) based peptide substrate, which results in the generation of AMC (λ excitation= 350/λ emission= 450 nm) proportional to the enzymatic activity present, measured by fluorometric multi well microplate reader (BioTek Instruments Ltd).

The assay was carried out at room temperature. 50 µl of standards and samples were pipetted per well in duplicates. Saliva samples were not listed among the samples to be assayed with this kit, therefore, multiple dilutions assays were performed to ensure that saliva samples readings are within linear range of the standard curve. All samples were diluted in 50 µl of uPA assay buffer or filtered PBS. Cell culture supernatants were used as neat samples. Inherent fluorescence in samples might result in sample background. To correct for the background, sample blanks were included for the samples. The sample blank was only uPA assay buffer or filtered PBS without uPA substrate. The sample blanks readings were subtracted from the samples readings for the background correction.

Fifty µl of appropriate reaction mix were added per well, to ensure mixing with standards and samples, the plate was placed immediately on horizontal shaker on low speed. The reaction mix was prepared within 10 minutes and kept protected from light till use. For standards and samples the reaction mix was 48 µl of uPA assay buffer + 2 µl of uPA substrate, whereas, only 50 µl of assay buffer were added to sample blanks. Initial readings were taken after 2-3 minutes of incubation with the reaction mix on the microplate shaker with protection from light. The initial fluorescence intensity ($FLU_{initial}$) was measured at (λ_{ex} = 350/λ_{em} = 450 nm) at the initial time ($T_{initial}$). The plate was incubated again at room temperature protected from light and measurements (FLU) were taken every 5 minutes. The total incubation time varies with the activity of the sample. Samples with low uPA activity occasionally needed a longer incubation in order to detect enough fluorescence within the range of the standard curve. In order to obtain accurate measurements, the ΔFLU (FLU_{final}

- $FLU_{initial)}$ must be in the linear range of the corrected fluorescence values used to create the standard curve. Therefore, readings were taken until the ΔFLU for saliva or cell culture supernatants samples fall within the standard curve range. For saliva samples 30-35 minutes incubation time with 6-7 readings were enough to fall within the standard curve range.

To calculate the results, first the changes in the fluorescence measurements were calculated from initial time ($T_{initial}$) to the final time (T_{final}) for all standards and samples to obtain the corrected fluorescence readings

$$\Delta FLU = FLU_{final} - FLU_{initial}$$

The background of the readings for the standards were corrected by subtracting the corrected fluorescence reading of the 0 blank standard ($\Delta FLU_{Blank\ Standard}$) from each standard

$$\Delta FLU - FLU_{Blank\ Standard}$$

The values obtained were used to plot the standard curve (Figure 2.13). A new standard curve was generated for each assay.

Following the determination of the standard curve, the background of the final readings for samples were corrected by subtracting the corrected fluorescence reading of the 0 blank sample ($\Delta FLU_{Blank\ Sample}$) from each sample

$$\Delta FLU - FLU_{Blank\ Sample}$$

The corrected readings were used in the standard curve equation to determine the uPA values for the samples. The values obtained from the equation were used to determine the uPA activity taking in consideration the dilution factor

$$\text{uPA Activity (IU/ml)} = S_a / S_v$$

S_a = Amount of uPA for each sample from the standard curve.

S_v = Sample volume (ml) added to well.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS 23.0 software (IBM, Portsmouth, UK), and Minitab 17 statistical software (Minitab Inc. Coventry, UK). Box and scatter plot graphs were created in SPSS. Bar graphs were created in Microsoft Excel 2013. All data were assessed for normality and homogeneity of variance using Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variance. When data

were normally distributed, means, standard errors of the means (SEM) and standard deviations (SD) of the means were calculated and presented, and parametric statistical tests were performed on the data. Parametric statistical tests included: paired samples t-test for related samples and independent samples t-test for non-related samples, whereas, for more than one group one-way ANOVA test was used. When the normal distribution of data was rejected, medians and interquartile ranges (IQR) were calculated and presented, and non-parametric statistical tests were performed on the data. Non-parametric statistical tests included: Wilcoxon signed rank test for dependent samples and Mann-Whitney U test for independent samples. Friedman test was used for more than 2 groups with dependent samples. When more than 2 independent samples groups with not-normally distributed data were presented, Kruskal-Wallis test was used. One sample t-test was used to determine the significance (p-value) for the logarithms of fold change in the PPA assays.

A p-value of <0.05 was considered significant. The p-values were corrected for multiple comparisons with the post hoc or Bonferroni correction tests. Spearman's coefficient correlation analysis was used to determine possible associations between pairs of parameters including: salivary ELISA results with periodontal indices scores, GCF ELISA results with periodontal indices scores, uPA activity assay results with uPA levels, and uPA activity assay results with periodontal indices scores. Spearman's correlations were considered to be significant when $p<0.05$. Using Sigma Plot 12.5 software (from Sigma Plot, Hounslow, London, UK), receiver operating characteristic curve analysis (ROC), was carried out to study the ability of the candidate biomarkers to discriminate between healthy and diseased status. The ROC curve, is a statistical test used to study the ability of a biomarker, assay or clinical procedure to differentiate between healthy and diseased status by the means of sensitivity (true positive rate) and 1-specificity (false positive rate) as measures of accuracy of the test or biomarker, results were presented as the area under the curve (AUC) value ranging between 0-1 (Fawcett 2006; Hajian-Tilaki 2013).

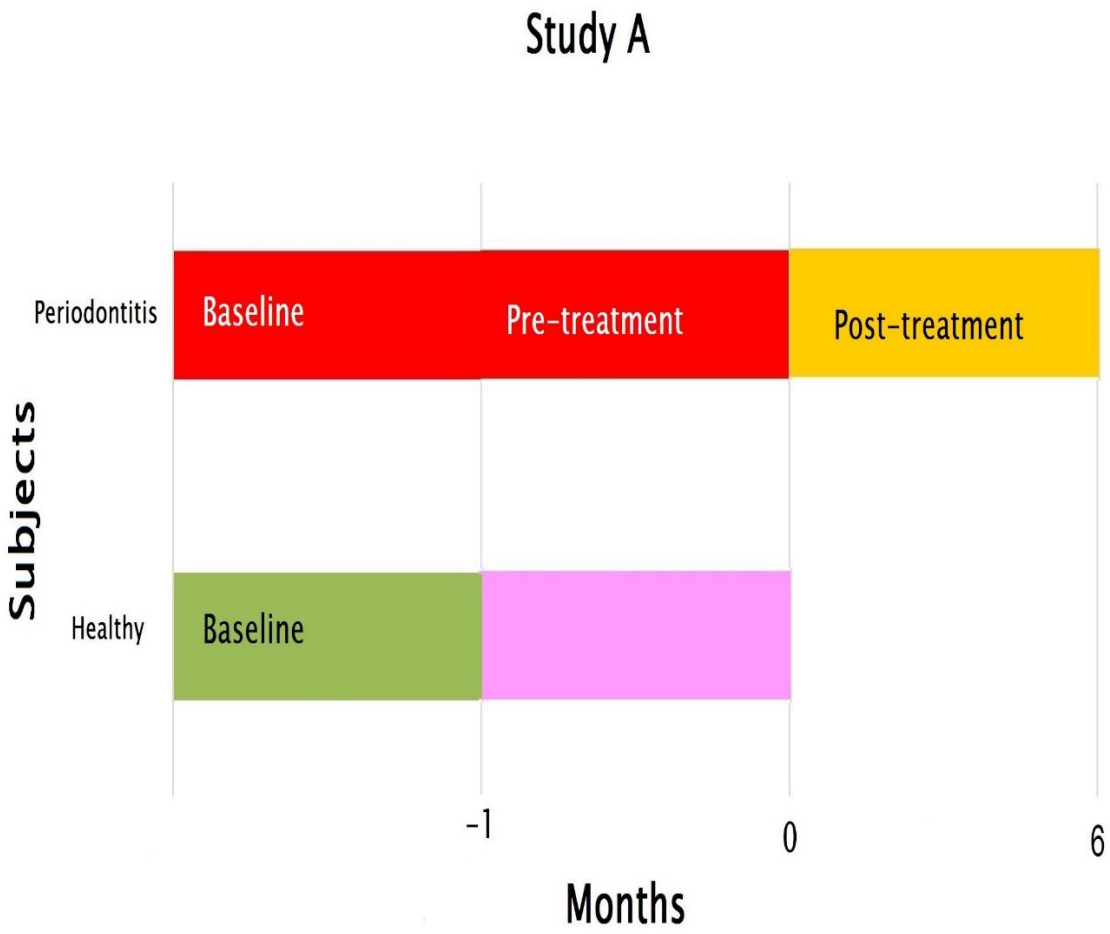

Figure 2.1: Clinical study A timetable.

The visits of clinical study A at which saliva samples were obtained. Saliva samples were obtained from healthy subjects at the Baseline month -1 visit (visit 1), and month 0 visit (visit 2). Saliva samples were obtained from periodontitis patients at the Baseline month -1 visit (visit 1), at the pre-treatment month 0 visit (visit 2) which was followed by a number of non-surgical periodontal treatment visits, and finally at the post-treatment month 6 visit (visit 6).

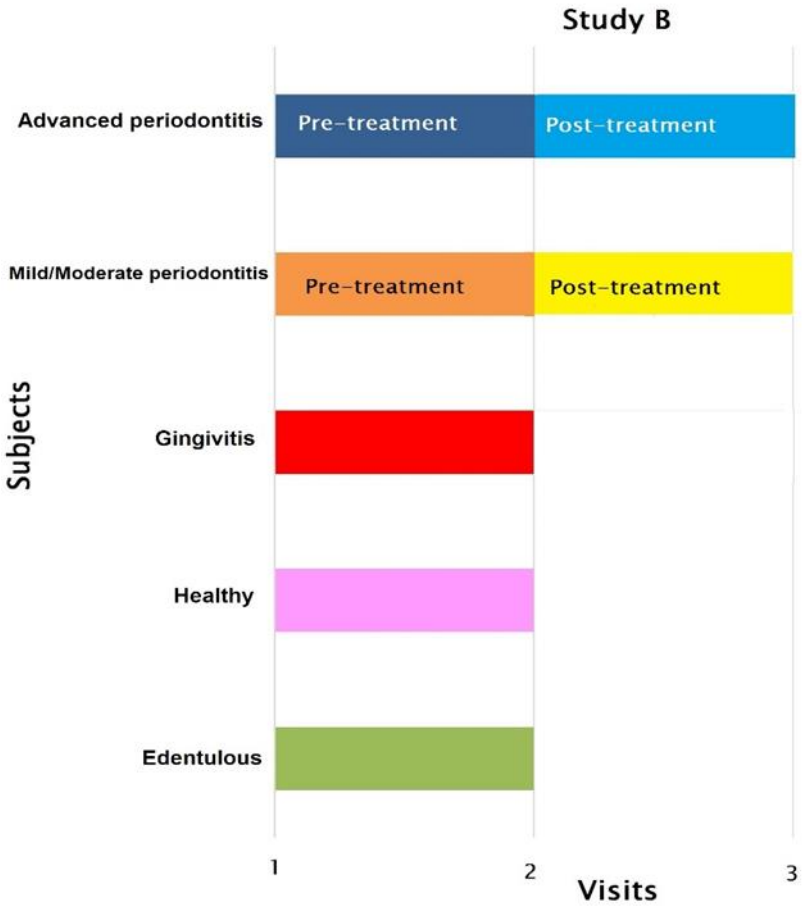

Figure 2.2: Clinical study B timetable.

The visits of clinical study B at which saliva samples were obtained. Saliva samples were obtained from edentulous subjects, dentulous healthy subjects and gingivitis patients at the visit 2 (which was 5-30 days from visit 1). Saliva samples were obtained from mild/moderate and advanced periodontitis patients at the visit 2 (pre-treatment), followed by a number of non-surgical periodontal treatment visits, and finally at the visit 3 (post-treatment) which was12 ± 2 weeks after treatment.

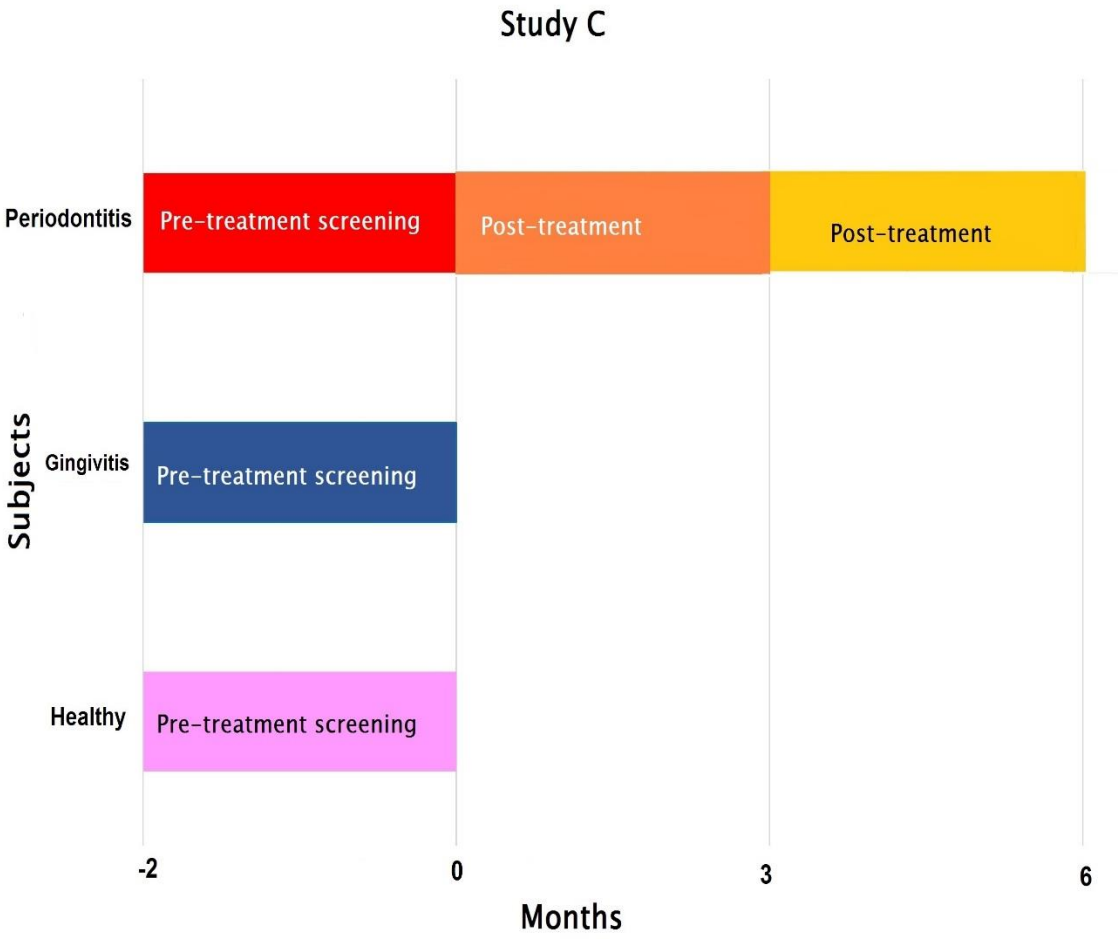

Figure 2.3: Clinical study C timetable.

The visits of clinical study C at which the GCF samples were obtained. GCF samples were obtained from healthy subjects, gingivitis patients and periodontitis patients at the pre-treatment screening visit (2 months before treatment). Non-surgical periodontal treatment was offered for the periodontitis patients at the treatment visit (month 0). GCF samples were obtained again from the same periodontitis patients at the post-treatment month 3 visit, and at the post-treatment month 6 visit.

Periodontal diagnosis	Criteria
Healthy	BOP≤15%
	No PD sites >4mm
	No LOA (Disregard any recession due to tooth brushing)
	No bone loss
Gingivitis	BOP≥15%
	No sites with PD >4 mm, except for sites at the distal surface of last standing molars (up to 5 sites with 5 mm PD accepted)
	No LOA (Disregard any recession due to tooth brushing)
Periodontitis	≥6 sites with PD of ≥5 mm
	LOA and/ or bone loss present.

Table 2.1: Periodontal diagnostic criteria.

Firm diagnostic criteria for periodontal status were used to classify the patients from healthy volunteers. To avoid any misdiagnosis of a difficult case for a patient or subject, decision was made by discussion between the clinicians involved.

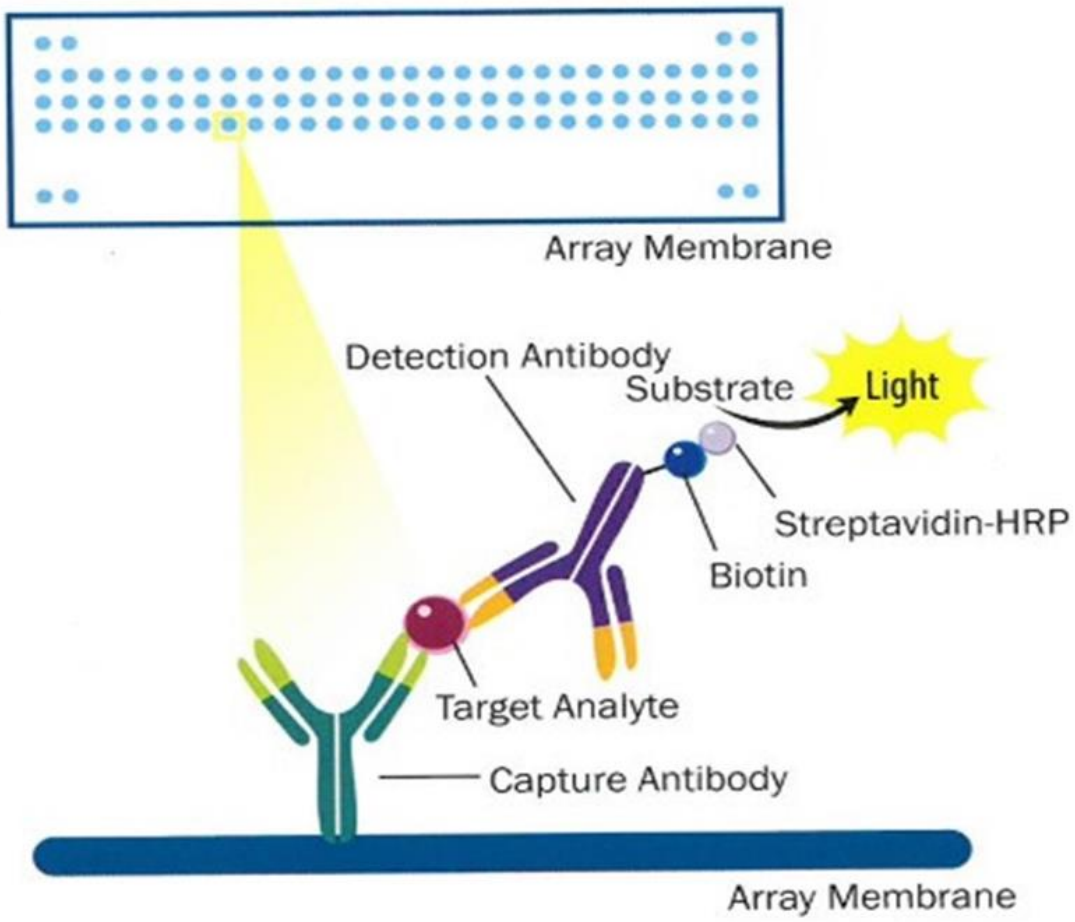

Figure 2.4: Assay principle of the proteome profiler arrays.

The two-site sandwich assay principle of proteome profiler arrays. The target analyte binds to the specific capture antibody on the array membrane on one site, and the biotinylated detection antibody on the other site, to be detected and visualised by Streptavidin-HRP and substrate. (R & D systems).

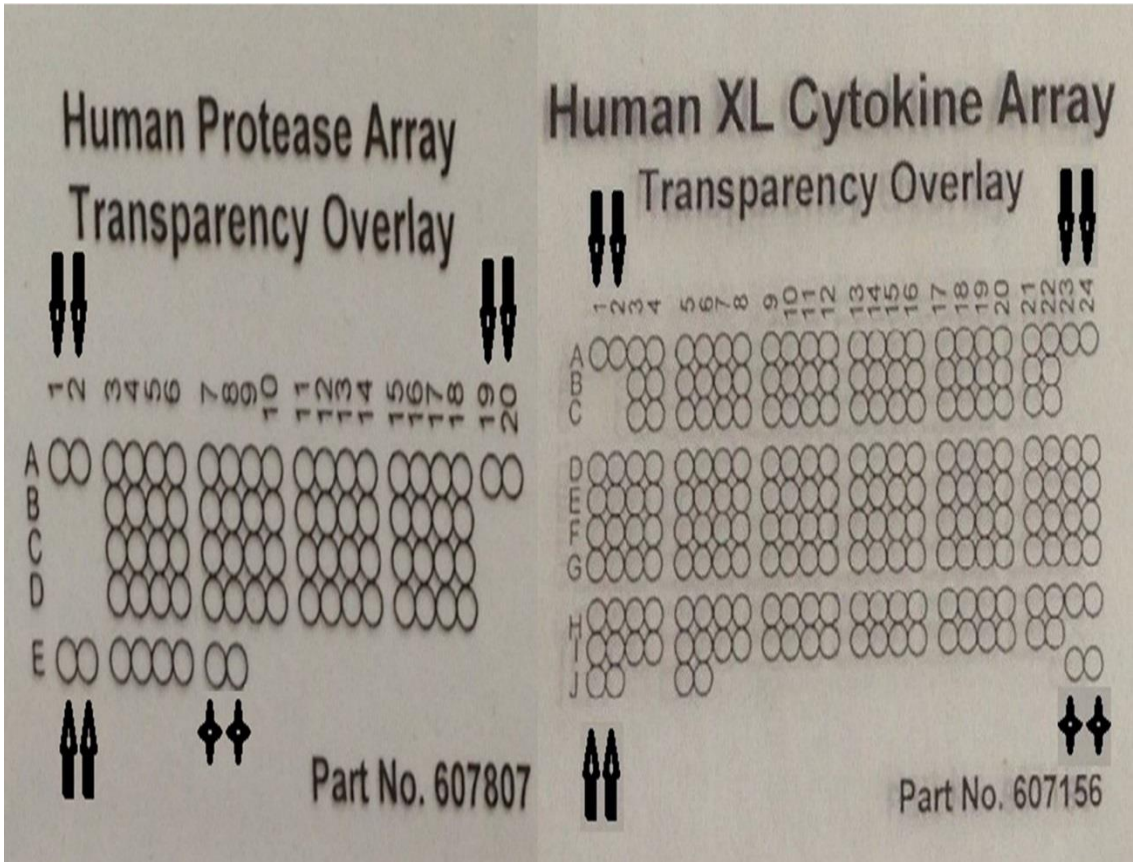

Figure 2.5: Proteome profiler arrays templates.

The transparency overlay templates for the protease and cytokine arrays, illustrating the positions of the reference spots. The arrows represent the three duplicate spot positive control references on three corners of each template. The stars represent the duplicate spot of the negative control reference on each template. Each protein identified by its precise duplicate spot on the template for instance the duplicate spot (C13, C14) on the protease template represent the MMP-1. The duplicate spot (D3, D4) on the cytokine template represent the growth hormone.

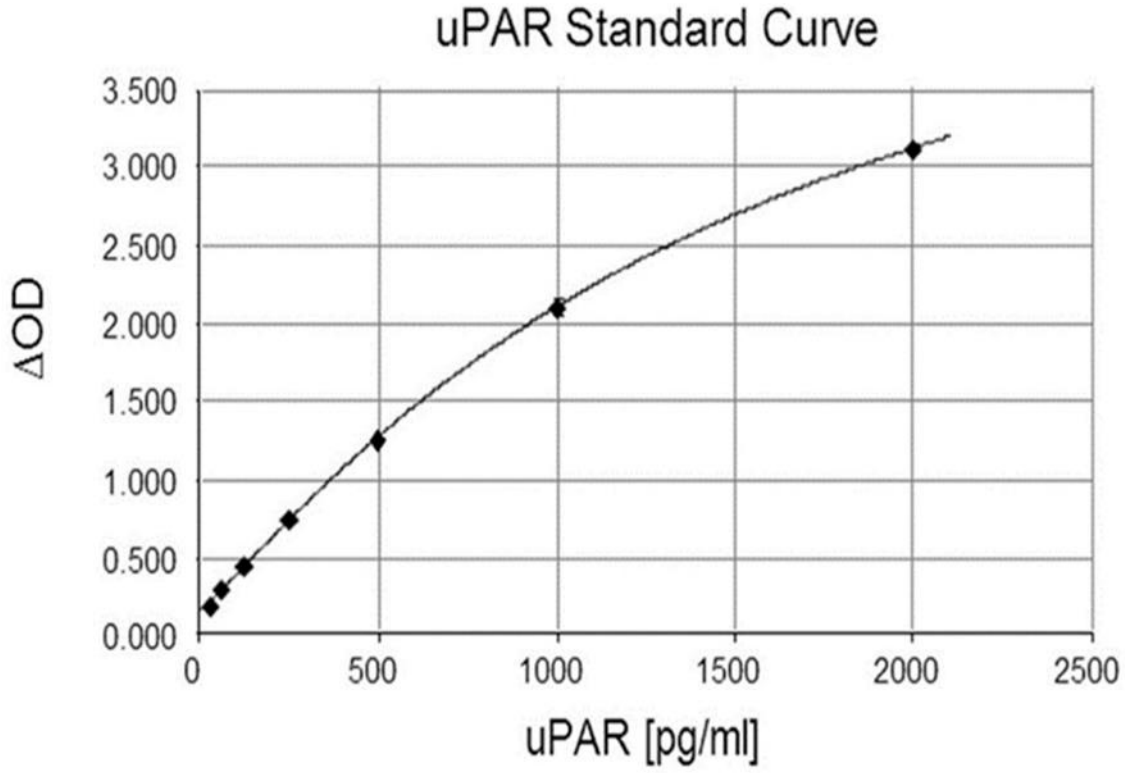

Figure 2.6: uPAR DuoSet ELISA standard curve.

Despite of the ideal standard curve detection range, an individual uPAR DuoSet ELISA standard curve was generated for each individual assay with little bit higher or lower ranges. The ΔOD values of the different uPAR concentrations were plotted against the uPAR concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the uPAR ELISA. ΔOD: OD 450 nm – OD 550 nm.

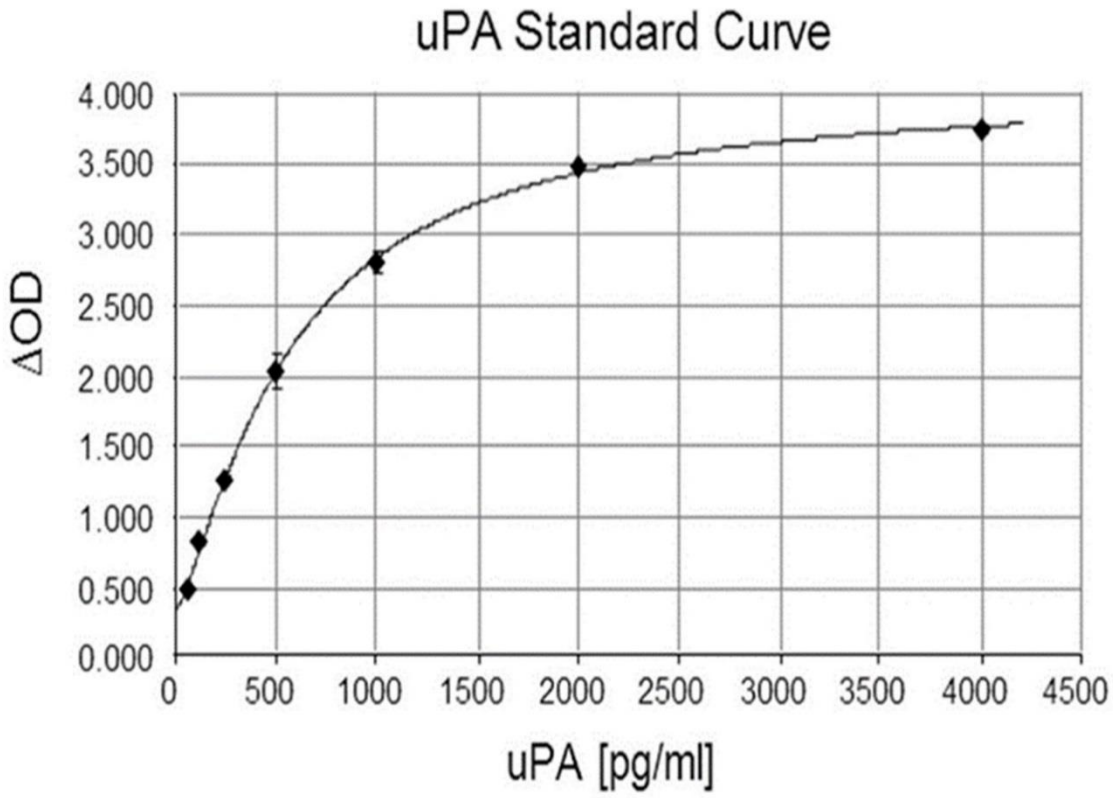

Figure 2.7: uPA DuoSet ELISA standard curve.

In spite of the ideal standard curve detection range, an individual uPA DuoSet ELISA standard curve was generated for each individual assay with little bit higher or lower ranges. The ΔOD values of the different uPA concentrations were plotted against the uPA concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the uPA ELISA. ΔOD: OD 450 nm – OD 550 nm.

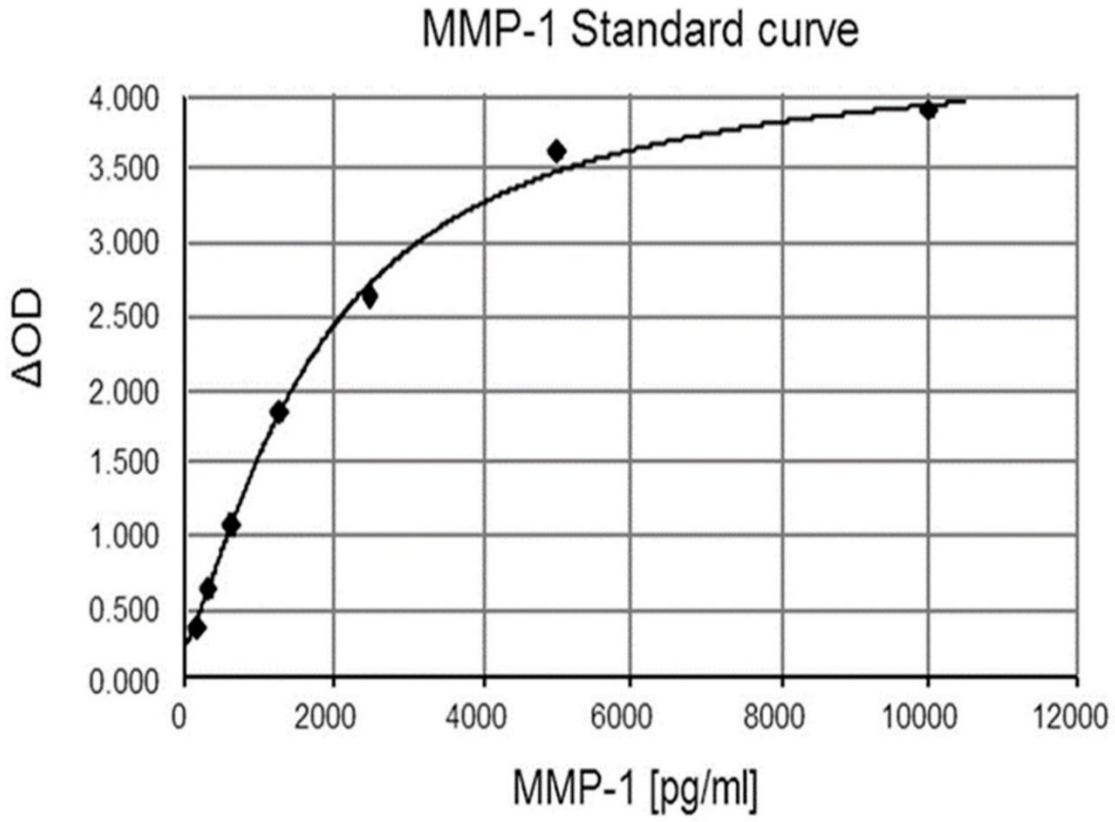

Figure 2.8: Human total MMP-1 DuoSet ELISA standard curve.

Regardless to the ideal standard curve detection range, an individual MMP-1 DuoSet ELISA standard curve was generated for each individual assay with little bit higher or lower ranges. The ΔOD values of the different MMP-1 concentrations were plotted against the MMP-1 concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the MMP-1 ELISA. ΔOD: OD 450 nm – OD 550 nm.

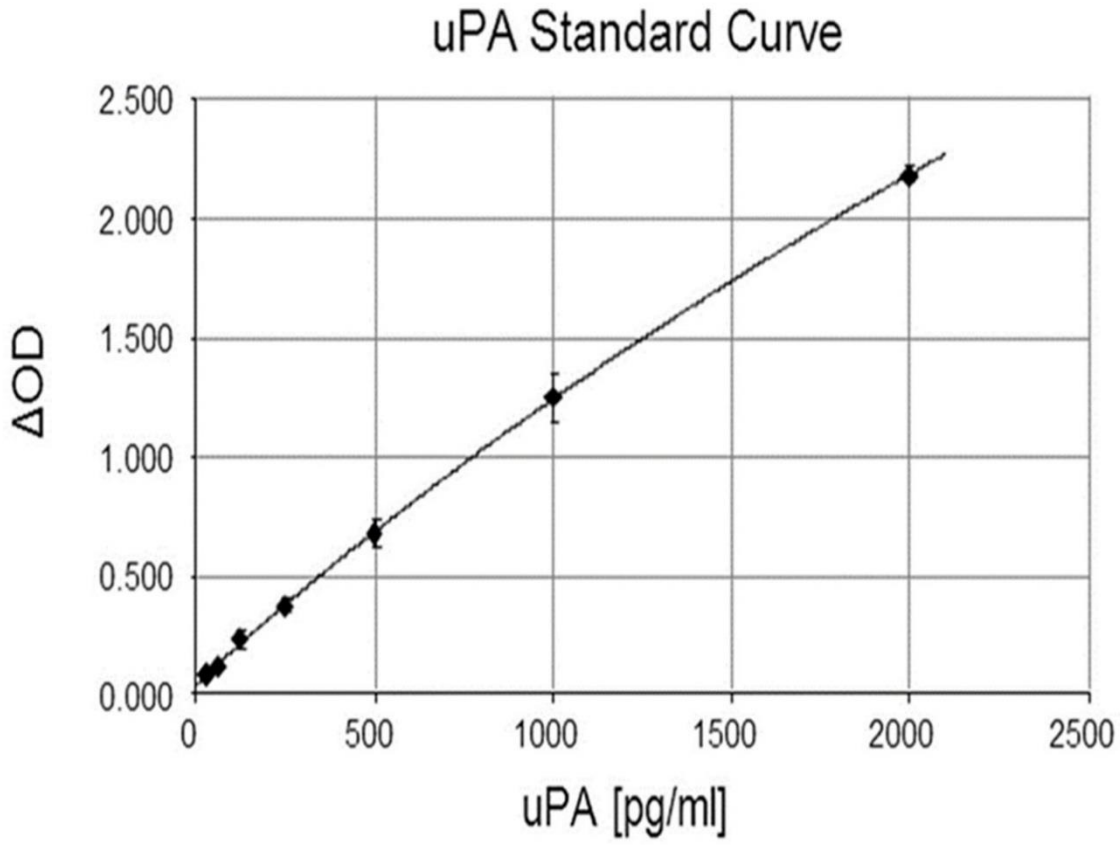

Figure 2.9: uPA Quantikine ELISA standard curve.

Aside from the ideal standard curve detection range, an individual uPA Quantikine standard curve was generated for each single assay. The ΔOD values of the different uPA concentrations were plotted against the uPA concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the uPA ELISA. ΔOD: OD 450 nm – OD 550 nm.

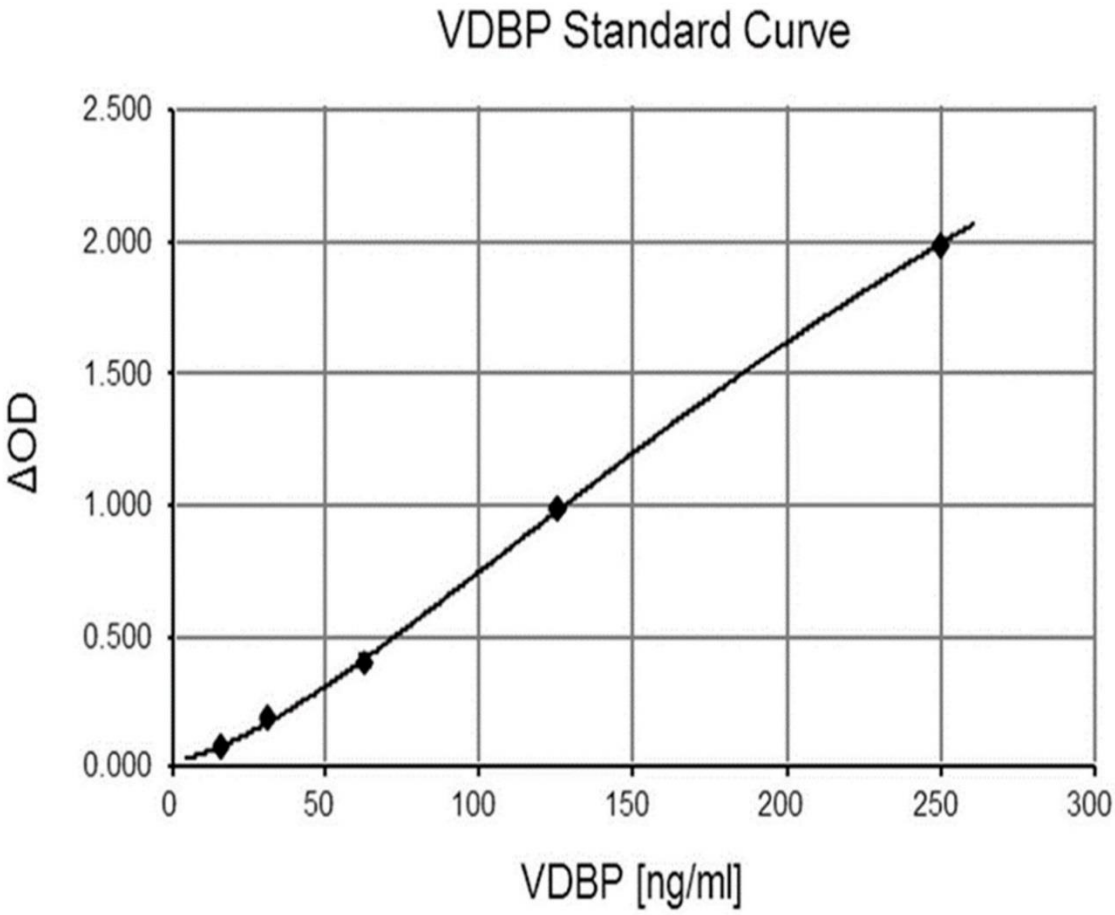

Figure 2.10: VDBP Quantikine ELISA standard curve.

In spite of the ideal standard curve detection range, an individual VDBP Quantikine ELISA standard curve was generated for each single assay. The ΔOD values of the different VDBP concentrations were plotted against the VDBP concertation. A 4-parameter curve fit was created to produce the 5-point standard curve for the VDBP ELISA. ΔOD: OD 450 nm – OD 550 nm.

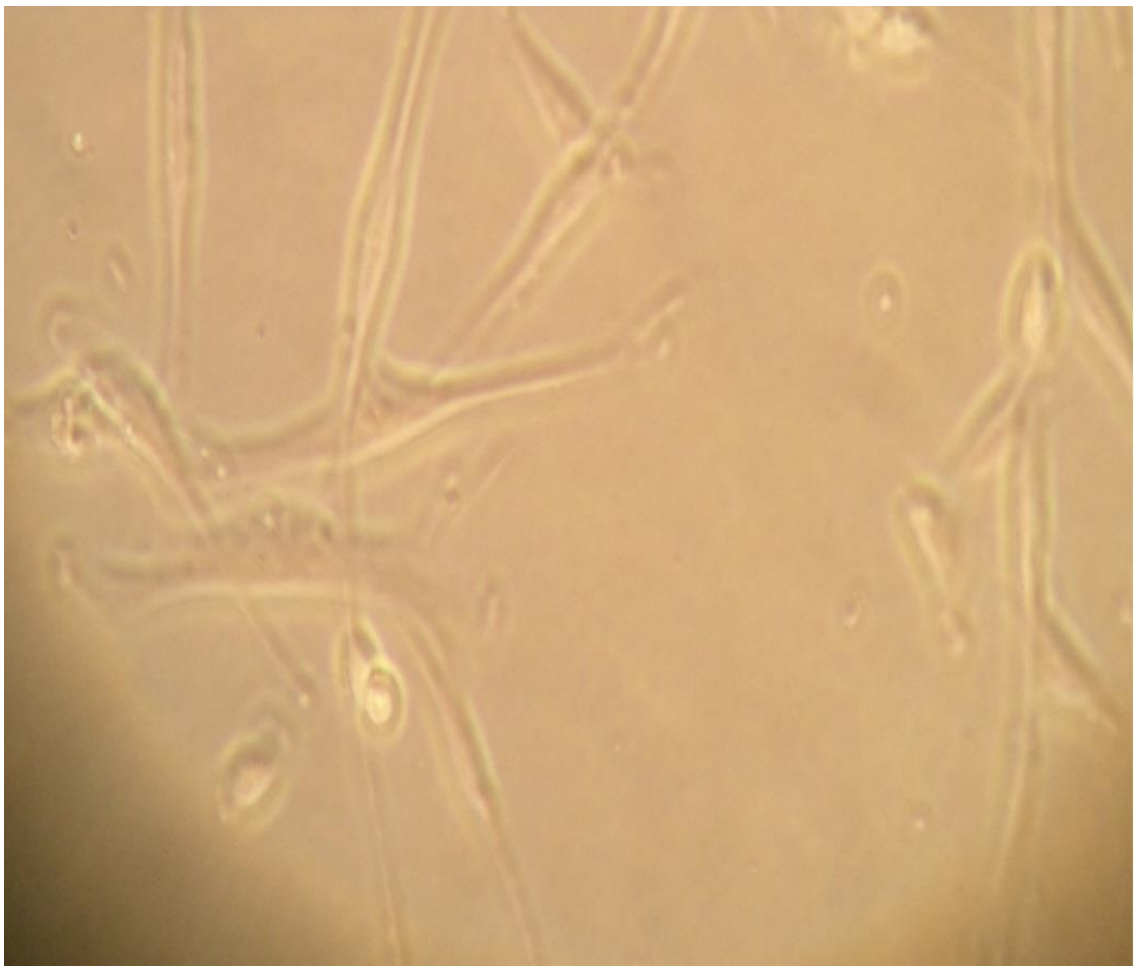

Figure 2.11: hGF cells during growth.

Spindle-shaped morphology of viable hGF cells under microscope. At low confluency during growth in $DMEM^{+}$ medium the cells were attached to the bottom of plastic-ware cell culture flask.100x magnification.

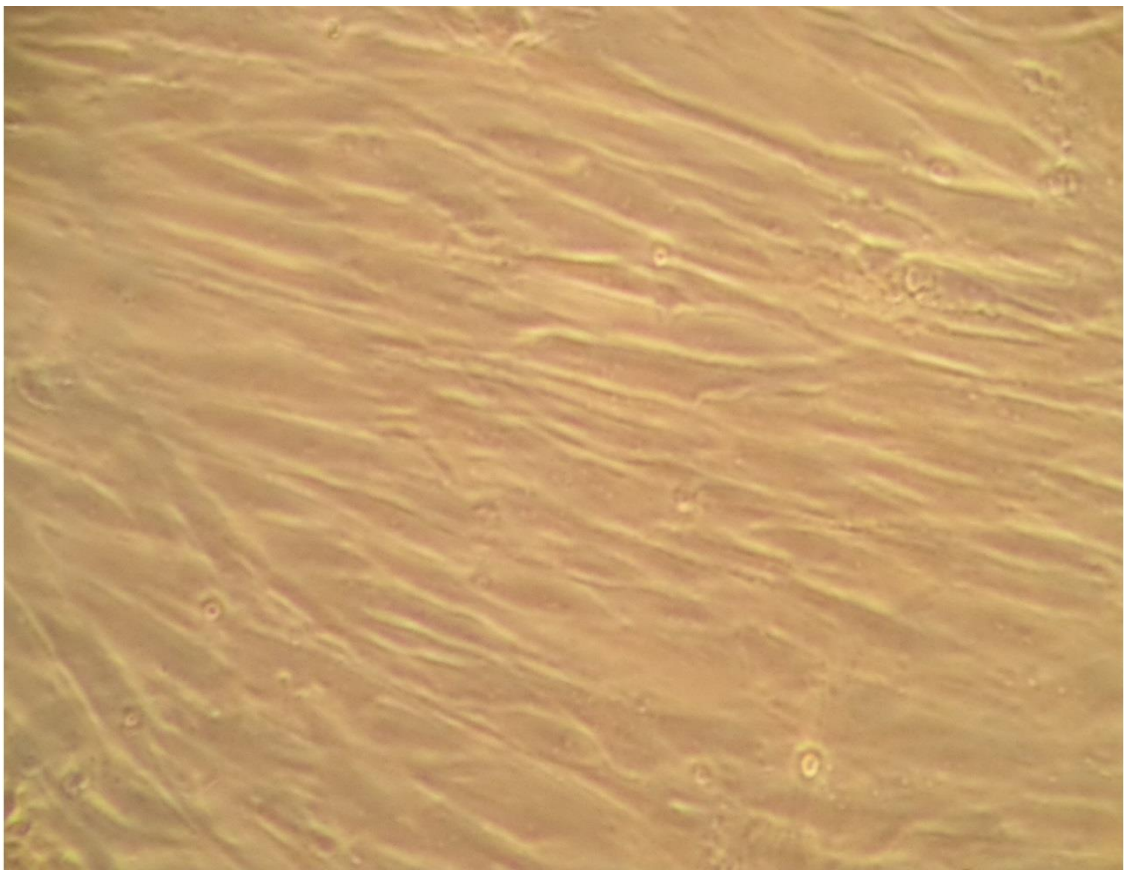

Figure 2.12: hGF cells on confluent growth.

The hGF cells in confluent growth under microscope. As the cells' growth reach 80 % confluency and above, they will appear aligned in the same orientation at the bottom of plastic-ware cell culture flask containing $DMEM^{+}$ medium.100x magnification.

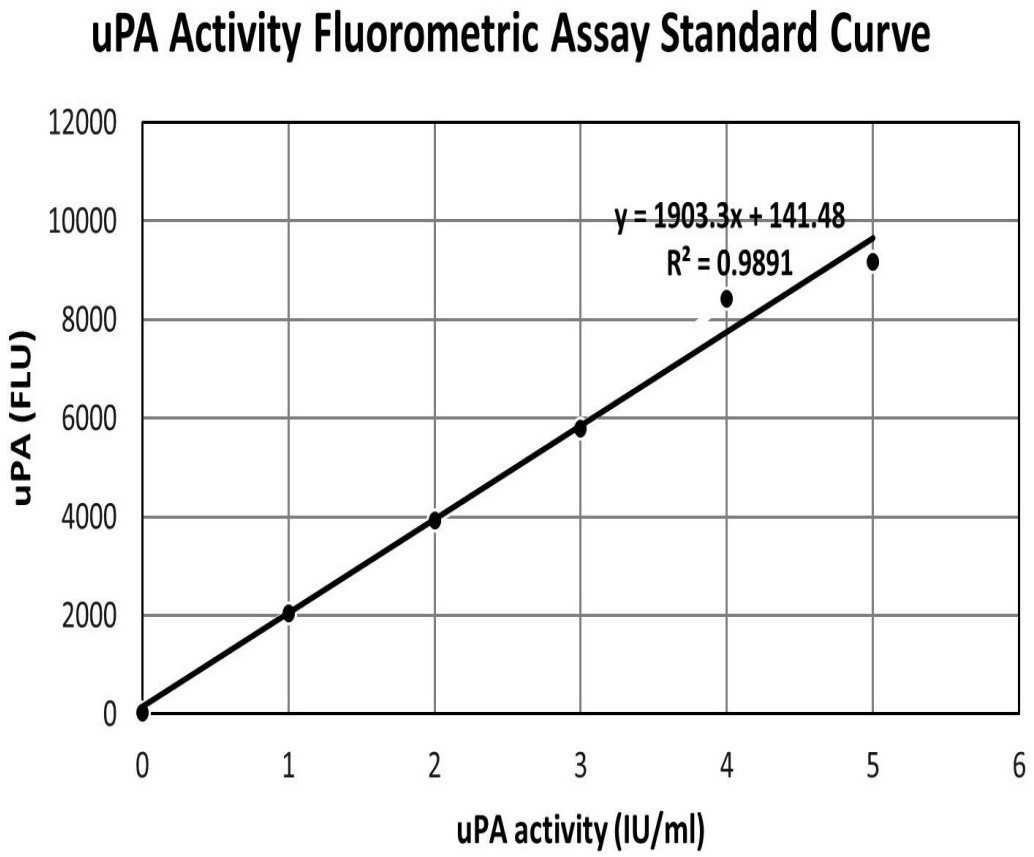

Figure 2.13: uPA activity fluorometric assay standard curve.

The corrected FLU values of the uPA standards were plotted against their known activity values (IU/ml). A 4-parameter curve fit was created to produce the 6-point standard curve of the uPA activity assay. The resultant equation was used to calculate the uPA activity for the samples.

Chapter 3 Identification of candidate salivary biomarkers

3.1 Introduction

Several clinical studies identified a number of biomarkers and inflammatory mediators associated with periodontitis. These biomarkers were correlated with the clinical measurements of the disease, and their levels were changed in parallel with the clinical course of the disease and in response to treatment (Buduneli and Kinane 2011; Taylor 2014). Salivary IL-1β has been suggested as a good biomarker for periodontitis in a number of studies as the measurement of IL-1β can discriminate periodontitis patients from healthy volunteers (Miller et al. 2006; Tobon-Arroyave et al. 2008; Gursoy et al. 2009; Mirrielees et al. 2010; Kaushik et al. 2011; Ebersole et al. 2013; Rathnayake et al. 2013; Javed et al. 2014). Numerous studies demonstrated that salivary matrix metallo-proteinase 8 (MMP-8) activity was elevated in periodontitis patients in comparison to healthy volunteers, and it was correlated with the clinical measures of periodontitis (Miller et al. 2006; Ramseier et al. 2009; Costa et al. 2010; Gursoy et al. 2010; Mirrielees et al. 2010; Ebersole et al. 2013; Rathnayake et al. 2013; Miricescu et al. 2014). MMP-8 appears to be the best biomarker in comparison to others such as IL-1β, as it not only identifies the presence of periodontal inflammation but also has the ability to determine the extent of tissue destruction (Rathnayake et al. 2013). Other salivary MMPs such as MMP-1, salivary gelatinases (MMP-2 and MMP-9) and elastase were significantly elevated in periodontitis patients as compared to healthy controls (Pietruska et al. 2009; Ramseier et al. 2009; Isaza-Guzman et al. 2011; Morelli et al. 2014). Significant association of salivary hepatocyte growth factor (HGF) with periodontitis was demonstrated in independent studies (Wilczynska-Borawska et al. 2006; Scannapieco et al. 2007; Rudrakshi et al. 2011; Wilczynska-Borawska et al. 2012).

Though IL-1β, MMPs, and HGF are good biomarkers for periodontitis, for an efficient diagnosis of a disease such as periodontitis with its complex pathogenesis, progression, and inter-individual variation, there is a need for multiple biomarkers (Giannobile et al. 2009; Kinney et al. 2011). This notion known as “biomarker signature” which was defined by Lagani et al. (2013) as “a minimal subset of molecular quantities that are maximally informative for a given task when considered jointly”. Therefore, investigation of the proteome profile of human saliva, with comprehensive analysis of salivary proteins, may help to understand oral diseases

pathogenesis and identify novel salivary biomarkers for human diseases such as periodontitis (Hu et al. 2007). On this basis, the first aim of the present study to be discussed in this chapter, is to identify novel salivary biomarkers for periodontitis.

In the diagnosis of periodontitis, proteomics is considered as a novel approach to identify salivary biomarkers associated with the disease. Proteomics can provide information about different proteins that cannot be obtained by ELISAs or western blot techniques (Haigh et al. 2010). One of the proteomic techniques is the proteome profiler array (PPA) system (R & D systems), which was employed for the first time in this study to identify candidate salivary biomarkers for periodontal diseases. Two types of arrays were used: a human protease array and a human cytokine array. The PPAs offer a wide panel of target antibodies per array membrane for sample screening. 34 and 102 specific capture antibodies are spotted in duplicate onto protease and cytokine PPAs respectively. The PPA procedure involved: an overnight incubation of the samples with the membranes at 2-8°C, followed by multiple steps of washing and incubation at room temperature with specific detection antibodies, and streptavidin. Finally, a chemiluminescent substrate mix was added to demonstrate the positive spots on the arrays, and to view these positive spots multiple radiographs were taken.

3.2 Results

In the present study, 6 PPA assays for salivary proteases and 6 assays for salivary cytokines, were performed on whole unstimulated saliva samples from the baseline visit of clinical study A, according to the previously described method (see chapter 2, section 2.3.1). 4-5 minutes was found to be the optimum exposure time to develop positive signals of the array membranes by autoradiography. Image analysis of the radiographs revealed that, the arrays identified a number of proteases and cytokines in saliva samples obtained from patients with untreated chronic periodontitis and healthy control volunteers. The relative expression of the proteins were measured by the means of pixel intensity (PI), the PI values were calculated by subtracting the mean of each protein duplicate spot from the mean of the negative reference duplicate spot on the PPA membrane for background correction as described by R & D systems. The changes in the relative expression of the proteases and cytokines were compared in periodontitis and control samples. These changes were illustrated by the means of bar graphs representing the means of the PI values as described by

R & D systems and (Westman et al. 2015; Tsuboki et al. 2016). Furthermore, the difference in the mean PI values for the relative expression of the detected proteases and cytokines, were compared by the means of fold change and p-value of the logarithm-fold change obtained by performing the one sample t-test on the logarithms of the fold change to test the null hypothesis H0: log (fold change) equal to 0 against the alternative H1: log (fold change) not equal to 0 (McCarthy and Smyth 2009).

The proteins described in the following sections were examples of proteases and cytokines selected according to their known roles in extra cellular proteolysis, bone metabolism and degradation, cellular signalling, and chemotaxis of inflammatory cells, or inflammatory events, which may take place in the pathogenesis of periodontitis. The remaining PPA data from both the protease and cytokine PPA arrays are presented in the Appendix tables (Appendix A. Table 1, Appendix A. Table 2, Appendix A. Table 3, & Appendix A. Table 4).

3.2.1 Salivary protease analysis

Twelve study samples obtained from untreated chronic periodontitis patients and 12 control samples obtained from healthy subjects, were assayed in 6 protease PPA assays. Three independent single sample protease PPA assays were performed on 6 samples (2 samples for each assay, one chronic periodontitis sample and one healthy sample). Also, 3 independent protease PPA assays were performed on 6 pooled samples (2 samples for each assay, each pooled sample consisted of 3 periodontitis or 3 healthy samples mixed in equal ratios).

Single sample assays

The overall analysis of the three assays in which individual saliva samples (rather than pooled samples) were analysed revealed that all the 34 proteases were expressed in both the periodontitis and healthy samples. The relative expression of 15 proteases were numerically higher in the periodontitis samples as compared to the expression in the periodontally healthy volunteers, but only 2 of these were statistically significant (Figure 3.1, Table 3.1 & Appendix A. Table 1). The 2 proteins, which were significantly elevated, were ADAM8 and uPA (Figure 3.1 & Table 3.1). In contrast, the relative expression of some 19 proteases was numerically higher in the healthy samples as compared to the periodontitis samples but in only one case was this difference statistically significant (Table 3.1 & Appendix A. Table 1). The protein,

which was significantly elevated in healthy samples, was MMP-9 (Figure 3.1 & Table 3.1).

Pooled sample assays

The overall analysis of the in which pooled saliva samples (rather than individual samples) were analysed demonstrated that all the 34 proteases on the array were expressed in saliva samples of both the periodontitis patients and healthy volunteers. The relative expression of 33 proteases (including ADAM8 and uPA) were numerically higher in the periodontitis samples as compared to the expression in the periodontally healthy volunteers and 17 of these differences were statistically significant (Figure 3.2, Table 3.2 & Appendix A. Table 2). In contrast to the data from the analysis of the single sample assays, the levels of MMP-9 were not significantly different between health and periodontitis. From the 3 pooled sample assays, only proteinase 3 was more highly expressed in the healthy samples in comparison to the periodontitis samples, but this difference was not statistically significant (Figure 3.2 & Table 3.2).

Summary of protease expression analysis

The results analysis of both the single and pooled sample protease assays revealed that, there was a difference in the protease profile for the whole unstimulated saliva obtained from the periodontitis patients and the healthy subjects. In the periodontitis samples, the relative expression for the salivary proteases was higher in the pooled sample assays in comparison to the single sample assays. Both the single and pooled sample assays were able to detect all the 34 listed proteases in both the healthy and periodontitis saliva samples. It is interesting that in both the single and pooled sample protease PPA assays, there was a statistically significantly higher expression of salivary uPA as well as ADAM8 in the periodontitis patients in comparison to the healthy subjects (Figure 3.3).

3.2.2 Salivary cytokine analysis

In a similar manner to the salivary protease PPA assays, 6 cytokine PPA assays were performed on 12 study samples obtained from untreated chronic periodontitis patients and 12 control samples obtained from healthy subjects. Three independent single sample cytokine PPA assays were performed on 6 samples (2 samples for each assay, one chronic periodontitis sample and one healthy sample). Also, 3

independent cytokine PPA assays were performed on 6 pooled samples (2 samples for each assay, each pooled sample consisted of 3 periodontitis or 3 healthy samples mixed in equal ratios).

Single sample assays

The assays in which individual saliva samples (rather than pooled samples) were analysed revealed that, from the total of 102 cytokines, 90 were detected by the arrays specific capture antibodies in the periodontitis samples, whereas only 30 cytokines were expressed in the healthy samples (Table 3.3 & Appendix A. Table 3). Among the detected cytokines, the relative expression of 26 cytokines were numerically higher in the periodontitis samples in comparison to the healthy samples, but these differences were only statistically significantly different in 4 cases: adiponectin, complement factor D, uPAR and retinol binding protein 4 (RBP4) (Figure 3.4, Table 3.3 & Appendix A. Table 3). In contrast, there were only 4 proteins numerically more highly expressed in the healthy samples in comparison to the periodontitis samples, but none of these differences were statistically significant (Table 3.3 & Appendix A. Table 3). The majority of proteins (n=60) detected in periodontitis samples, in addition to 12 other proteins (total 72) were not detected at all in saliva from healthy volunteers (Appendix A. Table 3).

Pooled sample assays

The results of assays in which pooled saliva samples (rather than individual samples) were analysed showed that, from the total of 102 cytokines, 95 were expressed in the periodontitis samples, and 93 cytokines were expressed in the healthy samples (Table 3.4 & Appendix A. Table 4). Among the 95 cytokines detected in the periodontitis samples, 55 were relatively more highly expressed in comparison to the healthy samples in numerical terms, but these differences were only significant with respect to 6 proteins (EGF, IL-8, resistin, uPAR, VEGF and IL-1Ra) (Figure 3.5, Table 3.4 & Appendix A. Table 4). The elevated expression of uPAR in periodontitis is in agreement with the data from the single sample protease PPA assays. In contrast, there were some 34 proteins numerically more highly expressed in the healthy samples in comparison to the periodontitis samples, but none of these differences were statistically significant (Figure 3.5, Table 3.4 & Appendix A. Table 4). The expression of 9 proteins was not detected in the pooled healthy saliva

samples and 7 proteins were not detected in the pooled periodontitis samples (Table 3.4 & Appendix A. Table 4).

Summary of cytokine expression analysis

The single and pooled sample cytokine PPA assays revealed that, there were differences in the cytokine profile for the whole unstimulated saliva obtained from the periodontitis patients and healthy subjects. The relative expression of the cytokines in both the healthy and periodontitis samples was higher in the pooled sample assays than the single sample assays. In contrast to the protease assays which detected all the 34 listed salivary proteases, a number of the 102 listed proteins were not detected at all by the cytokine arrays in both the periodontitis and healthy samples. . Significantly, both the single and pooled sample cytokine PPAs indicated that there was significantly higher expression of the salivary uPAR in the periodontitis patients in comparison to the healthy subjects (Figure 3.6).

3.3 Discussion

3.3.1 Proteome profiler arrays and proteomics

The diverse and powerful technologies of proteomics have opened the door for the identification of new biomarkers that will improve the diagnosis and monitoring of several oral and systemic diseases (Zhang et al. 2013a). Therefore, PPA assays were selected to be used for their ability to detect wide range of proteins in saliva samples and thus to identify candidate salivary biomarkers for chronic periodontitis. The results revealed that there was a difference in the proteome profile of the whole unstimulated saliva obtained from the periodontitis patients as compared to that of the healthy subjects.

Despite the fact that proteomics offers advantages when compared to other techniques that identify single or limited number of proteins, there are some problems or weakness points associated with proteomics. The global analysis of saliva using proteomics may represent a challenge to the researchers due to the wide range of abundances for the proteins of interest. For instance, though proteomics are able to detect the salivary protein profile; some proteomic approaches are only sensitive when the proteins are highly abundant, whereas other proteins with low abundance such as some cytokines and MMPs may not be detected, therefore, many studies working on expanding the range of proteome profile coverage and salivary protein

catalogue by combining different techniques together such as 2D & 3D gel electrophoresis, protein/peptide fractioning, and liquid chromatography-mass spectrometry / mass spectrometry (LC-MS/MS) (Xie et al. 2005; Guo et al. 2006; Bandhakavi et al. 2009; Wu et al. 2009; Goncalves Lda et al. 2010; Haigh et al. 2010; Salazar et al. 2013).

Although some salivary proteins such as MMP-8 and IL-1β are well known biomarkers for periodontal diseases (Kaushik et al. 2011; Ebersole et al. 2013; Rathnayake et al. 2013; Miricescu et al. 2014); there is a controversy about the detection of these proteins in proteomic studies. Haigh et al. (2010) reported that though the 2 dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE) can accurately quantify a wide range of proteins in one sample; proteins of less than 1 µg/ml concentration, and smaller than 10 kDa in size or of high hydrophobicity, are either poorly resolved or not detected. Some proteins such as cytokines and MMPs, are only presented in ng/ml concentrations in saliva, hence these proteins were not quantified by the 2D SDS-PAGE (Haigh et al. 2010). The use of other sensitive approaches such as more sensitive fluorescent stain, instead of Coomassie blue, may have increased the technique ability to detect these low abundance proteins (Haigh et al. 2010). This finding by Haigh et al. (2010) may explain why some proteases such as MMP-8 and MMP-9 were variably or not highly expressed by the protease PPA membranes, and cytokines namely IL-1β was either not detected by the single sample cytokine PPA assays or detected in low expression by the pooled sample PPA assays in saliva of periodontitis patients in the present study. However, in contrast to Haigh et al. (2010), using LC-MS/MS approaches enabled Salazar et al. (2013) to find that MMP-8 and MMP-9 were among the proteins detected in saliva of periodontitis patients that showed ≥1.5 fold change difference in abundance with $p<0.05$ significance as compared to healthy controls. Whereas, the present study protease PPA assays results revealed that the MMP-8 was numerically but not significantly highly expressed in saliva of the periodontitis patients as compared to the healthy controls. The MMP-9 was both numerically and significantly highly expressed by the single sample protease PPA assays in the healthy subjects as compared to the patients, but it was only numerically highly expressed in the periodontitis patients as compared to the healthy controls by the pooled sample PPA assays. In addition to their ng/ml concentrations

in saliva (Haigh et al. 2010), this inconstancy in the MMPs expression especially the MMP-9, may be due to inter-individual variation. Hence, regardless to the health or disease status, the expression of salivary MMPs and IL-1β in the PPA assays found by the present study indicates that these proteomic antibody membranes may be more sensitive to proteins presented in high concentrations in human saliva than to those of relatively low concentrations.

Vitorino et al. (2012), reported issues including sensitivity and reproducibility associated with proteomic techniques such as two-dimensional gel electrophoresis (2-DE), which may represent limitations to obtain reliable data. However, to bypass these challenges new approaches and alternative techniques are developing. For instance, for reasons of reproducibility, Vitorino et al. (2012) used proteomics analysis with iTRAQ (isobaric tags for relative and absolute quantification reagents) which identified higher number of various peptides and discrete quantities of many proteins in saliva samples (Vitorino et al. 2012). Reagent driven and protein driven cross reactivity in antibody microarrays and multiplexed sandwich assays with reagent mixing have been reported, such as the cross reactivity that occurs between the capture and detective antibodies of the assays, or between these antibodies and proteins other than the targets, the possibility of such cross reactivity might increase proportionally with the number of targets (Pla-Roca et al. 2012; Juncker et al. 2014). All these issues were considered when conducting the present study. Though, 34 specific antibodies for proteases and 102 specific antibodies for cytokines spotted in duplicate on each PPA membrane, cross reactivity may occur. This cross reactivity may be due to the antibodies spots are closely located to each other with no barrier or separation between them. As well as, some proteins may be genetically or structurally related. In addition to that, each reagent/sample was loaded on each membrane to be exposed to all the capture antibodies spots at the same time and the membranes were rocked on a platform shaker during the incubations, which may increase the chance for cross reactivity. Differences in the abundance of salivary proteins and inter/intra-individual variations may affect the detection of some proteins such as MMPs and interleukins. Also there is the possibility of immune complexes being formed in mixed saliva samples, which may compromise detection. Hence, to overcome cross reactivity and for the purposes of sensitivity, and reproducibility, the assays were repeated 6 times for each array. Three PPA assays were carried out

using individual healthy and individual periodontitis samples. As well as three assays with saliva samples, which comprised a pool of 3 individual healthy or 3 individual periodontitis samples. It is interesting to note that the relative expression of the proteins was higher in the pooled sample protease assays than the single sample assays for the periodontitis samples, and for both the healthy and periodontitis samples in the pooled cytokine assays than the single sample assays. It is not clear whether these differences are explained by technical artefact (and hence 'false positive' differences), variation between samples and/or genuine increased sensitivity to differences between health and disease when pooled samples are employed. Also, there were many instances of substantial numerical differences in proteins expression between health and disease but which were not statistically significantly different; these findings cannot be confirmed until further samples have been analysed (e.g. by ELISA). It is important to emphasise; however, that there was substantial consistency between analysis of individual saliva samples and pooled samples in as much as the majority of statistically significant differences detected by analysis of 3 individual saliva samples were confirmed by analysis of pooled samples. Thus, 2 of these markers (UPA and uPAR) were selected for further, detailed, analysis (see below).

PPA assays were used for the first time in this study for saliva proteome profiling in periodontitis. Previously published studies have used different proteomic techniques to identify candidate biomarkers in saliva or GCF for periodontal diseases. Using different techniques including: two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), n-terminal amino acid sequencing, MS, and immunostaining (western blot), Kojima et al. (2000) detected 2 members of calcium binding proteins of the s100 family in saliva and GCF of 10 periodontitis patients. The detection of high levels of both MRP8 (S100A8) and MRP14 (S100A9), led to the hypothesis that these two S100 proteins may play an important role in pathogenesis of periodontal diseases (Kojima et al. 2000). In agreement with (Kojima et al. 2000), the same S100 proteins were detected in whole unstimulated saliva of five patients with generalized aggressive periodontitis (GAgP) using 2-D PAGE and electrospray tandem MS (Wu et al. 2009). Third study identified the S100A9 protein in saliva of chronic periodontitis patients (Goncalves Lda et al. 2010). In addition to S100A8/A9 another S100 protein (A6) were identified in high abundance in saliva of periodontitis patients

(Haigh et al. 2010). The detection of S100 proteins, were confirmed by a fifth study using MS analysis of whole saliva from twenty periodontitis patients (Salazar et al. 2013).

MMPs, Kalllikreins and cystain C were among the salivary proteins identified by the PPA assays in the present study as potential biomarkers, which was in agreement with previous non-proteomic studies (Kathariya 2010). Besides VDBP (which was identified in the present study) and S100 proteins, 9 other proteins were identified in the proteome profile of saliva from GAgP patients, such as albumin, Igγ2 chain C region, Igα2 chain C region, α-amylase, zinc-α2 glycoprotein and lactoferrin (Wu et al. 2009). In agreement with the Wu et al. (2009) study, Goncalves Lda et al. (2010) also used 2-D gel electrophoresis but with LC techniques to identify a number of proteins in whole unstimulated saliva of 10 chronic periodontitis patients, including along with S100A, other proteins such as serum albumin, haemoglobin, α-amylase, cystatins, and transferrin, these findings gave a novel perception about the salivary proteins alterations in periodontal diseases (Goncalves Lda et al. 2010). Haigh et al. (2010), used quantitative proteomics (2-D PAGE and MS) to investigate the salivary proteome profile in samples of 9 patients with severe periodontitis before and after treatment, the study demonstrated that among 128 proteins identified, 15 proteins were significantly altered after treatment including S100 proteins, haptoglobin, prolactin inducible protein and parotid secretory protein. The results were in agreement with previous studies regarding the involvement of S100 proteins in the pathogenesis of periodontitis, as well as, led to the identification of new potential biomarkers to be used in monitoring the disease progression (Haigh et al. 2010).

In their search for biomarkers that have the ability to predict periodontal diseases, Bostanci et al. (2010) used the quantitative proteomic approach LC-MS to investigate the GCF proteome profile in 5 patients with aggressive periodontitis in comparison to healthy controls. The results revealed that the GCF proteins cystatin-B and alpha defensin-1 were detected only in the healthy subjects and the annexin-1 was 5 fold higher in the healthy subjects' samples as compared to the patients. Whereas the actin bundling protein L-plastin was only detected in the GCF of the patients (Bostanci et al. 2010). L-plastin, which is exclusively expressed by leukocytes, plays a crucial role in immune-mediated events (Ozturk et al. 2015). A recent study used quantitative real-time PCR and ELISA approaches, identified high levels of L-plastin

in GCF samples of chronic and generalized aggressive periodontitis patients (Ozturk et al. 2015).

Pursuing studies to analyse salivary proteome using 2-D PAGE, LC and MS techniques, one study found that there was a difference in the salivary proteome profile of 10 patients with gingivitis in comparison to 10 healthy controls (Goncalves Lda et al. 2011). High levels of proteins such as α-amylase, albumin, haemoglobin, immunoglobulin peptides and keratins were detected in saliva of the gingivitis patients, whereas salivary cystatins were higher in the controls, the study results highlighted a new salivary proteome profile which may aid in the diagnosis and monitoring of gingivitis (Goncalves Lda et al. 2011). MS proteome analysis of whole saliva from obese patients with and without periodontitis detected 8 candidate biomarkers such as albumin, α and β haemoglobin chains and α-defensins (Range et al. 2012). The study concluded that periodontal inflammation may modify the whole saliva proteome profile in obese patients, and that α-defensins may be associated with gingival inflammation, therefore, might explain the high susceptibility of obese patients to periodontitis (Range et al. 2012). A study was carried out to investigate the salivary proteome profile in type 2 diabetes mellitus (T2DM) patients with and without periodontitis (Chan et al. 2012). The authors used 2-D PAGE and LC-MS/MS techniques to investigate the changes in the salivary proteome profile of the patients and found that seven proteins, including polymeric immunoglobulin receptor, plastin-2, actin related protein 3, leukocyte elastase inhibitor, carbonic anhydrases 6, immunoglobulin J and interleukin-1 receptor antagonist, to be differentially and significantly expressed ($p<0.01$) in the T2DM patients with periodontitis as compared to the periodontally healthy patients. The study suggested that these proteins may have the potential to be used as biomarkers for the prediction of T2DM patients who are at risk of periodontitis (Chan et al. 2012). Using LC-MS/MS analysis of whole unstimulated saliva from 20 patients with periodontitis in comparison to 20 healthy controls demonstrated that, including the previously mentioned S100 proteins, a total of 344 proteins were detected in both groups, with 152 proteins identified with more than one unique peptide, of which 20 proteins were significantly higher in the periodontitis patients such as lacto-peroxidase, catalase, MMP-9, neutrophil collagenase, neutrophil defensin, complement C3 and others (Salazar et al. 2013). The study concluded that the proteome profile analysis of whole unstimulated saliva

is an efficacious means to characterize and differentiate between periodontitis patients (during acute phase of the disease) and healthy subjects (Salazar et al. 2013).

Gel free and gel-based electrophoresis along with LC-MS/MS techniques were used in the proteomic analysis of GCF for discovery of novel periodontal disease biomarkers (Tsuchida et al. 2012). A total of 327 proteins were identified in GCF of healthy subjects as compared to supra-gingival saliva. Among these proteins, some of which were found to be significantly expressed in GCF especially superoxide dismutase 1 (SOD1), apolipoprotein A-I (ApoA-I), and dermcidin (DCD). Suggesting future proteomic studies for these proteins as potential GCF biomarkers for periodontal diseases (Tsuchida et al. 2012). Baliban et al. (2013), used high-performance LC, tandem MS, and the pilot protein algorithm, with a mixed-integer linear optimization (MILP) model to identify GCF biomarker combinations of which can distinguish a blind subject sample as healthy or diseased. Glyceraldehyde 3-phosphate dehydrogenase, thymidine phosphorylase and Ig kappa chain V-I region AG, were identified as protein biomarkers for periodontally healthy status. The study reported that this novel biomarkers combination have greater than 95% predictive accuracy in the diagnosis of periodontal status; however, the authors recommended further investigations regarding the roles of these proteins (Baliban et al. 2013).

In respect to the protein ADAM8 (A disintegrin and metalloproteinase domain-containing protein 8), the present study was the first to detect this protein in saliva of periodontitis patients. The single and pooled sample protease PPA assays revealed that this protein was both numerically and significantly highly expressed in saliva of periodontitis patients as compared to healthy volunteers, this finding was in harmony with 3 recent studies which investigated this protein in relation to periodontal diseases. Though these 3 studies used techniques other than PPA assays, all of them reported high levels of ADAM8 in patients with periodontal diseases. Using sodium dodecyl sulphate polyacrylamide gel electrophoresis with immunoblotting and ELISA assays, Khongkhunthian et al. (2013) detected significantly high levels of ADAM8 in GCF of patients with gingivitis, chronic and aggressive periodontitis as compared to healthy control subjects and these high levels of ADAM8 were positively correlated with the clinical periodontal measures. The second study used ELISA assay to detect significantly elevated levels of ADAM8 in GCF samples of patients

with chronic periodontitis in comparison to healthy controls, and these high GCF ADAM8 levels were positively associated with the clinical parameters of periodontitis (Elavarasu et al. 2015). Finally, ADAM8 expression was investigated in gingival epithelial cells obtained from 33 patients with chronic periodontitis in comparison to 23 healthy subjects (Aung et al. 2017). The real time PCR, immunoblotting, immunohistochemistry and flow cytometry investigations of the epithelial cells reported significantly elevated mRNA and protein expression of ADAM8 in the periodontitis patients as compared to the healthy volunteers. Moreover, the study reported consistent upregulation of ADAM8 expression in the gingival epithelial cells but not gingival fibroblasts in response to stimulation with *F. nucleatum* bacteria (Aung et al. 2017). Therefore, these studies may justify the detection and the significantly high expression of ADAM8 in saliva of periodontitis patients by the present study.

Many of the proteins detected in the previously mentioned salivary proteomic studies were also identified in the present study, which may justify the use of the PPA assays to identify candidate biomarkers in saliva of chronic periodontitis patients. Furthermore, the difference in the proteome profile of whole unstimulated saliva from the periodontitis patients and healthy controls found by the present study, was in agreement with many of the above studies in regard to the ability of periodontal inflammation to alter the salivary proteins profile. However, with an exception for Salazar et al. (2013) who identified MMP-8 and MMP-9, and the present study which detected the same MMPs (though they were variably expressed) as well as MMP-2, -3, & -12, and number of interleukins (such as IL-8), up to the time conducting the present study, no other proteomic study was able to detect MMPs and IL-1β in saliva and GCF of patients with periodontal diseases (Haigh et al. 2010; Amado et al. 2013).

3.3.2 Identified salivary biomarkers and periodontitis

Based on the results of the protease and cytokine PPA assays and in a review of the previous clinical and experimental studies, three candidate salivary biomarkers for periodontitis were identified including: uPA, uPAR and VDBP. uPA and uPAR were consistently elevated in periodontitis samples as compared to healthy samples and these differences were statistically significantly different. VDBP was also selected as good analysis reagents were available for this protein and there were substantial

numerical differences between the patients and healthy samples although these were not statistically significantly different.

Gustafsson et al. (2011), carried out the only published study on salivary uPAR and they have reported the analysis of soluble uPAR (suPAR) in saliva and plasma of healthy non-smoking adults. Their detection of uPAR in saliva was in agreement with the present study for the identification of uPAR in human saliva by PPAs; however, Gustafsson et al. (2011) results were not correlated with any oral or systemic disease. The present study was the first that identified uPA in saliva of periodontitis patients and investigated both its levels and enzymatic activity. There was a single study carried out by Virtanen et al. (2006) who investigated uPA activity along with other plasminogen activators (PAs) and inhibitors (PAIs) in human saliva and salivary gland tissues, but their study had no relation with any periodontal disease. However, the presence of an active uPA and other PA activators in human saliva found by Virtanen et al. (2006), might explain the identification of salivary uPA by the PPA protease assays in the present study.

Though they were not identified or measured in saliva of chronic periodontitis patients, uPA/uPAR and other components of the PA system were investigated in GCF and gingival epithelium of patients with different forms of periodontal diseases (Ogura et al. 1995; Kinnby et al. 1996; Ogura et al. 1999; Ogura et al. 2001; Buduneli et al. 2004; Buduneli et al. 2005; Smith and Martinez 2006; Sulniute et al. 2011; Fleetwood et al. 2015). These studies related uPA/uPAR and other PA system components to gingivitis and periodontitis, and studied those PA system proteins in response to inflammatory or periodontal bacterial stimuli. Therefore, as whole unstimulated saliva contains traces of GCF, and inflammatory exudate from all sites of periodontal disease, this might explain the identification of uPA and uPAR in saliva of chronic periodontitis patients by the present study.

In regard to the identification of salivary VDBP, few studies investigated this protein in oral fluids (saliva and GCF) of patients with periodontal diseases. In agreement with the present study, a clinical study carried out in 1987 detected VDBP in saliva samples of periodontitis patients; however, the study didn’t measure the protein levels following treatment (Krayer et al. 1987). Though it was carried out on GAgP patients, using techniques other than PPA assays including 2D gel electrophoresis

and electrospray ionization tandem mass spectrometry, the protein profile analysis of whole unstimulated saliva carried out by Wu et al. (2009), revealed that, VDBP was one of 11 proteins identified in the GAgP patients in significantly elevated levels as compared to healthy controls (the VDBP was 1.7 folds higher in the GAgP patients), this finding is in agreement with the present study for the identification of VDBP in saliva of chronic periodontitis patients.

All the studies focusing on the identified biomarkers and their possible roles in periodontitis will be discussed in details in their related chapters.

3.3.3 Summary of findings

The PPA assays demonstrated that: the proteome profile of whole unstimulated saliva obtained from patients with untreated chronic periodontitis, differs from that of healthy control subjects, this finding suggested that periodontitis may affect the proteome profile of saliva. Referring to the PPA assays and in review of previous studies, three candidate salivary biomarkers for periodontitis were identified including: uPA, uPAR and VDBP, though the latter was identified in 1987, it was not studied following periodontal treatment. The PPA assays, are simple, readily usable, with reasonable costs (i.e. do not need expensive or sophisticated equipment when compared to other proteomic techniques), able to detect wide range of proteins and to investigate the proteome profile of saliva. However, it is recommended to perform multiple assays for the reasons of reproducibility and to over-come cross reactivity and sensitivity issues.

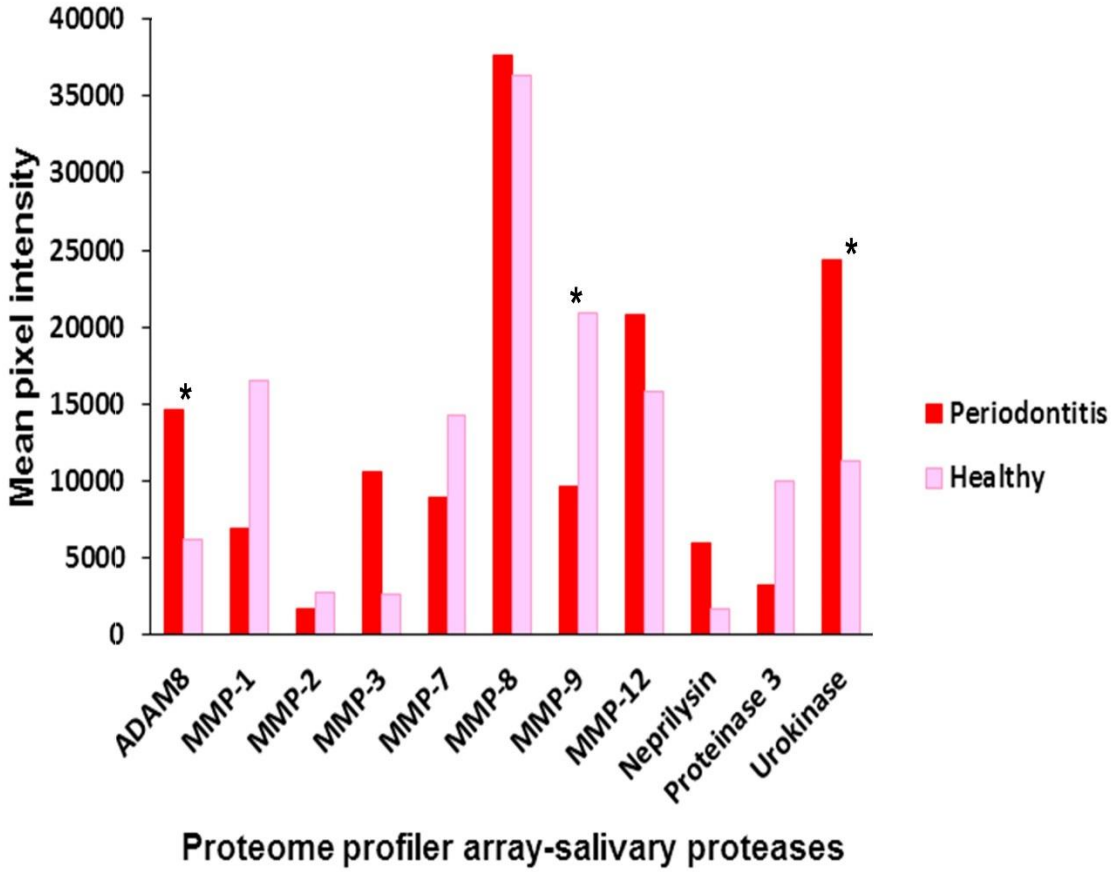

Figure 3.1: The analysis of three single saliva samples using proteome profiler arrays for salivary proteases.

Three independent single sample protease arrays were performed on 6 whole unstimulated saliva samples obtained from 3 untreated chronic periodontitis patients and 3 healthy volunteers. Two samples were analysed in each assay, one of periodontitis patient and one of healthy subject. The bars represent the mean pixel intensity values for the relative expression of 11 proteins obtained from the analysis of the three single sample assays. Statistics: One sample t-test was used to determine the p-value of the log-fold change (*=$p<0.05$).

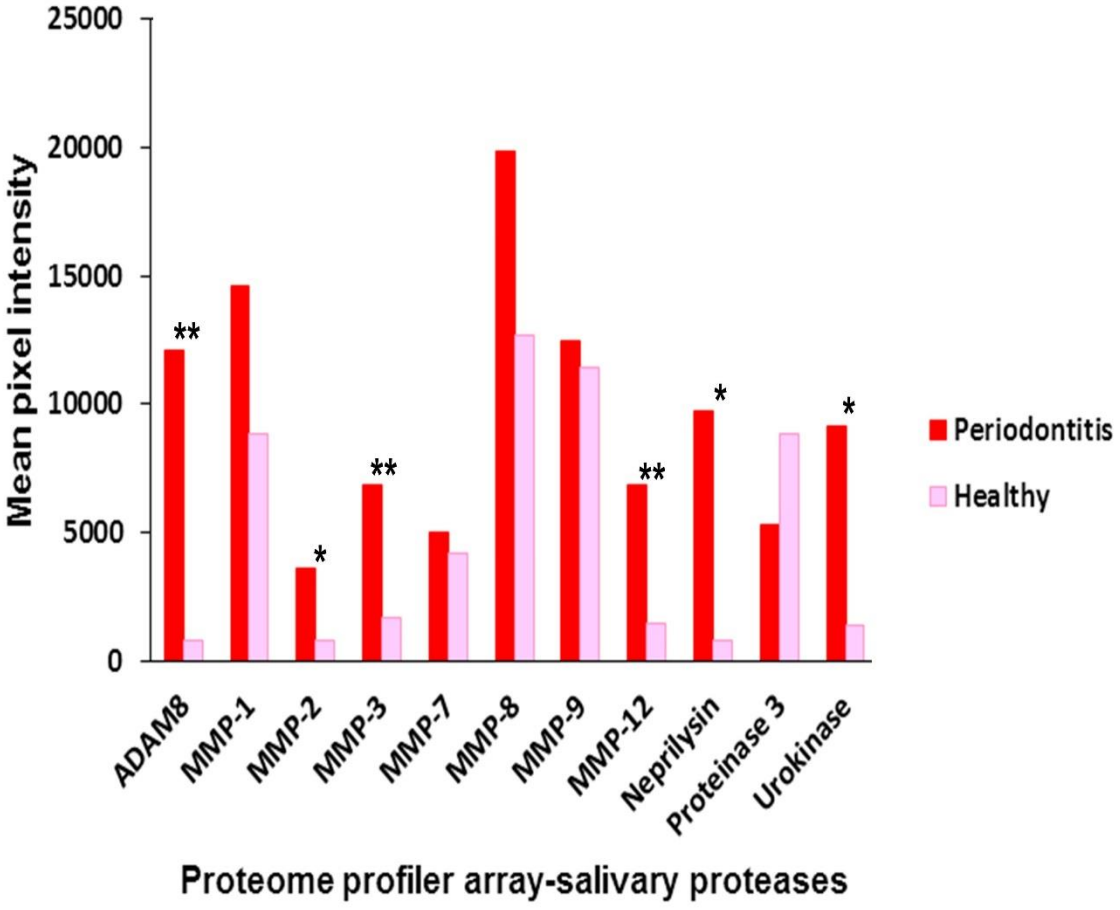

Figure 3.2: The analysis of three pooled saliva samples using proteome profiler arrays for salivary proteases.

Three independent pooled sample protease arrays were performed on pooled saliva samples. Two pooled samples were analysed in each assay (and the assay repeated twice further using the same pools), each pooled sampled consisted of 3 periodontitis or 3 healthy samples mixed in equal volumes. The bars represent the mean pixel intensity values for the relative expression of 11 proteins obtained from the analysis of the three pooled sample assays. Statistics: One sample t-test was used to determine the p-value of the log-fold change (*=$p<0.05$, and **=$p<0.01$ as indicated).

Salivary proteases	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
ADAM8	6177 ±1266	14659 ±4958	+ 2.37	<0.05
MMP-1	16586 ±4263	6894 ±1572	- 2.41	NS
MMP-2	2805 ±2116	1750 ±260	- 1.6	NS
MMP-3	2699 ±1106	10656 ±5540	+ 3.95	NS
MMP-7	14219 ±12614	8881 ±5481	- 1.6	NS
MMP-8	36296 ±9667	37598 ±11532	+ 1.03	NS
MMP-9	20909 ±10961	9619 ±4173	- 2.17	<0.05
MMP-12	15781 ±7305	20775 ±9153	+ 1.32	NS
Neprilysin	1742 ±722	5959 ±3361	+ 3.42	NS
Proteinase 3	10059 ±7727	3262 ±1282	- 3.08	NS
Urokinase uPA	11350 ±4723	24370 ±6599	+ 2.15	<0.05

Table 3.1: Relative expression of salivary proteases in periodontitis samples as compared to healthy samples.

The relative expression of 11 salivary proteases were identified in 3 single sample protease PPA assays. Data are presented as mean and standard error of the mean (SEM) of the pixel intensity (PI) values. Proteins were compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant).

Salivary proteases	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
ADAM8	830 ±36	12059 ±945	+ 14.52	<0.01
MMP-1	8809 ±3045	14609 ±1755	+ 1.66	NS
MMP-2	808 ±24	3618 ±925	+ 4.48	<0.05
MMP-3	1679 ±495	6831 ±1822	+ 4.07	<0.01
MMP-7	4183 ±2134	4967 ±914	+ 1.19	NS
MMP-8	12659 ±2399	19843 ±1289	+ 1.57	NS
MMP-9	11411 ±893	12487 ±1633	+ 1.09	NS
MMP-12	1463 ±176	6823 ±770	+ 4.66	<0.01
Neprilysin	798 ±272	9739 ±801	+ 12.19	<0.05
Proteinase 3	8846 ±1036	5295 ±999	- 1.67	NS
Urokinase uPA	1363 ±364	9102 ±563	+ 6.68	<0.05

Table 3.2: Relative expression of salivary proteases in periodontitis samples as compared to healthy samples.

The relative expression of 11 salivary proteases identified in 3 pooled sample protease PPA assays. Data are presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant).

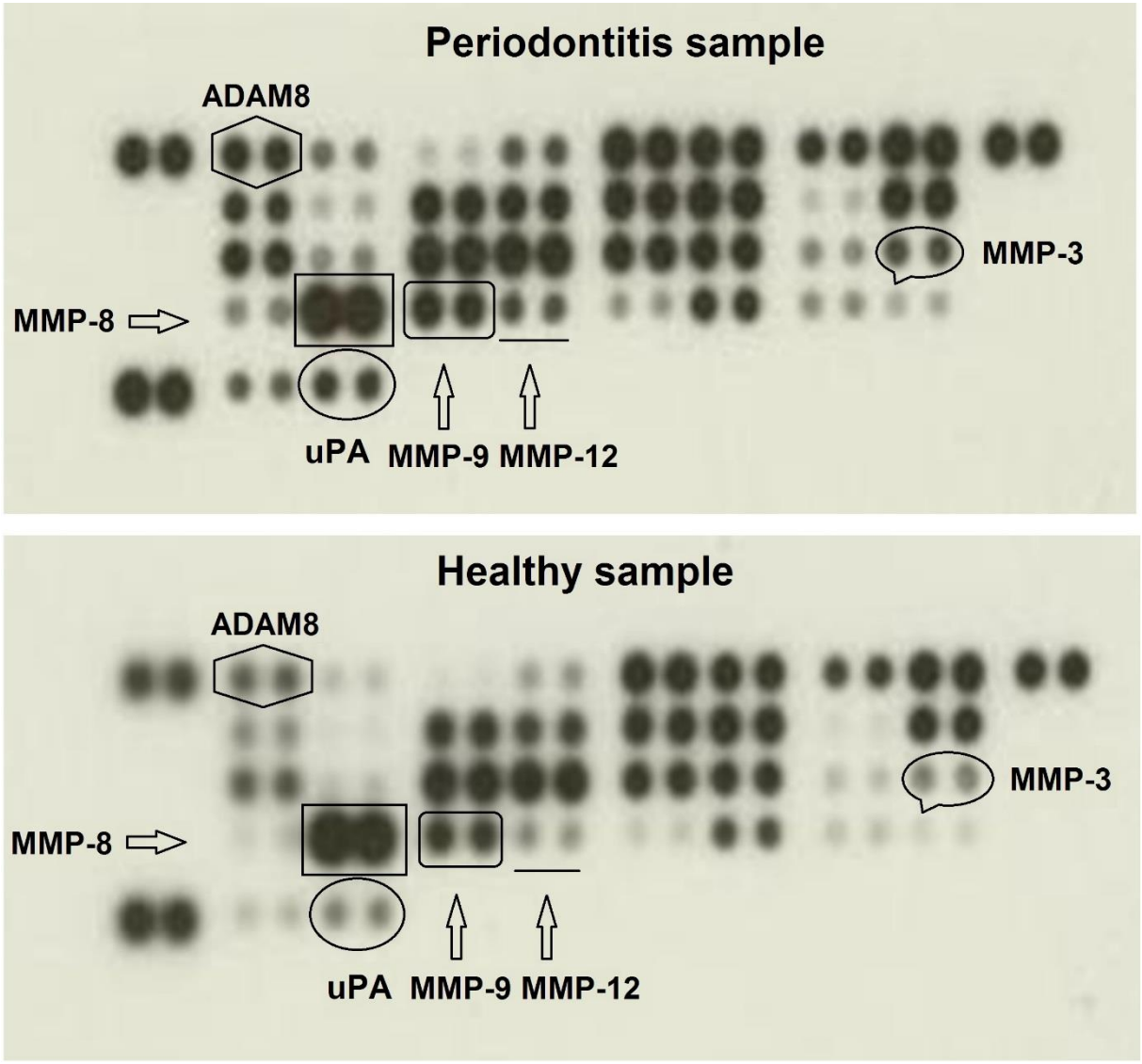

Figure 3.3: Radiography for protease proteome profiler arrays.

Following the radiography of the protease PPA assays, the radiographs were scanned for image analysis. These are scanned images of the radiographs for one of the single sample protease PPA assays for periodontitis and healthy samples. On each image, each duplicate spot represents a precise protein, and there are 3 duplicate spot positive control references on the 3 corners of the image with one duplicate spot negative control reference located near to the last protein. The marks represent the positions of the identified candidate salivary biomarker urokinase (uPA), the well-known biomarkers MMP-8 and MMP-9, and other proteases such as ADAM8, MMP-3, and MMP-12. The expression of the labelled proteins in this assay was relatively higher in the periodontitis samples as compared to the healthy sample.

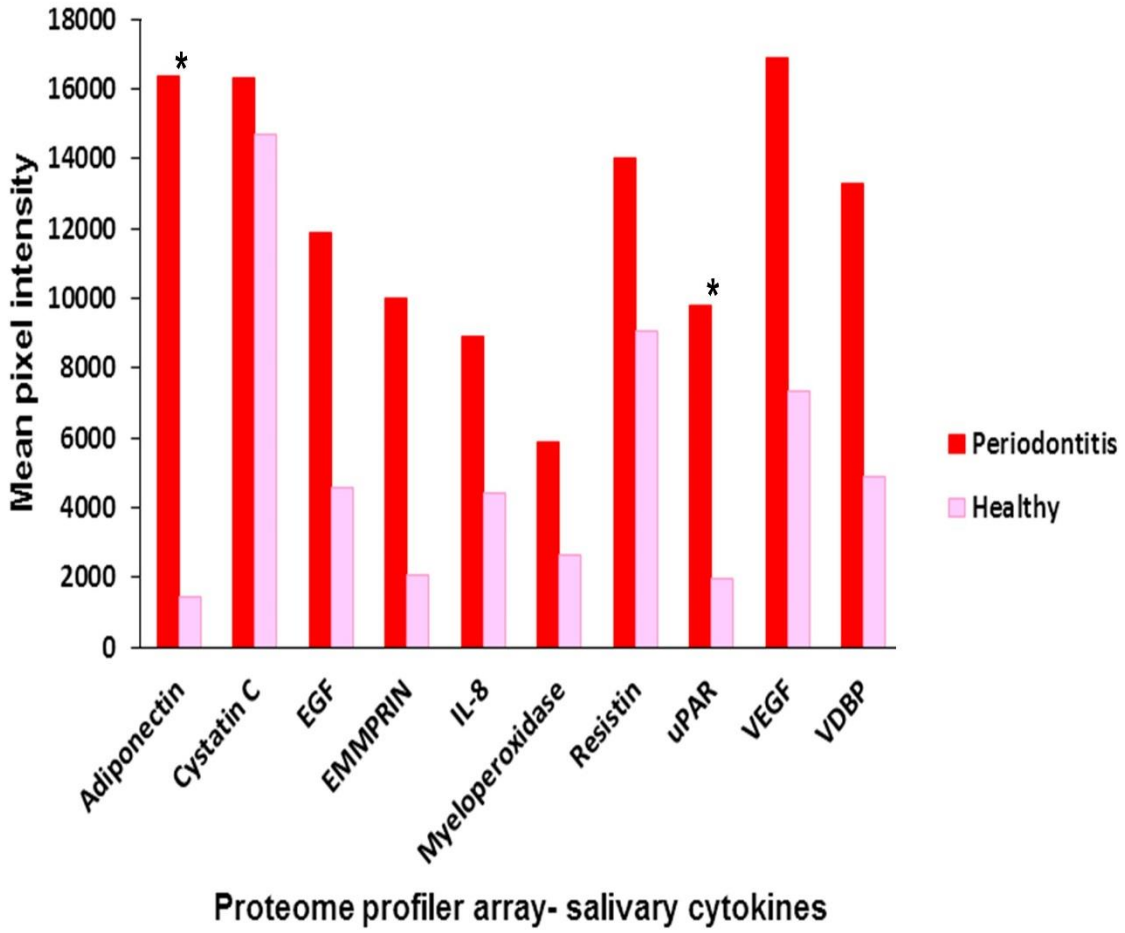

Figure 3.4: The analysis of three single saliva samples using proteome profiler arrays for salivary cytokines.

Three independent single sample cytokine arrays were performed on 6 whole unstimulated saliva samples obtained from 3 untreated chronic periodontitis patients and 3 healthy volunteers. Two samples were analysed in each assay, one of periodontitis patient and one of healthy subject. The bars represent the mean pixel intensity values for the relative expression of 10 proteins obtained from the analysis of the three single sample assays. Statistics: One sample t-test was used to determine the p-value of the log-fold change (*=$p<0.05$).

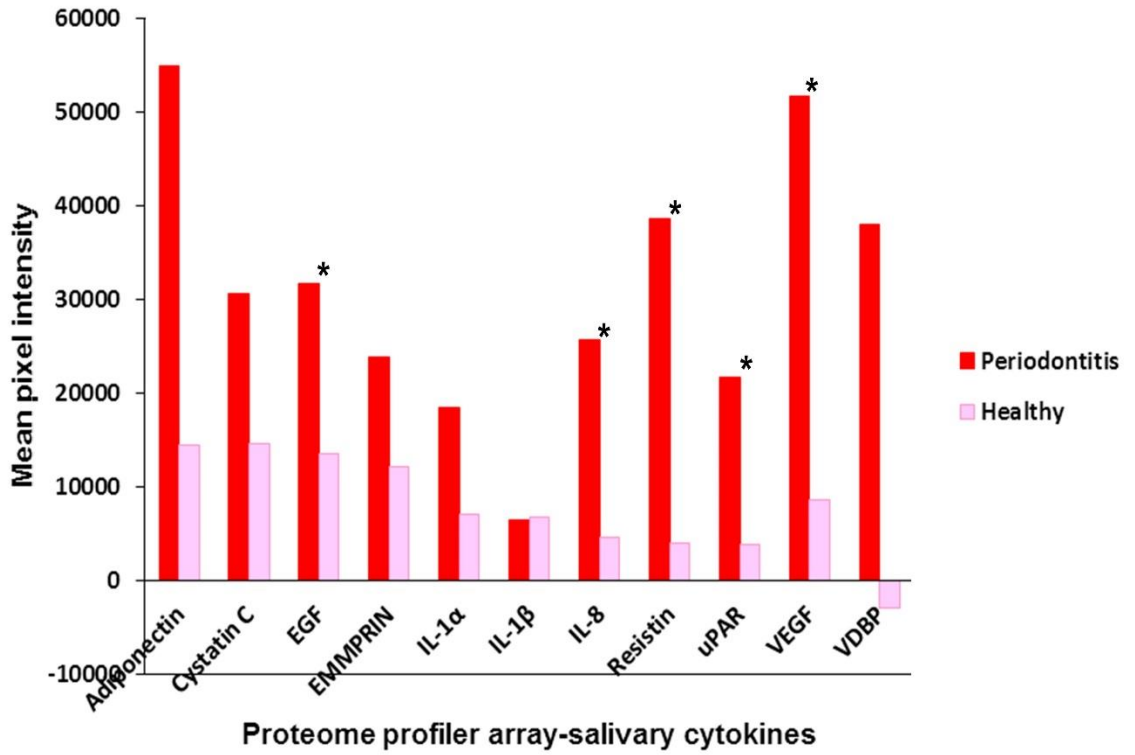

Figure 3.5: The analysis of three pooled saliva samples using proteome profiler arrays for salivary cytokines.

Three independent pooled sample cytokine arrays were performed on pooled saliva samples. Two pooled samples were analysed in each assay (and the assay repeated twice further using the same pools), each pooled sampled consisted of 3 periodontitis or 3 healthy samples mixed in equal volumes. The bars represent the mean pixel intensity values for the relative expression of 11 proteins obtained from the analysis of the three pooled sample assays. Statistics: One sample t-test was used to determine the p-value of the log-fold change (*=$p<0.05$).

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Adiponectin	1455 ±348	16358 ±9770	+ 11.24	<0.05
Cystatin C	14711 ±6523	16338 ±8525	+ 1.11	NS
EGF	4553 ±2066	11868 ±9000	+ 2.61	NS
EMMPRIN	2071 ±1665	10009 ±8962	+ 4.83	NS
IL-8	4434 ±4197	8917 ±7184	+ 2.01	NS
Myeloperoxidase	2631 ±2788	5866 ±4943	+ 2.23	NS
Resistin	9039 ±8517	13996 ±8541	+ 1.55	NS
uPAR	1962 ±1543	9811 ±7743	+ 5	<0.05
VEGF	7321 ±6861	16900 ±15147	+ 2.31	NS
VDBP	4898 ±786	13290 ±7078	+ 2.71	NS

Table 3.3: Relative expression of salivary cytokines in periodontitis samples as compared to healthy samples.

The relative expression of 10 salivary cytokines identified in 3 single sample cytokine PPA assays. Data are presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant).

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Adiponectin	14406 ±13661	54877 ±46998	+ 3.81	NS
Cystatin C	14533 ±9730	30613 ±22256	+ 2.11	NS
EGF	13569 ±11214	31755 ±25132	+ 2.34	<0.05
EMMPRIN	12093 ±11661	23814 ±20195	+ 1.97	NS
IL-1α	7044 ±6673	18526 ±15308	+ 2.6	NS
IL-1β	6722 ±6825	6395 ±5766	- 1.05	NS
IL-8	4650 ±3622	25759 ±20951	+ 5.54	<0.05
Resistin	3921 ±2796	38600 ±28020	+ 9.84	<0.05
uPAR	3844 ±3117	21691 ±17627	+ 5.64	<0.05
VEGF	8623 ±7651	51721 ±47079	+ 6	<0.05
VDBP	-2891 ±7659	37974 ±28669	NA	NA

Table 3.4: Relative expression of salivary cytokines in periodontitis samples as compared to healthy samples.

The relative expression of 11 salivary cytokines identified in 3 pooled sample cytokine PPA assays. Data presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant). NA not applicable.

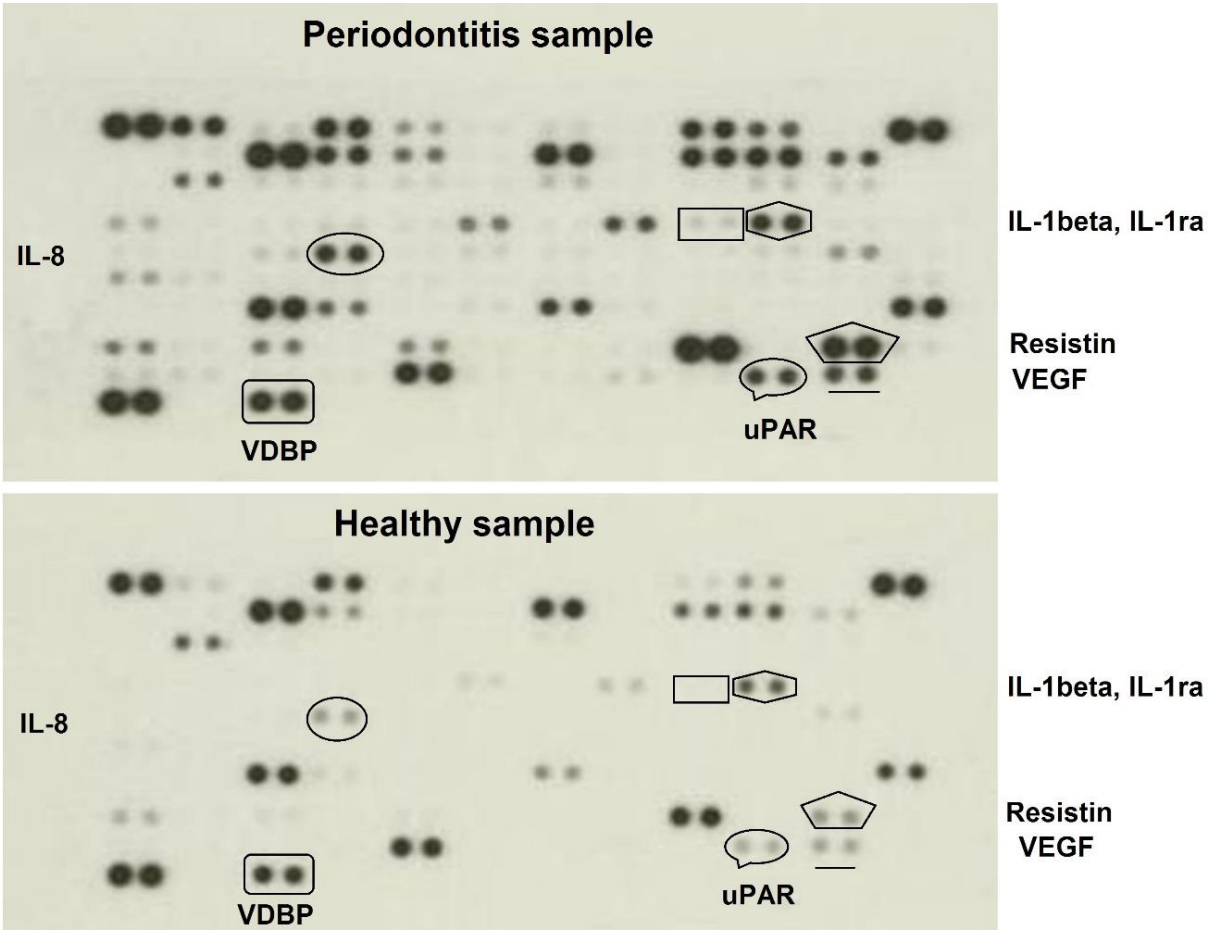

Figure 3.6: Radiography for cytokine proteome profiler arrays.

The radiographs of the cytokine PPA assays were scanned for image analysis. These are scanned images of the radiographs for one of the pooled sample cytokine PPA assays for periodontitis and healthy samples. On each image, each duplicate spot represents a precise cytokine, and there are 3 duplicate spot positive control references on the 3 corners of the image with one duplicate spot negative control reference located on the lower right corner of the image. The marks represent the positions of the identified candidate salivary biomarkers urokinase receptor (uPAR) and vitamin D binding protein (VDBP), the well-known biomarker IL-1β, and other cytokines such as IL-1ra, IL-8, resistin, and VEGF. In this assay, the labelled cytokines' expressions were relatively higher in the periodontitis sample as compared to the healthy sample.

Chapter 4 Investigation of the association of salivary and GCF uPA and uPAR with periodontitis

4.1 Introduction

As the results of experiments described in the previous chapter, a number of proteins have been identified by the protease and cytokine PPA assays in saliva of patients with untreated chronic periodontitis. Among the identified proteins were the urokinase plasminogen activator (uPA) and its receptor (uPAR). Both the uPA and uPAR belong to the plasminogen activating (PA) system. In the presence of a stimulus (host-derived or bacterial), uPA binds to its receptor uPAR, this binding activates and localizes uPA on the cell surface, and in turn uPA converts plasminogen (the key component of the PA system) into active plasmin, thereby initiating the activities of the PA system (Crippa 2007; Blasi and Sidenius 2010; Smith and Marshall 2010). Once the PA system has been activated, the system will carry out a number of functions including, inflammatory cells chemotaxis, activation of matrix-metalloproteases and subsequently activation of extra-cellular matrix proteolysis (Ogura et al. 1999; Ogura et al. 2001; Buduneli et al. 2004; Buduneli et al. 2005; Crippa 2007; Blasi and Sidenius 2010; Smith and Marshall 2010). These pro-inflammatory activities take place during the pathogenesis of periodontitis. Therefore, on this basis, uPA and uPAR have been selected as candidate salivary biomarkers for periodontitis. The PA system proteins were investigated by a number of studies which detected the tissue plasminogen activator (tPA), plasminogen inhibitors (PAI-1 & PAI-2) and uPA in the GCF of patients with periodontal diseases (Kinnby et al. 1991; Kinnby et al. 1996; Yin et al. 2000; Buduneli et al. 2005). Positive correlations were found between these GCF PA proteins and different periodontal clinical measures. However, salivary uPA has not been investigated in periodontal diseases with the exception of a single study which investigated the uPA and tPA activities along with PAI-1 and 2 in the saliva and salivary gland tissues of patients with neurological conditions but the results were not related to any periodontal, oral or systemic disease (Virtanen et al. 2006). In a similar manner to uPA, a single study detected uPAR in saliva of healthy young adults (Gustafsson et al. 2011). The research presented in this chapter aimed to investigate whether uPA and uPAR levels elevated in saliva of patients with periodontal diseases and whether or not there is a relationship between these two salivary proteins levels and periodontitis.

4.2 Results

Following the identification of uPA and uPAR as candidate salivary biomarkers for periodontitis, ELISA assays were carried out to confirm the results of the PPA assays and to quantify the biomarkers in saliva and GCF samples of patients with periodontal diseases. Experiments were carried out on saliva samples obtained from 2 clinical studies (study A and study B, see Chapter 2, section 2.1.3), and on GCF samples obtained from the clinical study C (see Chapter 2, section 2.1.3). Human uPAR DuoSet ELISA, and human uPA Quantikine ELISA kits were used to measure the biomarkers levels in saliva and GCF samples obtained from the 3 studies. ELISA validation assays were carried out on each ELISA assay prior to use on the study samples in order to confirm the efficacy of these assays in measuring the desired mediators in saliva. The ELISA results were compared between the patients and control groups, and in the patients groups before and after non-surgical treatment. The results were correlated with the periodontal indices used for the clinical assessment of the patients and control subjects. Finally, the salivary uPA enzymatic activity was investigated using uPA activity fluorometric assay kits (Sigma-Aldrich), and the relationship of salivary uPA activity with both the salivary uPA levels and the periodontal disease indices was determined.

The results of the ELISA assays (concentrations pg/ml) and the uPA enzymatic activity assays (IU/ml), are presented either as median and interquartile ranges (IQR) [median (upper quartile-lower quartile)] when data were not normally distributed, or as mean and standard error of the mean SEM (mean ±SEM) when data were normally distributed.

All GCF samples were obtained from a previous project of the periodontal research group/School of Dental Sciences/Newcastle University, which may explain the limited number of GCF samples and volumes as compared to saliva samples.

4.2.1 ELISA validation assays

As saliva was not listed by the manufacturer among the samples that can be assayed by the uPAR DuoSet, the uPA DuoSet, and the uPA Quantikine ELISA assays and for the most precise quantification of the biomarkers, validation assays were carried out prior to use on the study samples. The validation assays included: determination of optimal dilution, recovery and linearity measurements, intra- and inter-assay

variations, and sensitivity of the assay (see Chapter 2, section 2.3.2, ELISA validation assays).

Determination of optimal dilution

Three saliva samples obtained from 3 healthy volunteers were used for each ELISA assay. For the uPA and uPAR DuoSet ELISA kits, the samples were assayed in 4 dilutions for each sample (neat, 1:10, 1:100, and 1:500). Whereas, for the uPA Quantikine ELISA, samples were assayed as neat, 1:2, and 1:3 dilutions. For each ELISA, the assay gave an indication for which dilution to be used in the next steps of the validation assays, and at which the desired biomarker falls within the range of the standard curve. The assay results revealed that the optimal dilutions for the ELISA assays were 1:100 for uPAR DuoSet ELISA, neat (no dilution) for uPA DuoSet ELISA, and 1:2 for uPA Quantikine ELISA.

Recovery and linearity measurements

For each ELISA assay, one saliva sample was used as the neat sample to be spiked in the recovery and linearity assays. The sample to be spiked was diluted according to the optimal dilution factor for each ELISA. In addition to the saliva sample, a control was also used in both recovery and linearity assays, and the control was reagent or calibrator diluent only. Both assays were carried out on the same plate. Recovery was useful to check the ability of each ELISA assay to measure the desired biomarker in saliva samples. Recovery was carried out by spiking both the sample and control with the same known amount of the standard recombinant protein supplied with each assay kit selected from or near to the middle of the standard curve, and the resultant concentrations were expected to be same as or near to that used for spiking. In the uPA DuoSet ELISA, both the sample and control were spiked with 1000 pg/ml of the uPA standard. In the uPAR DuoSet ELISA, both the sample and control were spiked with 500 pg/ml of the uPAR standard. In the uPA Quantikine ELISA, both the sample and control were spiked with 500 pg/ml of the uPA standard. Recovery values between 80 and 120% generally acceptable (Jaedicke et al. 2012), results presented in Table 4.1.

For each ELISA assay, linearity was determined in the same saliva sample (as neat and spiked) along with the spiked control in different dilutions. In both the uPA and uPAR DuoSet ELISAs: the neat, spiked sample and spiked control were assayed in 4

dilutions (1:2, 1:4, 1:8, and 1:16). Whereas in the Quantikine uPA ELISA: the neat, spiked sample and spiked control were assayed in 2 dilutions only (1:2 and 1:4). Same as recovery, linearity values between 80 and 120% were considered to be acceptable, results are presented in Table 4.1.

Intra- and inter-assay variations

Usually, the intra- and inter-assay variations (precision) are listed by the manufacturer, if not they should be determined along with the validation assays. The intra- and inter-assay variations values determined with the coefficient of variation CV%, values less than 10-15% generally acceptable (R & D systems) and (Hanneman et al. 2011). For the uPAR DuoSet ELISA assay, the intra-assay variation was carried out on three different saliva samples obtained from three healthy volunteers, assayed in triplicates on the same plate. The values were: 7.2, 2.4, and 5.3 CV% respectively (Table 4.2). Whereas, the inter-assay variations analysed three different saliva samples in three different plates on three separate days. The values were: 3.9, 4.4, and 2.8 CV% respectively (Table 4.3). For the uPA Quantikine ELISA the values were obtained from the manufacturer (R & D systems), the intra-assay variation values were: 2.1, 1.4, and 2.4 CV% respectively, and the inter-assay variation values were: 7.1, 6.5, and 6.9 CV% respectively. The assays were not carried out on the uPA DuoSet ELISA. These variation assays gave an indication about the reproducibility of the data within one assay and between assays performed on different days.

Assay sensitivity

ELISA manufacturers usually list information about the assay sensitivity in their kits leaflets, which indicates the smallest concentration of the desired protein the precise assay can measure in a sample. The assay was carried out on the uPAR DuoSet ELISA by assaying 20 replicates of reagent diluent in one plate. For the uPA Quantikine ELISA, the sensitivity values were obtained from R & D systems. The assay was not performed for the uPA DuoSet ELISA. Assay sensitivity values obtained as “the mean of assay results for 20 zero standard replicates + 2 standard deviations of the mean”. The resultant value presented as optical density (OD), which was used in the standard curve equation to calculate the minimum concentration of the desired protein to be detected by the precise ELISA assay.

For the uPAR DuoSet ELISA, the sensitivity was:
0.175+ (2 × 0.00831) = 0.192 this value represents the OD value at which the ELISA assay can detect minimum concentration of uPAR (1 pg/ml).

For the uPA Quantikine ELISA, values obtained from R & D systems: sensitivity was 0.931, and the minimum detectable concentration was 1.61 pg/ml.

Summary of ELISA validation assays

The results of ELISA validation assays revealed that the uPA DuoSet ELISA assay was not able to measure uPA in saliva samples, therefore, the assay was excluded and replaced by the uPA Quantikine ELISA assay. The results of the validation assays carried out on the uPA Quantikine ELISA along with the values obtained from R & D systems, revealed that the assay can measure uPA in saliva samples. For the uPAR DouSet ELISA, the validation assays revealed that the assay can measure uPAR in saliva samples. Hence, uPAR DuoSet ELISA and uPA Quantikine ELISA were used to measure the uPAR and uPA levels respectively in saliva samples of the present study. Validation assays were not performed on the GCF samples as they are treated same as cell culture supernatants and serum samples which are already validated by the manufacturer.

4.2.2 Investigations of salivary uPAR levels and relationship with periodontitis

The uPAR DuoSet ELISA kits were used to measure the uPAR levels in saliva samples obtained from the clinical study A. Investigations were then carried out to study the relationships of the uPAR levels in saliva samples with the periodontal disease indices used for the clinical assessment of periodontitis.

Salivary uPAR levels in untreated periodontitis patients

Experiments were carried out to measure the uPAR levels in saliva of 30 patients with untreated chronic periodontitis and 34 healthy control subjects (cross-sectional investigation). All samples were obtained at the baseline visit (visit 1) of the clinical study A (see Chapter 2, section 2.1.3, study A). Salivary uPAR levels were higher in the periodontitis patients [10672 (6918-22133) pg/ml], than in the healthy subjects [5296 (3528-9028) pg/ml] and the difference was highly significant (Mann-Whitney U test, $p<0.001$), (Figure 4.1).

Salivary uPAR levels in pre- and post-treatment samples of periodontitis patients

The uPAR levels were measured in saliva of 45 pre-treatment periodontitis patients and 40 healthy control subjects (cross-sectional investigation). All samples were obtained at visit 2 of the clinical study A (see Chapter 2, section 2.1.3, study A). Salivary uPAR levels were measured again in the same 45 periodontitis patients following non-surgical periodontal treatment (longitudinal investigation), the post treatment periodontitis samples were obtained at visit 6 which was 6 months after visit 2 of the clinical study A (see Chapter 2, section 2.1.3, study A). Salivary uPAR levels in the pre-treatment periodontitis patients [9540 (4482-16814) pg/ml] were higher than in the healthy subjects [4058 (1870-6651) pg/ml] and the difference was highly significant (Mann-Whitney U test, $p<0.001$), (Figure 4.2). However, the difference in the salivary uPAR levels of the periodontitis patients at the pre-treatment visit [9540 (4482-16814) pg/ml] and the post-treatment visit [8842 (6805-14269) pg/ml], was not statistically significant (Wilcoxon signed rank test, $p>0.05$).

Salivary uPAR correlations with periodontal disease indices

Determination of Spearman's rank correlation coefficient (rho) was used to investigate the relationships of the salivary uPAR levels in 45 pre-treatment periodontitis patients and 40 healthy controls with the periodontal disease indices used for the clinical assessment of periodontitis in the clinical study A. These correlations gave information about the potential of salivary uPAR as a biomarker that can indicate the severity of chronic periodontitis. The statistical analysis of the salivary uPAR correlations revealed that there was a significant positive correlation between the salivary uPAR levels and each of: modified gingival index (MGI) ($rho=0.316$, $p<0.01$), probing depth (PD) ($rho=0.294$, $p<0.01$), percentage of bleeding on probing (%BOP) ($rho=0.380$, $p<0.001$), and clinical attachment loss (CAL) ($rho=0.361$, $p<0.01$), (Figure 4.3, Figure 4.4, Figure 4.5 & Figure 4.6).

Summary of the investigation of salivary uPAR

The salivary uPAR investigations revealed that the uPAR levels were significantly higher in the whole unstimulated saliva of patients with untreated chronic periodontitis as compared to the healthy control subjects, and these elevated levels were positively correlated with the periodontal disease indices of periodontitis. Though the uPAR levels in the whole unstimulated saliva of the periodontitis patients

were reduced following treatment, the difference was not statistically significant as compared to the pre-treatment status.

4.2.3 Investigations of the uPA levels in saliva and GCF and relationship with periodontitis

The uPA Quantikine ELISA kits were used to measure the uPA levels in saliva samples obtained from the clinical studies A and B, as well as to measure the uPA levels in GCF samples obtained from the clinical study C. Investigations were then carried out to study the relationships of the uPA levels in both saliva and GCF samples with the periodontal disease indices used for the clinical assessment of periodontitis.

Salivary uPA levels in pre- and post-treatment samples of periodontitis patients in study A

ELISA assays were carried out to measure the uPA levels in saliva samples of 45 pre-treatment periodontitis patients and 40 healthy control subjects (cross-sectional investigation), the samples were obtained at visit 2 of the clinical study A (see Chapter 2, section 2.1.3, study A). Assays were also carried out to measure the salivary uPA levels in the same 45 periodontitis patients following non-surgical treatment (longitudinal investigation), the post treatment periodontitis samples were obtained at visit 6 which was 6 months after visit 2 of the clinical study A (see Chapter 2, section 2.1.3, study A). Salivary uPA levels were higher in the pre-treatment periodontitis patients [910 (615-1866) pg/ml] as compared to the healthy subjects [206 (129-356) pg/ml] and the difference was highly significant (Mann-Whitney U test, $p<0.001$), (Figure 4.7). Salivary uPA levels were significantly reduced from [910 (615-1866) pg/ml] at the pre-treatment visit to [609 (461-1346) pg/ml] following non-surgical treatment (Wilcoxon signed rank test, $p<0.05$), (Figure 4.8).

Salivary uPA correlations with periodontal disease indices in study A

Determination of the Spearman's rank correlation coefficient was used to investigate the relationships of the salivary uPA levels in 45 pre-treatment periodontitis patients and 40 healthy control subjects with the periodontal disease indices used for the clinical assessment of periodontitis in the clinical study A. These correlations gave information about potential of salivary uPA as a biomarker that can indicate the severity of chronic periodontitis. The statistical analysis of the salivary uPA

correlations revealed that, there was a highly significant positive correlation between the salivary uPA levels and each of the indices: MGI (rho=0.716, p<0.001), PD (rho=0.770, p<0.001), %BOP (rho=0.781, p<0.001), and CAL (rho=0.724, p<0.001), (Figure 4.9, Figure 4.10, Figure 4.11, & Figure 4.12).

Salivary uPA levels in pre- and post-treatment samples of periodontitis patients in study B

Salivary uPA levels were measured in 5 groups of samples (cross-sectional investigation) obtained at visit 2 of the clinical study B (see Chapter 2, section 2.1.3, study B). The groups comprised samples from: 26 edentulous subjects, 29 dentulous healthy subjects, 25 gingivitis patients, 31 pre-treatment mild/moderate periodontitis patients, and 27 pre-treatment advanced periodontitis patients. The uPA levels were also measured in post-treatment saliva samples obtained from the same periodontitis patients at visit 3 of the clinical study B which was12±2 weeks after treatment (see Chapter 2, section 2.1.3, study B), (longitudinal investigation). The post-treatment samples comprised 31 saliva samples obtained from the mild/moderate periodontitis patients, and 27 saliva samples obtained from the advanced periodontitis patients. Salivary uPA levels were higher in all the groups [healthy 139 (55-238) pg/ml, gingivitis 318 (172-636) pg/ml, pre-treatment mild/moderate periodontitis 395±36 pg/ml, and pre-treatment advanced periodontitis 495 (430-948) pg/ml] as compared to the edentulous subjects [0.9 (0-60) pg/ml] and the difference was highly significant (Kruskal-Wallis with Mann-Whitney U test and Bonferroni correction, p<0.001), (Figure 4.13). Salivary uPA levels were higher in the gingivitis and the both pre-treatment periodontitis groups [318 (172-636) pg/ml, 395±36 pg/ml, and 495 (430-948) pg/ml respectively] as compared to the healthy subjects [139 (55-238) pg/ml] and the difference was highly significant (Kruskal-Wallis with Mann-Whitney U test and Bonferroni correction, p<0.001), (Figure 4.13). Salivary uPA levels were significantly higher in the pre-treatment advanced periodontitis patients [495 (430-948) pg/ml] than in the pre-treatment mild/moderate periodontitis patients (395±36 pg/ml) (Mann-Whitney U test, p<0.01), (Figure 4.13). There was no statistically significant difference in the salivary uPA levels between the gingivitis patients [318 (172-636) pg/ml] and the pre-treatment mild/moderate periodontitis patients (395±36 pg/ml) (Mann-Whitney U test, p>0.05), (Figure 4.13). Whereas, the uPA levels were higher in saliva of the pre-treatment advanced periodontitis patients [495 (430-948) pg/ml] than in saliva of the gingivitis patients [318 (172-636) pg/ml] and the difference

was statistically significant (Mann-Whitney U test, p<0.01), (Figure 4.13). Salivary uPA levels were significantly reduced in the post-treatment samples of the mild/moderate periodontitis patients (280±31 pg/ml) as compared to their pre-treatment levels (395±36 pg/ml) (Paired samples t-test, p<0.01), (Figure 4.14). The advanced periodontitis patients demonstrated significantly reduced levels of salivary uPA following non-surgical treatment [344 (221-609) pg/ml] as compared to their pre-treatment levels [495 (430-948) pg/ml] (Wilcoxon signed rank test, p<0.01), (Figure 4.15).

Salivary uPA correlations with periodontal disease indices in study B

Determination of the Spearman's rank correlation coefficient was used to investigate the relationships of salivary uPA levels in 31 pre-treatment mild/moderate periodontitis patients, 27 pre-treatment advanced periodontitis patients, and 29 healthy control subjects with the periodontal disease indices used for the clinical assessment of periodontitis in the clinical study B. These correlations gave information about the potential of salivary uPA as a biomarker that can indicate the severity of mild/moderate and advanced periodontitis. The statistical analysis of the salivary uPA correlations revealed that, there was a highly significant positive correlation between the salivary uPA levels in the pre-treatment mild/moderate periodontitis patients and each of the indices: MGI (rho=0.524, p<0.001), PD (rho=0.574, p<0.001), %BOP (rho=0.510, p<0.001), and CAL (rho=0.578, p<0.001), (Figure 4.16, Figure 4.17, Figure 4.18, & Figure 4.19). There was a highly significant positive correlation between the salivary uPA levels in the pre-treatment advanced periodontitis patients and each of the indices: MGI (rho=0.675, p<0.001), PD (rho=0.711, p<0.001), %BOP (rho=0.719, p<0.001), and CAL (rho=0.689, p<0.001), (Figure 4.20, Figure 4.21, Figure 4.22, & Figure 4.23).

The uPA levels in pre- and post-treatment GCF samples of periodontitis patients

The uPA levels were measured in 3 groups of GCF samples obtained at visit 1 of the clinical study C (see Chapter 2, section 2.1.3, study C), (cross-sectional investigation). The groups were: the healthy subjects' group which comprised 7 GCF samples, the gingivitis patients' group which comprised 13 GCF samples, and the chronic periodontitis patients' group which comprised 9 pre-treatment GCF samples. The uPA levels were measured again in post-treatment GCF samples obtained from

the same periodontitis patients (longitudinal investigation) including: 9 post-treatment GCF samples obtained at visit 5 (3 months after treatment), and 9 post-treatment GCF samples obtained at visit 6 (6 months after treatment), (see Chapter 2, section 2.1.3, study C). There was a statistically significant difference in the GCF uPA levels among the healthy, gingivitis and pre-treatment periodontitis groups (Kruskal-Wallis test with Mann-Whitney U test and Bonferroni correction, $p<0.001$), (Figure 4.24). The GCF uPA levels were higher in the pre-treatment periodontitis patients [257 (199-265) pg/ml] than in both the healthy subjects (0 pg/ml) and gingivitis patients (0 pg/ml), and the difference was highly significant (Mann-Whitney U test, $p<0.01$ and $p<0.001$ respectively), (Figure 4.24). There was no statistically significant difference in the GCF uPA levels between the healthy and gingivitis groups (Man-Whitney U test, $p>0.05$), (Figure 4.24). The GCF uPA levels were significantly decreased in the periodontitis patients following non-surgical treatment at the both post-treatment visits 5 and 6 [61 (0-201) pg/ml and 0 (0-46) pg/ml respectively] as compared to their pre-treatment levels [257 (199-265) pg/ml] (Friedman test with Wilcoxon signed rank test and Bonferroni correction, $p<0.05$), (Figure 4.25). Though the GCF uPA levels were lower in the post-treatment visit 6 [0 (0-46) pg/ml] in comparison to the post-treatment visit 5 [61 (0-201) pg/ml], the difference was not statistically significant (Wilcoxon signed rank test, $p>0.05$), (Figure 4.25).

The GCF uPA correlations with periodontal disease indices

Determination of the Spearman's rank correlation coefficient was used to study the relationships of the uPA levels in GCF samples of 9 pre-treatment periodontitis patients and 7 healthy subjects with the periodontal disease indices used for the clinical assessment of periodontal health status in the clinical study C. These correlations gave information about the potential of GCF uPA as a biomarker that can indicate the severity of periodontitis. The statistical analysis of the GCF uPA correlations revealed that there was a significant positive correlation between the GCF uPA levels and each of the indices: MGI ($rho=0.862$, $p<0.001$), PD ($rho=0.690$, $p<0.01$), %BOP ($rho=0.704$, $p<0.01$), and CAL ($rho=0.743$, $p<0.01$), (Figure 4.26, Figure 4.27, Figure 4.28, & Figure 4.29).

Summary of the investigation of uPA levels

The investigations of the uPA levels in studies A, B, and C revealed that the uPA levels were significantly higher in the whole unstimulated saliva, whole stimulated

saliva and GCF samples of the pre-treatment chronic, mild/moderate, and advanced periodontitis patients in comparison to both gingivitis patients and healthy control subjects. The uPA levels were higher in the whole stimulated saliva of the gingivitis patients as compared to the healthy controls in study B. However, there was no statistically significant difference in the GCF uPA levels between the gingivitis patients and healthy controls in study C. The uPA levels were significantly reduced in the whole unstimulated saliva, whole stimulated saliva, and GCF samples of all periodontitis patients following non-surgical periodontal treatment. In conclusion, the uPA levels were elevated in both the whole unstimulated and stimulated saliva and in the GCF of the periodontitis patients and reduced after treatment.

4.2.4 Investigation of the relationship of salivary uPA activity with periodontitis

The uPA activity fluorometric assay kits were used to measure the uPA enzymatic activity in saliva samples obtained from the clinical studies A and B (see Chapter 2, section 2.1.3, study A & study B). Correlations of the uPA activity with both its levels in saliva and the periodontal disease indices used for the clinical assessment of periodontal health status were also determined. The uPA activity assay was validated prior to use on study samples, validation was carried out by assaying three volunteer samples in different dilutions (neat, 10:100, 10:500, and 10:1000) using three diluting agents (the kit assay buffer, reagent diluent, and PBS). The validation revealed that, 10:1000 was the dilution factor at which the uPA activity in saliva samples falls within the range of the assay standard curve, and that PBS was the suitable diluting agent to replace the limited volume of the assay buffer supplied by the manufacturer. It was not possible to investigate the uPA activity in the GCF samples due to the limited volumes of GCF available.

Salivary uPA activity in the periodontitis patients of study A

The salivary uPA activity was assayed in 45 samples of pre-treatment periodontitis patients and 40 samples of healthy control subjects, all samples were obtained at visit 2 of the clinical study A (see Chapter 2, section 2.1.3, study A). Salivary uPA activity was higher in the pre-treatment periodontitis patients [242 (142-337) IU/ml] as compared to the healthy subjects [117 (76-205) IU/ml] and the difference was highly significant (Mann-Whitney U test, $p<0.001$), (Figure 4.30).

Salivary uPA activity correlations with periodontal disease indices in study A

Determination of the Spearman's rank correlation coefficient was used to study the relationships of the uPA activity with both its levels in saliva samples of 45 pre-treatment periodontitis patients and 40 healthy control subjects, and with the periodontal disease indices used for the clinical assessment of periodontitis in the clinical study A. These correlations gave information about the relationship of salivary uPA activity with salivary uPA levels as measured by ELISA, and the severity of periodontitis. The statistical analysis of the salivary uPA activity correlations revealed that, there was a significant positive correlation between the uPA activity and the uPA levels in saliva (rho=0.497, $p<0.001$), (Figure 4.31). Also, there was a significant positive correlation between salivary uPA activity and each of the indices: MGI (rho=0.247, $p<0.05$), PD (rho=0.366, $p<0.01$), %BOP (rho=0.298, $p<0.01$), and CAL (rho=0.367, $p<0.01$), (Figure 4.32, Figure 4.33, Figure 4.34, & Figure 4.35).

Salivary uPA activity in the periodontitis patients of study B

Experiments were carried out to measure the uPA activity in 4 groups of saliva samples obtained at visit 2 of the clinical study B (see Chapter 2, section 2.1.3, study B). The groups comprised: 31 pre-treatment mild/moderate periodontitis patients, 27 pre-treatment advanced periodontitis patients, 25 gingivitis patients and 29 healthy control subjects. There was a statistically significant difference in the salivary uPA activity among the groups (Kruskal-Wallis test with Mann-Whitney U test and Bonferroni correction, $p<0.001$), (Figure 4.36). Salivary uPA activity was higher in the pre-treatment mild/moderate periodontitis patients [75 (56-141) IU/ml] than in both the gingivitis patients [24 (14-48) IU/ml] and healthy subjects [41 (8-63) IU/ml] and the difference was highly significant (Mann-Whitney U test, $p<0.001$ respectively), (Figure 4.36). Salivary uPA activity was higher in the pre-treatment advanced periodontitis patients [57 (31-123) IU/ml] as compared to both the healthy subjects [41 (8-63) IU/ml] and the gingivitis patients [24 (14-48) IU/ml] and the difference was statistically significant (Mann-Whitney U test, $p<0.05$, and $p<0.001$ respectively), (Figure 4.36). Though the salivary uPA activity appeared to be higher in the pre-treatment mild/moderate periodontitis patients [75 (56-141) IU/ml] than in the pre-treatment advanced periodontitis patients [57 (31-123) IU/ml], the difference was not statistically significant (Mann-Whitney U test, $p>0.05$), (Figure 4.36). There was no statistically significant difference in the salivary uPA activity between the healthy

subjects [41 (8-63) IU/ml] and gingivitis patients [24 (14-48) IU/ml] (Mann-Whitney U test, p>0.05), (Figure 4.36).

Salivary uPA activity correlations with periodontal disease indices in study B

Determination of the Spearman's rank correlation coefficient was used to study the relationships of the uPA activity with both its levels in saliva samples of the pre-treatment periodontitis patients and the healthy subjects, and with the periodontal disease indices used for the assessment of periodontitis in the clinical study B. These correlations gave information about the relationship of salivary uPA activity with salivary uPA levels as measured by ELISA, and the severity of periodontitis. The statistical analysis of the salivary uPA activity correlations demonstrated that there was a significantly positive correlation between the uPA activity and its high levels in saliva of the mild/moderate periodontitis patients (rho=0.467, p<0.001), (Figure 4.37). Also, there was a significant positive correlation between the salivary uPA activity in the mild/moderate periodontitis patients and each of the indices: MGI (rho=0.525, p<0.001), PD (rho=0.480, p<0.001), %BOP (rho=0.482, p<0.001), and CAL (rho=0.512, p<0.001), (Figure 4.38, Figure 4.39, Figure 4.40, & Figure 4.41). The salivary uPA activity was positively correlated with its high levels in saliva of the advanced periodontitis patients (rho=0.295, p<0.05), (Figure 4.42). Furthermore, there was a significant positive correlation between the salivary uPA activity in the advanced periodontitis patients and each of the indices: MGI (rho=0.409, p<0.01), PD (rho=0.333, p<0.05), %BOP (rho=0.424, p<0.01), and CAL (rho=0.365, p<0.01), (Figure 4.43, Figure 4.44, Figure 4.45, & Figure 4.46).

Summary of salivary uPA activity investigations

The investigations of uPA activity in saliva samples of studies A and B revealed that, the uPA activity was significantly higher in the whole unstimulated saliva of the pre-treatment periodontitis patients in comparison to the healthy controls in study A, and in the whole stimulated saliva of the pre-treatment mild/moderate and advanced periodontitis patients as compared to both gingivitis patients and healthy control subjects in study B. The uPA activity in the whole stimulated saliva of the gingivitis patients was not statistically different in comparison to the healthy controls in study B. There were significant positive correlations for the salivary uPA activity with both the salivary uPA levels and the periodontal disease indices in both studies. In conclusion,

the uPA activity was elevated in both the whole stimulated and whole unstimulated saliva of periodontitis patients but not in gingivitis patients.

4.3 Discussion

4.3.1 Salivary uPAR levels and relationship with periodontitis

The present study was the first to detect and investigate uPAR in saliva of chronic periodontitis patients in comparison to healthy control subjects. The present study was in agreement with Gustafsson et al. (2011) in regard to detecting considerable amounts of uPAR in human saliva, using ELISA they detected soluble uPAR with the median value of 17100 pg/ml (17.1 ng/ml) in saliva of 20 healthy young adults, and they found that uPAR levels in saliva were higher than serum/plasma levels. However, Gustafsson et al. (2011) did not relate the high levels of salivary uPAR to any oral or systemic disease, and they explained that these high levels may be as a consequence of periodontal inflammation, therefore, they suggested that salivary uPAR should be studied in relation to periodontal diseases which was carried out and investigated by the present study. Another explanation for the high levels of salivary uPAR by Gustafsson et al. (2011) was, that the uPAR molecules could be actively transported into saliva from the nearby blood supply. These authors also suggested that, the elevated levels of salivary uPAR might be attributed to local production by the gingival tissues or the salivary glands. These explanations may also clarify the high levels of salivary uPAR in the periodontitis patients found by the present study.

Despite the fact that uPAR levels in saliva of periodontitis patients have not been previously analysed, some studies investigated different components of the PA system in GCF samples from patients with different forms of periodontal diseases. Kinnby et al. (1991), investigated the PA system proteins including the tissue plasminogen activator (tPA), uPA, and the plasminogen activator inhibitors PAI-1, PAI-2 in GCF samples of gingivitis patients before and after treatment. The same components of the PA system were detected again in GCF samples of pregnant women with symptoms of gingival inflammation (Kinnby et al. 1996). Buduneli et al. (2005), detected high levels of the same PA system proteins in GCF samples of smokers and non-smoking adults with chronic gingivitis and chronic periodontitis as compared to healthy subjects. Hence, as uPAR is one of the essential components of the PA system and it is responsible for binding and localizing the activity of uPA that

will initiate proteolysis (Ploug and Ellis 1994; Crippa 2007; Blasi and Sidenius 2010; Smith and Marshall 2010; Fleetwood et al. 2014), and as saliva contains traces of GCF, these GCF studies and their findings may explain the high levels of uPAR found in saliva of patients with untreated chronic periodontitis by the present study. These studies will be discussed in further details in the next section (4.3.2).

Using the Spearman's correlation coefficient, positive correlations were found between the high levels of salivary uPAR in the periodontitis patients and the periodontal indices used for the clinical assessment of periodontitis. These positive correlations suggested that an increase in the salivary uPAR levels can be associated with an increase in the severity of periodontitis and vice versa. Once again, the aforementioned studies regarding the PA system components in GCF, were all carried out on patients and all their results were related to periodontal diseases, which may justify the positive relationship between salivary uPAR levels and periodontitis found by the present study. Therefore, the high levels of salivary uPAR in the periodontitis patients and their positive correlations with the periodontal disease indices, suggested that the salivary uPAR is a good biomarker for the diagnosis of chronic periodontitis and can indicate the severity of the disease.

Following non-surgical treatment of the periodontitis patients, the salivary uPAR levels were reduced at the post-treatment visit as compared to the pre-treatment visit, but the difference was not statistically significant. The presence of considerable levels of salivary uPAR in the periodontitis patients following treatment may be explained by the fact that, though the periodontal health status was improved clinically after treatment which was obvious by the reduction in the mean values of the periodontal disease indices (such as PD from 3.6 mm to 2.9 mm, and MGI from 2.8 to 1.1); unlike gingivitis, once they are destroyed and pockets formed, periodontal supporting tissues in chronic periodontitis will never return back to the normal healthy status (Pihlstrom et al. 2005), but their conditions can be improved and controlled to retain teeth in position, this means that sites of mild inflammation may still exist in the periodontium from which uPAR may be expressed into saliva. On the other hand, the improvement in the periodontal health status of the periodontitis patients was associated with significant reduction in the salivary uPA levels in response to non-surgical treatment, whereas the salivary uPAR retained considerable levels in the same patients. Refer to the biology and function of uPA/uPAR, during inflammation

uPAR binds and localizes the uPA activity initiating proteolysis, which is proposed to occur during periodontitis, by the end of proteolysis the PA inhibitor (PAI-1) will inactivate the uPA-uPAR active complex and block the uPA activity. In cooperation with members of the low density lipoprotein receptor family (LDL), uPAR will mediate the internalization of this inactive complex (uPA-uPAR-PAI-1) which will finally result in the degradation of uPA by the PAI-1 inhibitor and the release of uPAR to be available again for another proteolysis activation (Conese and Blasi 1995; Ghosh et al. 2000; Preissner et al. 2000; Crippa 2007; Blasi and Sidenius 2010; Smith and Marshall 2010), this may explain why the salivary uPAR levels were not reduced after treatment and the significant reduction in the salivary uPA levels of the patients following treatment and improvement in their periodontal health status. Therefore, the persistence of considerable levels of uPAR in saliva of the periodontitis patients following non-surgical treatment, indicates that salivary uPAR may not be a good biomarker for following the clinical course of periodontitis in response to treatment.

4.3.2 The uPA levels, activity, and relationships with periodontal diseases

As with uPAR, the present study was the first to investigate the uPA levels in saliva of patients with gingivitis, chronic periodontitis, mild/moderate periodontitis and advanced periodontitis. The uPA levels in GCF samples of patients with gingivitis and chronic periodontitis were also investigated.

The very first mention of the plasminogen activating system in relation to saliva reported the lysis of plasminogen containing bovine-fibrin plates following the addition of stimulated mixed human saliva (Albrechtsen and Thaysen 1955). Albrechtsen and Thaysen (1955), proposed the presence of an enzyme known as fibrinokinase which activates the plasminogen into plasmin in the presence of another enzyme lysokinase (or streptokinase). However, this study was not able to know what was the exact enzyme that activated the plasminogen, and decades later it was found that there were two plasminogen activators: the uPA and tPA enzymes and these two enzymes are activated by the plasmin itself and require binding to their receptors such as uPAR for the uPA (Ploug and Ellis 1994; Crippa 2007; Blasi and Sidenius 2010; Smith and Marshall 2010). Moody (1982b), reported the presence of the substrate plasminogen in the human saliva suggesting that it was due to the tissue plasminogen content of leucocytes and epithelial cells present in saliva as well as due to the fibrinolysis of the surrounding oral mucosa. Nevertheless, the author

did not carry out any experimental investigation, Page (1991), was another study to suggest that the uPA and the PA system may play a role in the pathogenesis of periodontal diseases; that study is consistent with the present study in terms of the detection of high levels of uPA, as well as uPAR in saliva and GCF of periodontitis and gingivitis patients, and the proposed roles of these PA proteins in the pathogenesis of periodontal diseases (Page 1991).

In their study, Schmid et al. (1991) reported that the PA system proteins in the human GCF have 100-fold greater concentration as compared to their levels in plasma, this may explain the high uPA levels in the GCF of the periodontitis patients found by the present study. Kinnby et al. (1996), investigated PA system proteins in GCF samples of females with gingivitis during pregnancy and postpartum. They found high levels of the PA system proteins (tPA, uPA, PAI-2 and PAI-1) in the GCF samples of 14 pregnant women with gingivitis, and these high GCF levels of PA proteins were positively correlated with the periodontal disease indices plaque index (PI) and gingival index (GI), as well as with the pregnancy status. The study implicated a role for the PA system in gingivitis and in the aggravation of gingival inflammatory symptoms during pregnancy. However, in contrast to Kinnby et al. (1996), the present study didn't find high levels of uPA in the GCF samples of the gingivitis patients. Though it was carried out on pregnant women with gingivitis, this data is consistent with the presence of high levels and the positive correlations of uPA in the GCF samples of the periodontitis patients, as well as, in the saliva samples of the gingivitis and periodontitis patients found by the present study (Kinnby et al. 1996).

The activator tPA and the inhibitor PAI-2 were investigated in GCF samples of gingivitis and periodontitis patients in comparison to healthy subjects (Yin et al. 2000). The study found that, the tPA and PAI-2 levels were higher in the GCF samples of the patients in comparison to the control subjects, and these high levels were positively correlated with the periodontal disease indices (PD and GI). Yin et al. (2000), suggested that both tPA and PAI-2 may play significant roles in the periodontal tissue destruction and remodelling that occur in periodontal diseases, and that tPA and PAI-2 in GCF may be useful biomarkers for periodontal diseases. Although they did not investigate uPA, the Yin et al. (2000) study can be considered to be consistent with the findings of the present study in regard to the high levels of

uPA in both the GCF and saliva samples of the periodontitis patients and their positive correlations with the periodontal indices, because uPA belongs to the PA system same as tPA and PAI-2 which are both more related to the fibrinolytic activity of the PA system in the blood and vascular endothelium than to the proteolysis activity which is related to uPA (Ogura et al. 1995; Ogura et al. 1999; Sulniute et al. 2011).

Buduneli et al. (2004), measured the PA system proteins (tPA, PAI-2, uPA, and PAI-1) in the gingival sites and total GCF samples of 18 renal transplant patients with gingival overgrowth induced by cyclosporine-A, and 16 chronic gingivitis patients in comparison to control subjects (16 healthy, and 10 renal transplant patients with no signs of gingival inflammation). High levels of tPA and PAI-2 were found in the gingival overgrowth sites, whereas uPA and PAI-1 levels showed no significant difference between the patients and controls. However, when the total amounts of GCF were considered for each patient, uPA levels were significantly higher in both the gingival overgrowth and chronic gingivitis patients in comparison to the control subjects. Hence, the data of Buduneli et al. (2004) were in contrast with those of the present study results which revealed that there was no significant difference in the GCF uPA levels between the gingivitis patients and control subjects; however, Buduneli et al. (2004) study may support the present study in regard to the significantly high levels of uPA found in the GCF of the periodontitis patients and saliva of the gingivitis and periodontitis patients in comparison to the control subjects. In addition to that, Buduneli et al. (2004) found positive correlations between the GCF PA proteins levels and the periodontal indices PI, papillary bleeding index (PBI), hyperplastic index (HI), and PD, a finding that may explain the positive correlations between the salivary/GCF uPA levels and the periodontal indices found by the present study. Thus, Buduneli et al. (2004) is another study that backs up the proposed role of the PA system proteins in the pathogenesis of periodontal diseases.

Another study carried out by Buduneli et al. (2005), measured the same PA system proteins (tPA, PAI-2, uPA, and PAI-1) in GCF and serum samples of 20 patients with chronic gingivitis, and 20 patients with chronic periodontitis in comparison to 20 healthy control subjects (all groups were divided into 10 smokers and 10 non-smoking subjects). The results suggested that all the PA system proteins (tPA, PAI-2, uPA, and PAI-1) were significantly elevated in the GCF samples of the patients in

comparison to the control subjects, and that smoking had a minor effect on the GCF PA system proteins levels with an exception for the PAI-2 protein. In contrary to the results of Buduneli et al. (2005), the present study revealed that there was no difference in the GCF uPA levels between the gingivitis patients and the healthy subjects; however, both the present study and Buduneli et al. (2005) study were in agreement in regard to the chronic periodontitis, as the present study results revealed that the GCF uPA levels were significantly higher in the chronic periodontitis patients than in the healthy control subjects. Buduneli et al. (2005), also found positive correlations between the GCF PA proteins levels and the periodontal disease indices (PD, GI, and PBI), again this finding was in agreement with the present study which also demonstrated positive correlations between the GCF uPA levels and the periodontal indices. As whole unstimulated saliva contains traces of GCF from all periodontal sites, therefore, the GCF uPA results of Buduneli et al. (2005) study are consistent with the high levels of salivary uPA in the gingivitis and periodontitis patients and their positive correlations with the periodontal disease indices found by present study. Hence, Buduneli et al. (2005) study is a further evidence that supports the role of the PA system proteins especially uPA in the pathogenesis of periodontal diseases.

Once again, the present study was the first to measure the salivary uPA levels in periodontitis patients following non-surgical treatment. In regard to the GCF uPA, the present study was also the first to investigate the uPA levels in the GCF of periodontitis patients in response to treatment; however, there were 2 studies that measured uPA in the GCF of gingivitis patients before and after treatment (Kinnby et al. 1994; Kinnby et al. 1996), and only one study that measured other PA system proteins in the GCF of periodontitis patients before and after treatment (Yin et al. 2000) (discussed below). The investigation of longitudinal changes in biomarkers as part of the clinical study A revealed that, the salivary uPA levels were significantly reduced in the periodontitis patients following non-surgical treatment. Similar data from studies of clinical study B samples showed that, the salivary uPA levels dropped significantly in both the mild/moderate and advanced periodontitis patients after treatment. In the clinical study C, the GCF uPA levels were significantly reduced in the periodontitis patients at the post-treatment visits in comparison to the pre-treatment visit. This reduction of both the salivary and GCF uPA levels following

treatment, may be explained by the improvement in the periodontal health status of the periodontitis patients in response to treatment which was evident by the improvement in the periodontal disease indices records, for instance the %BOP index in the periodontitis patients of the study A dropped from 52% to 18%. In the study B, the %BOP index in the mild/moderate periodontitis patients dropped from 47% to 15%, and in the advanced periodontitis patients the %BOP dropped from 67% to 24%. In the study C, the %BOP index of the periodontitis patients was reduced from 62% at the pre-treatment visit to 30% and 23% respectively at the post-treatment visits.

The Kinnby et al. (1994) study, was the first to measure the uPA along with other PA system proteins levels in GCF samples of patients with gingivitis before and after treatment. Before treatment, they found high levels of the PA system proteins (uPA, tPA, and PAI-2) in the GCF of 8 gingivitis patients with positive correlations of these GCF PA proteins levels with the periodontal disease indices (PI, GI, and PD), these findings were in contrast to the present study which didn't find significant levels of uPA in the GCF of gingivitis patients. However, the Kinnby et al. (1994) pre-treatment results may support the present study results which found high levels of uPA in the GCF samples of periodontitis patients with their positive correlations with periodontal disease indices, as well as, the high levels of salivary uPA in gingivitis and periodontitis patients which positively correlated with clinical periodontal indices. The post-treatment results of Kinnby et al. (1994) demonstrated reduced levels of tPA and PAI-2 in the GCF samples of gingivitis patients, whereas the post-treatment GCF uPA levels didn't show significant difference in comparison to the pre-treatment status. The post-treatment results of Kinnby et al. (1994) were in contrast to the results of the present study, which showed significantly reduced uPA levels in both the GCF and saliva samples of periodontitis patients following non-surgical treatment.

The other study carried out by Kinnby et al. (1996) , in which they followed up women with gingivitis during and after pregnancy. The postpartum results revealed that the GCF levels of the PA system proteins (tPA, PAI-2, and PAI-1) were reduced in comparison to the pregnancy period, whereas the uPA levels didn't show significant difference. Despite the fact that Kinnby et al. (1996) study was carried out on pregnant women with gingivitis, their results were in contrast to the present study

which found that the uPA levels in both GCF and saliva of periodontitis patients were significantly reduced at post-treatment visits in comparison to the pre-treatment visits. Yin et al. (2000), measured the PA system proteins (tPA and PAI-2) in patients with periodontitis before and after treatment. The results revealed that the PAI-2 levels were significantly decreased following treatment, in a similar manner to what happened in the present study when the levels of uPA were reduced significantly in both saliva and GCF of periodontitis patients following non-surgical treatment. Yin et al. (2000), also found that the GCF tPA levels were lower at the post-treatment visit but the difference was not statistically significant in comparison to the pre-treatment visit, this was consistent with the findings of the present study in which the salivary uPAR levels were reduced at the post-treatment visit as compared to the pre-treatment visit but the difference was not statistically significant.

The present study was the first to investigate the uPA activity in the whole unstimulated and stimulated saliva of untreated periodontitis patients and its relationships with both the salivary uPA levels and the periodontal disease indices. The results (for both studies A and B) revealed that, the salivary uPA activity was significantly higher in the untreated periodontitis patients as compared to both the gingivitis patients and healthy control subjects. Furthermore, significant positive correlations were found between the high salivary uPA activity in the periodontitis patients with both the high salivary uPA levels and the periodontal indices. This high activity of salivary uPA may simply reflect the high levels of uPA found in saliva of the periodontitis patients as they were positively correlated. The positive correlations of salivary uPA activity with the periodontal diseases indices may relate to the proposed role of uPA/uPAR in the pathogenesis of periodontitis.

The Moody (1982a) study, investigated the plasminogen activity in healthy human stimulated whole, parotid, and submandibular saliva using human fibrin plates. However, the activity that Moody (1982a) found was fibrinolytic activity which was only detected in the presence of epithelial cells and epithelial fragments in saliva, and this activity totally disappeared when the saliva was centrifuged. Therefore, Moody (1982a) related this activity in saliva to its epithelial cells' content and considered this fibrinolytic activity as the only plasminogen activator activity in saliva. In contrast to Moody (1982a), the present study proved that the plasminogen activator activity in the centrifuged stimulated and unstimulated whole saliva is also related to uPA which

is responsible for the proteolytic activity (matrix proteolysis) of the PA system in addition to cells migration, signalling and other functions of the PA system whereas tPA which also known as blood vessel type PA is responsible for the fibrinolytic activity of PA system in the blood and vascular endothelium (Kinnby et al. 1996; Ogura et al. 1999; Ogura et al. 2001; Buduneli et al. 2004; Buduneli et al. 2005; Virtanen et al. 2006; Blasi and Sidenius 2010; Sulniute et al. 2011; Montuori et al. 2012; Fleetwood et al. 2014).

In their study, Watanabe et al. (1987) investigated the PA activity in 3 GCF samples obtained from 2 patients representing 3 different periodontal sites. The molecular weights of the PA protein tPA was determined by zymography, and the PA activity was determined by the digestion of plasminogen in fibrin gels. This PA activity was inhibited when anti-tPA antibodies were added to the GCF samples. Though it was a fibrinolytic activity related to the tPA activator, the detection of PA activity in GCF by Watanabe et al. (1987) supports the role of the PA system in periodontitis and may justify the detection of uPA in the saliva and GCF samples of periodontitis patients as well as the uPA activity in their saliva found by the present study.

Another study carried out by Sindet-Pedersen et al. (1987), investigated the fibrinolytic activity in human stimulated and unstimulated saliva obtained from 10 healthy non-smoking subjects, using plasminogen-free and plasminogen-rich fibrin plates. They found that the fibrinolytic activity zones generated by saliva were inhibited by the addition of anti-tPA antibodies, whereas this fibrinolytic activity remained unchanged when anti-uPA antibodies were added. From their results, Sindet-Pedersen et al. (1987) assumed that the tPA is the only type of plasminogen activator present in normal human saliva, in fact this assumption was not right because they neither measured the uPA levels nor investigated its activity. Furthermore, the present study detected the presence as well as the activity of uPA in healthy subjects' saliva and in significantly higher levels in periodontitis patients' saliva; hence the tPA is not the only plasminogen activator present in saliva.

In a similar manner to (Moody 1982a; Sindet-Pedersen et al. 1987; Watanabe et al. 1987), Schmid and Chambers (1989) investigated the presence and activity of the plasminogen activator in the human supra-gingival plaque and saliva. Although they detected proteolytic zones on the plasminogen-dependent indicating plates, Schmid

and Chambers (1989) did not relate this activity to uPA. Instead of the proteolysis, Schmid and Chambers (1989) carried out further investigation on the fibrinolytic activity, in which fibrinolytic zones were detected on the fibrin-agar gel, and this fibrinolytic activity was higher in the plaque samples than the proteolytic activity. Furthermore, Schmid and Chambers (1989), added anti-tPA antibodies to their fibrin-agar gel which inhibited the fibrinolysis, they also added anti-uPA antibodies which didn't affect the fibrinolytic activity and this is true as uPA is related to the proteolysis whereas tPA is related to the fibrinolysis (Moody 1982a; Crippa 2007; Montuori et al. 2012). Moreover, the incubation of human unstimulated parotid and mixed saliva on the fibrin-agar gel demonstrated high tPA related fibrinolytic activity (Schmid and Chambers 1989). Schmid and Chambers (1989), suggested that the PA system role in periodontal diseases is attributed to its fibrinolytic activity which is related to tPA. Despite the fact that they did not carry out any investigation about uPA or its activity and role in proteolysis; the Schmid and Chambers (1989) study was a further evidence for the PA system involvement in the periodontal diseases pathogenesis.

Talonpoika et al. (1991), investigated the plasmin activity in 152 GCF samples obtained from 12 subjects with different clinical conditions and after periodontal treatment. The plasmin activity was measured in plasminogen-free fibrin plates by weighing the fibrin liquefied by the GCF samples. Talonpoika et al. (1991), found that there was a variation in the total GCF plasmin activity among the subjects and among the different periodontal pockets from which the GCF samples were collected within the subjects. There was a weak positive correlation between the GCF plasmin activity and each of the plaque amount, bleeding tendency, pocket depth, and bone loss. The Talonpoika et al. (1991) GCF plasmin activity results were in contrary with the present study which found that there was a significantly high salivary uPA activity in the periodontitis patients as well as significantly positive correlations between the salivary uPA activity and the periodontal disease indices. Furthermore, Talonpoika et al. (1991) found that the plasmin levels in the GCF didn't correlate with the clinical parameters, whereas the present study found that the uPA levels in both GCF and saliva were positively correlated with the periodontal disease indices. An interesting finding by Talonpoika et al. (1991) was the significant reduction in the GCF plasmin activity of the patients following periodontal treatment, this finding may explain the

significantly reduced levels of GCF uPA in the periodontitis patients following treatment found by the present study.

Brown et al. (1995) investigated the PA proteins (tPA, uPA, PAI-1, and PAI-2) in GCF samples obtained from healthy adults. Fibrin zymography was used to determine the PA activity in the GCF samples, and the assay detected tPA activity but not uPA activity. This finding may explain the lower uPA activity measured in saliva of the healthy subjects as compared to the periodontitis patients found by the present study. Moreover, Brown et al. (1995) immunoblotting and ELISA assays revealed that the tPA and PAI-2 levels were higher than the uPA and PAI-1 levels. The detection of low GCF uPA levels by Brown et al. (1995), was in agreement with the present study which also detected very low GCF uPA levels in the healthy subjects as compared to the periodontitis patients and may explain the lower salivary uPA levels in the healthy subjects of the present study as well.

The Virtanen et al. (2006) was the first study which investigated the uPA activity along with the activity and expression of other plasminogen activators and inhibitors in human saliva and salivary gland tissues. However, though the study was carried out on 34 patients attending the neurological department of Helsinki University Central Hospital, the results were not related to any periodontal, oral, or systemic disease. The activity of both the tPA and uPA, in addition to the relative inhibition of tPA were measured in whole saliva samples obtained from the patients using microtiter plate assays. The salivary levels of PAI-1 and PAI-2 were measured using ELISA assays. The expression of tPA, uPA, PAI-1 and PAI-2 proteins were investigated by the immunohistochemistry of 6 salivary gland tissue specimens (4 parotid, 1 submandibular, and 1 sublingual samples). The results of Virtanen et al. (2006) study, revealed that both the tPA and its inhibition activities were higher in all patients in comparison to the uPA activity which was observed in 9 patients only. Though it was found in the salivary gland tissue samples, the uPA expression was lower than that of tPA, PAI-1, and PAI-2 proteins. Furthermore, Virtanen et al. (2006) ELISA assays detected PAI-2 but not PAI-1 in the saliva samples. The present study results were in agreement with the Virtanen et al. (2006) results in respect to the low uPA activity measured in saliva of the healthy control subjects. However, the expression of uPA in salivary gland tissues and the detection of active uPA in human

saliva by Virtanen et al. (2006), may justify the detection of active uPA in saliva samples of periodontitis patients by the present study.

4.3.3 Summary of findings

The investigations carried out on the clinical study A samples revealed that, the salivary uPAR levels were significantly elevated in the chronic periodontitis patients in comparison to the healthy control subjects. There were positive correlations between the high salivary uPAR levels and the periodontal disease indices used for the clinical assessment of periodontitis. Therefore, salivary uPAR is suggested as a good biomarker for the diagnosis of periodontitis, the indication of the disease severity, and the differentiation between periodontitis patients and healthy subjects. Though salivary uPAR levels were reduced in the periodontitis patients following treatment, the difference was not statistically significant as compared to their pre-treatment levels. Therefore, salivary uPAR may not be a good biomarker that can follow the clinical course of periodontitis in response to treatment.

The results of the clinical studies A, B and C revealed that, the uPA levels were significantly higher in both the saliva and GCF samples of the periodontitis patients in comparison to the healthy subjects. Significant positive correlations were found between the high salivary/GCF uPA levels and the periodontal disease indices used for the clinical assessment of periodontal health status. Therefore, uPA in both saliva and GCF is suggested as a good biomarker for the diagnosis of periodontitis, the indication of the disease severity, and it is able to discriminate between periodontitis patients and healthy subjects. Moreover, the uPA levels were higher in both the saliva and GCF samples of the periodontitis patients as compared to the gingivitis patients, these findings suggested that uPA is more related to periodontitis than gingivitis and supported the proposed role of uPA in the proteolysis and destruction of the supporting periodontal tissues in periodontitis.

Both the salivary and GCF uPA levels were significantly reduced following non-surgical treatment in the periodontitis patients. Therefore, uPA in both saliva and GCF is suggested as a good biomarker that can predict and follow the clinical course of periodontitis in response to treatment. The salivary uPA activity was significantly higher in the periodontitis patients as compared to both the healthy subjects and the gingivitis patients, and the uPA activity was positively correlated with both its levels in

saliva and the periodontal disease indices. Consequently, in addition to its levels, the salivary uPA activity is also suggested as another useful biomarker for the diagnosis of periodontitis, indication of the disease severity, and distinguishing periodontitis patients from healthy subjects. Furthermore, the results indicated that the uPA activity is more related to periodontitis than gingivitis which may explain the high salivary uPA levels and support its proteolytic role during periodontitis. Hence, both the salivary uPA levels and activity can differentiate periodontitis patients from gingivitis patients.

	uPAR DuoSet (n=1)	uPA DuoSet (n=1)	uPA Quantikine (n=1)
Neat sample	**NA**	**NA**	**NA**
1:2 neat sample	**140.29%**	**NA**	**94.6%**
1:4 neat sample	**141.3%**	**NA**	**89.1%**
1:8 neat sample	**126.62%**	**NA**	**NA**
1:16 neat sample	**107.21%**	**NA**	**NA**
Spiked sample	**96.67%**	**26.65%**	**83.95%**
1:2 spiked sample	**112.62%**	**68.7%**	**97.81%**
1:4 spiked sample	**122.48%**	**66.45%**	**95.55%**
1:8 spiked sample	**123.13%**	**88.87%**	**NA**
1:16 spiked sample	**107.55%**	**104.14%**	**NA**
Spiked control	**83.26%**	**98.42%**	**94.77%**
1:2 spiked control	**101.28%**	**129.44%**	**94.76%**
1:4 spiked control	**100.47%**	**130.53%**	**NA**
1:8 spiked control	**134.05%**	**192%**	**NA**
1:16 spiked control	**193.86%**	**577.63%**	**NA**

Table 4.1: Recovery and linearity results for the uPAR and uPA ELISAs.

The results represent the recovery values for the spiked samples and spiked controls in the recovery assays for each ELISA assayed in duplicate. As well as, the recovery values for the neat sample dilutions, spiked sample dilutions, and spiked control dilutions in the linearity assays for each ELISA assayed in duplicate. The undiluted neat (unspiked) samples have no recovery or linearity measures. Recovery values presented as %, values between 80 and 120% generally acceptable (R & D systems) (Jaedicke et al. 2012). NA (not applicable because the sample/control was not assayed). Number=n.

	Sample 1	Sample 2	Sample 3
n	3	3	3
Mean (pg/ml)	135.9	95.7	184.2
SD	9.8	2.3	9.8
CV%	7.2	2.4	5.3

Table 4.2: Intra-assay variation values for the uPAR DuoSet ELISA.

The intra-assay variation test was carried out on the uPAR DuoSet ELISA, using three different saliva samples obtained from three healthy volunteers, assayed in triplicates on the same plate. Results presented as CV %, mean and standard deviation (SD). Number=n.

	Sample 1	Sample 2	Sample 3
n	9	9	9
Mean (pg/ml)	138	82.4	180.7
SD	5.4	3.6	5.1
CV%	3.9	4.4	2.8

Table 4.3: Inter-assay variation values for the uPAR DuoSet ELISA.

The inter-assay variation test was carried out on the uPAR DuoSet ELISA, using three different saliva samples assayed in triplicates in three different plates on three separate days (one plate per day). Results presented as CV %, mean and SD.

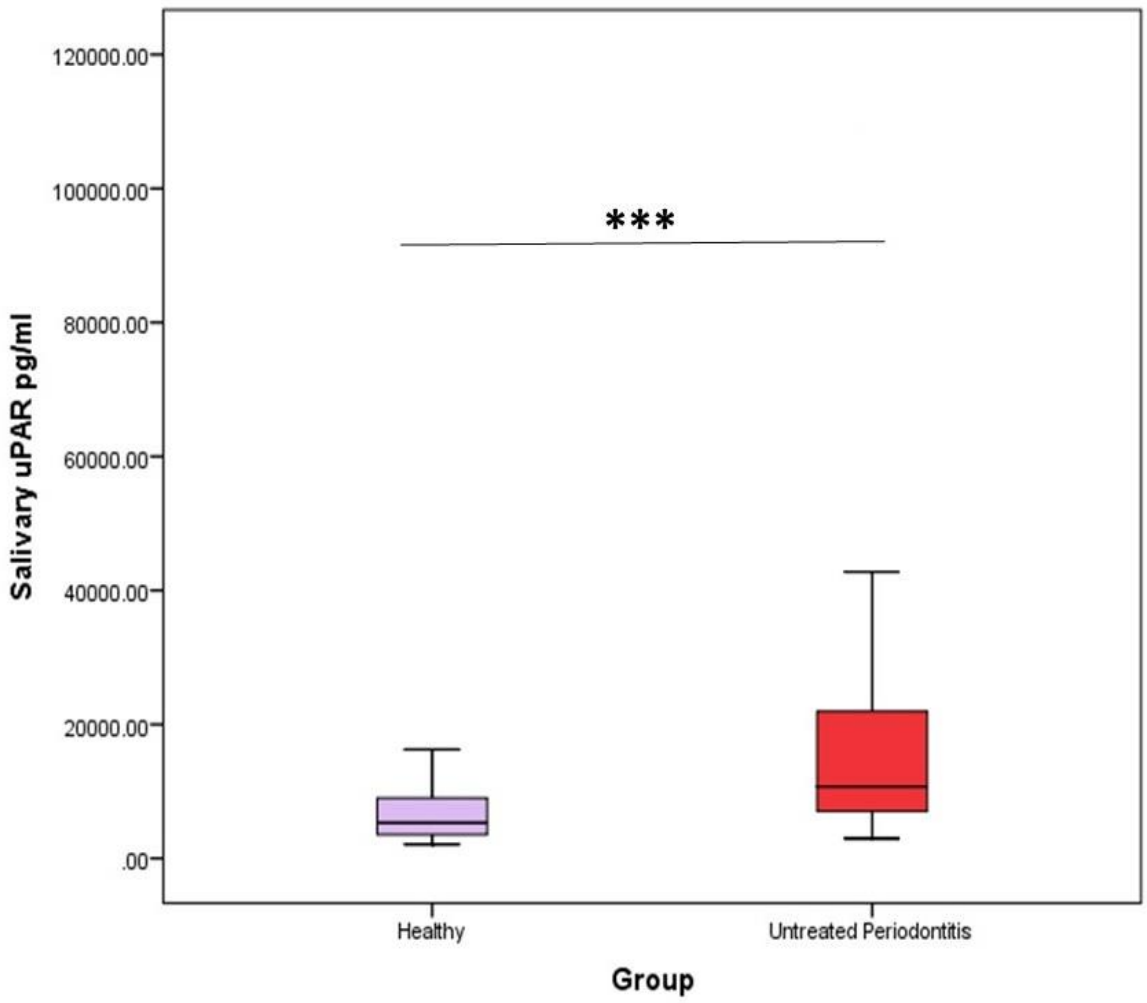

Figure 4.1: Salivary uPAR levels in untreated periodontitis patients of study A.

ELISA was carried out to measure the salivary uPAR levels in samples obtained at the baseline visit of clinical study A from 30 untreated periodontitis patients in comparison to 34 healthy subjects. The box plots represent the median and IQR for each group. ***=p<0.001 (Mann-Whitney U test).

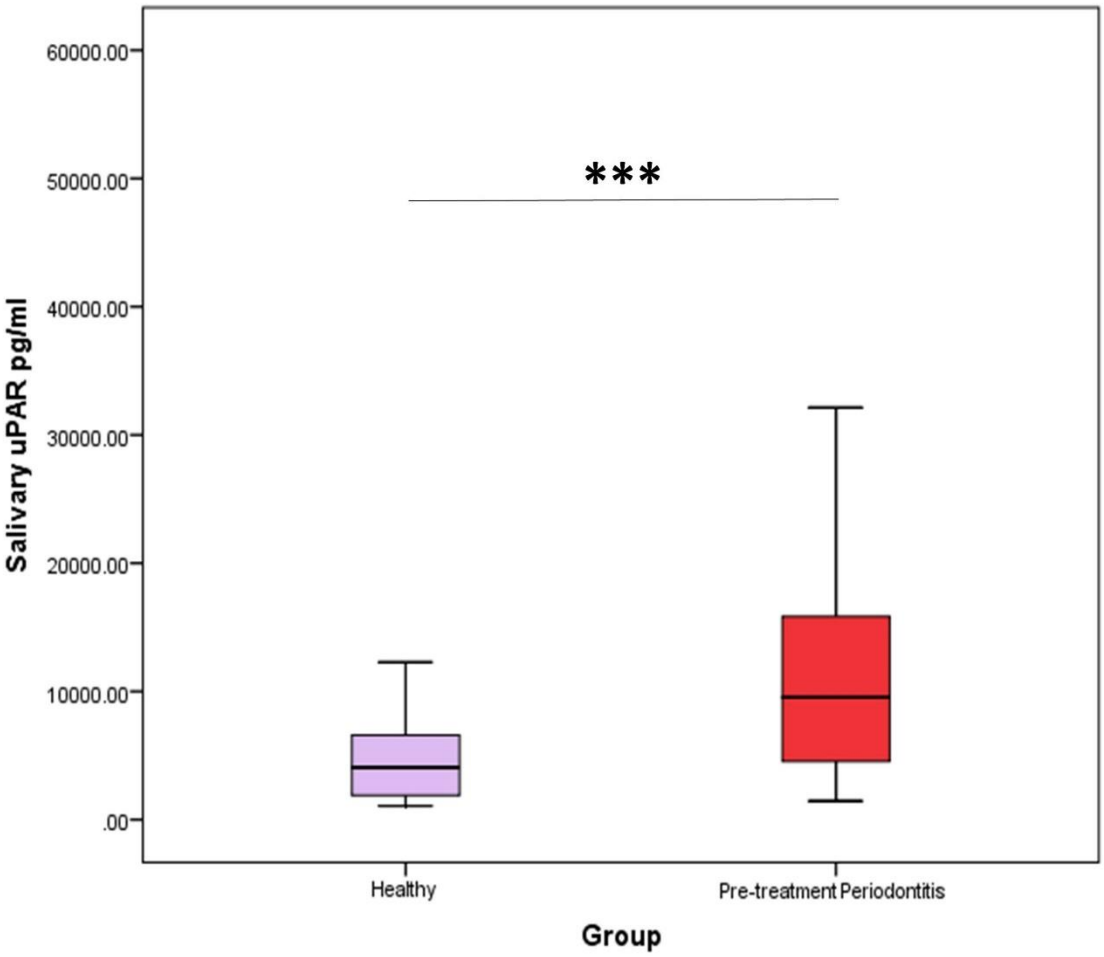

Figure 4.2: The pre-treatment salivary uPAR levels in the periodontitis patients of study A.

Salivary uPAR levels were measured by ELISA in samples obtained from 45 pre-treatment periodontitis patients in comparison to 40 healthy subjects. The box plots represent the median and IQR for each group. ***=p<0.001 (Mann-Whitney U test).

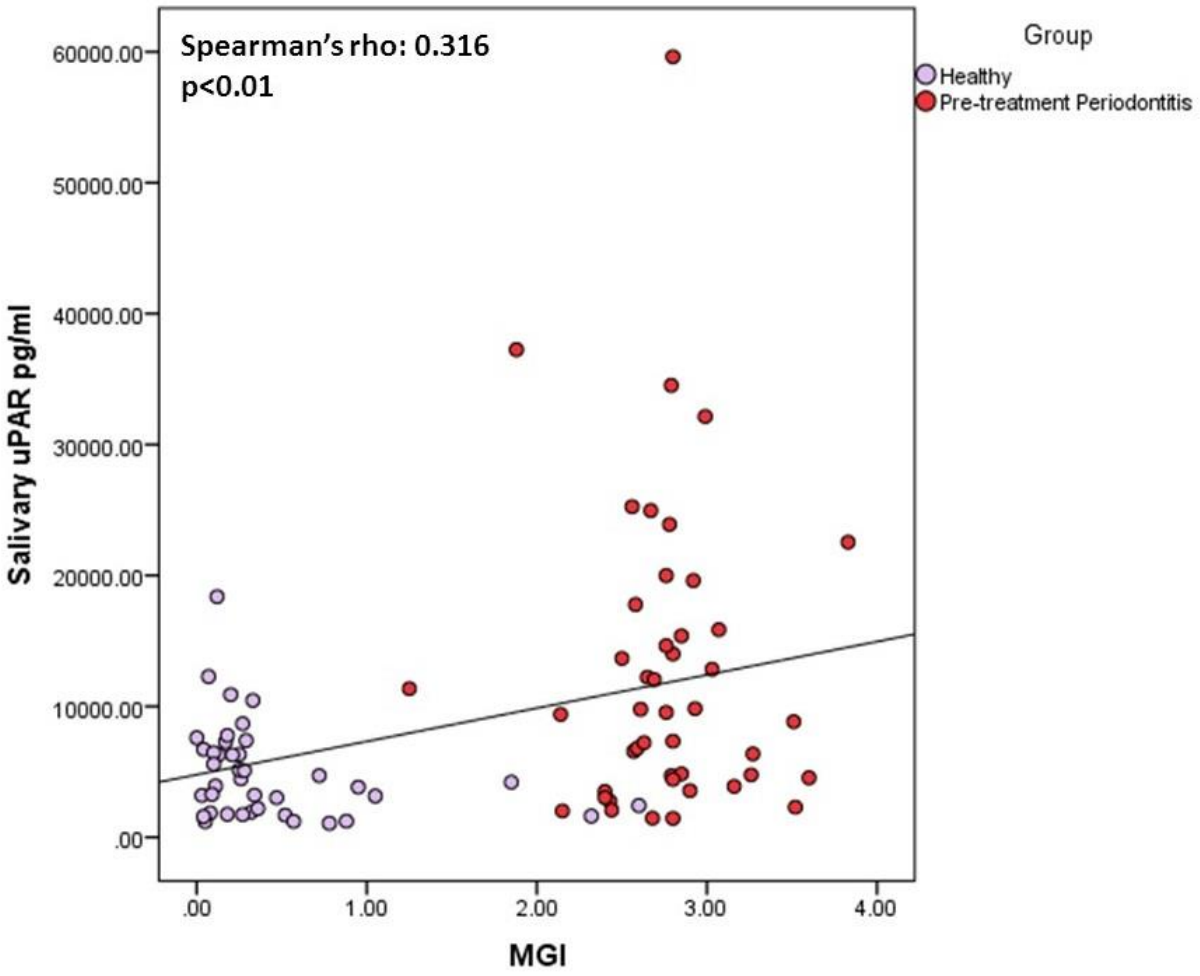

Figure 4.3: Relationship of salivary uPAR levels with MGI in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPAR levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the MGI index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

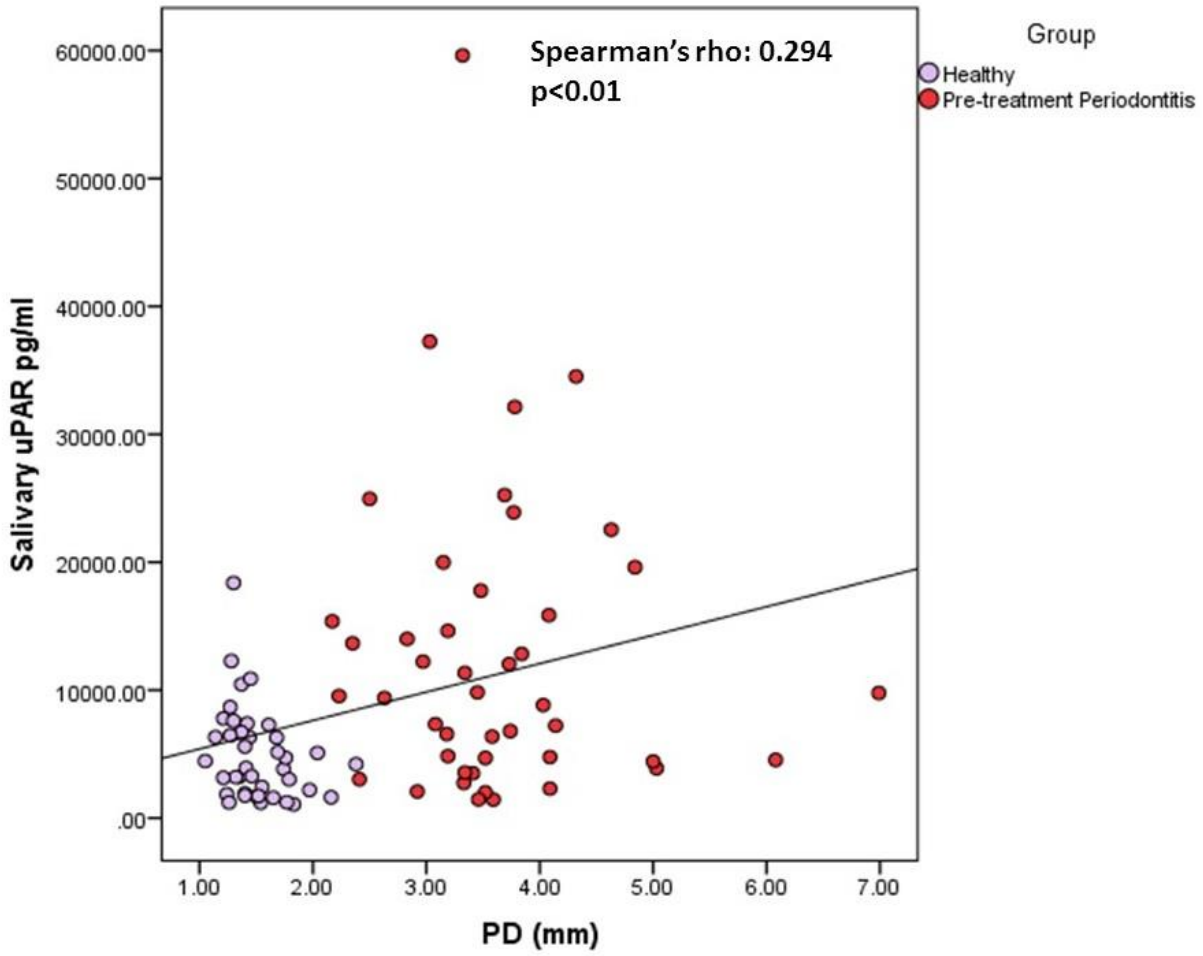

Figure 4.4: Relationship of salivary uPAR levels with PD in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPAR levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the PD index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

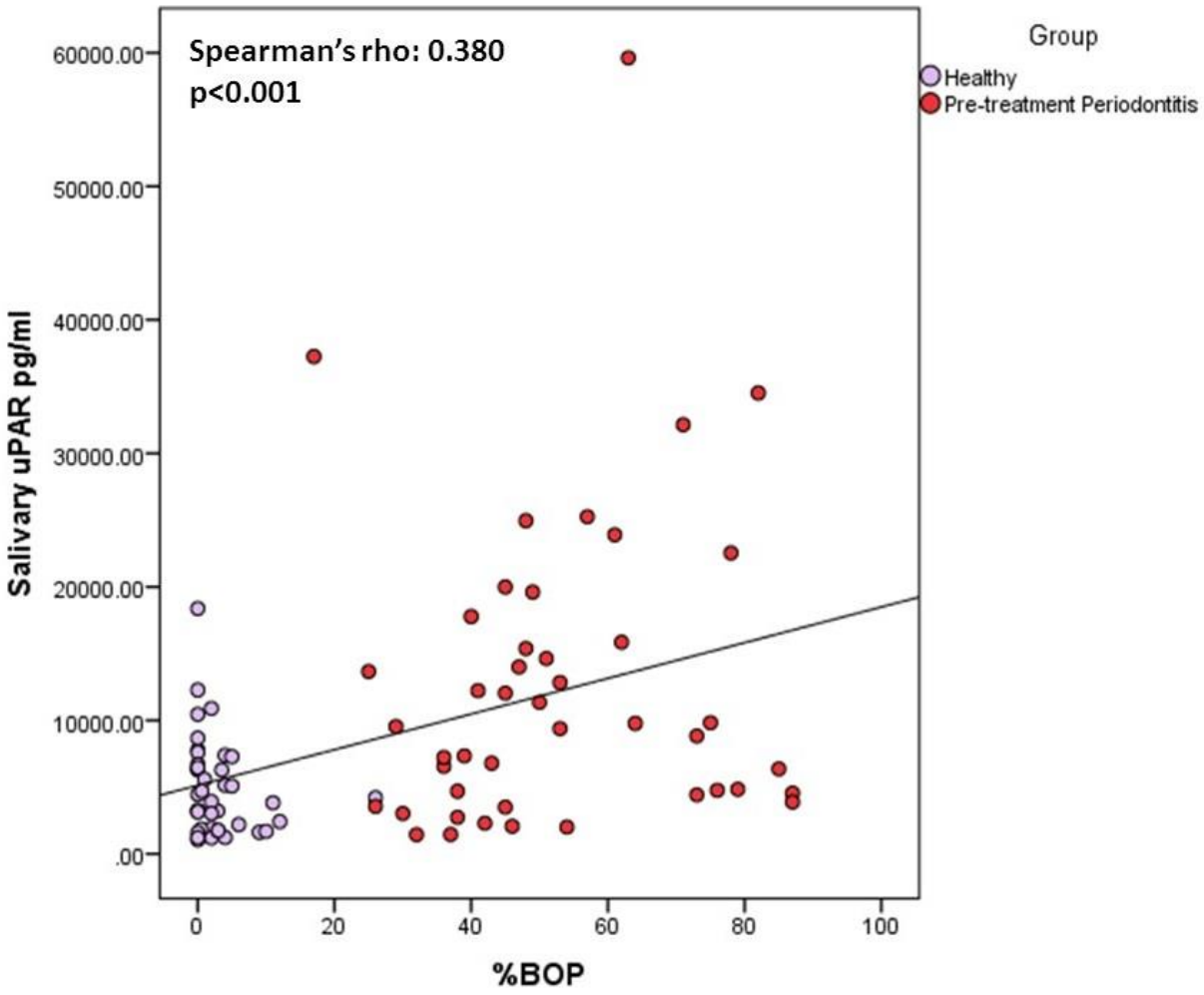

Figure 4.5: Relationship of salivary uPAR levels with %BOP in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPAR levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the %BOP index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

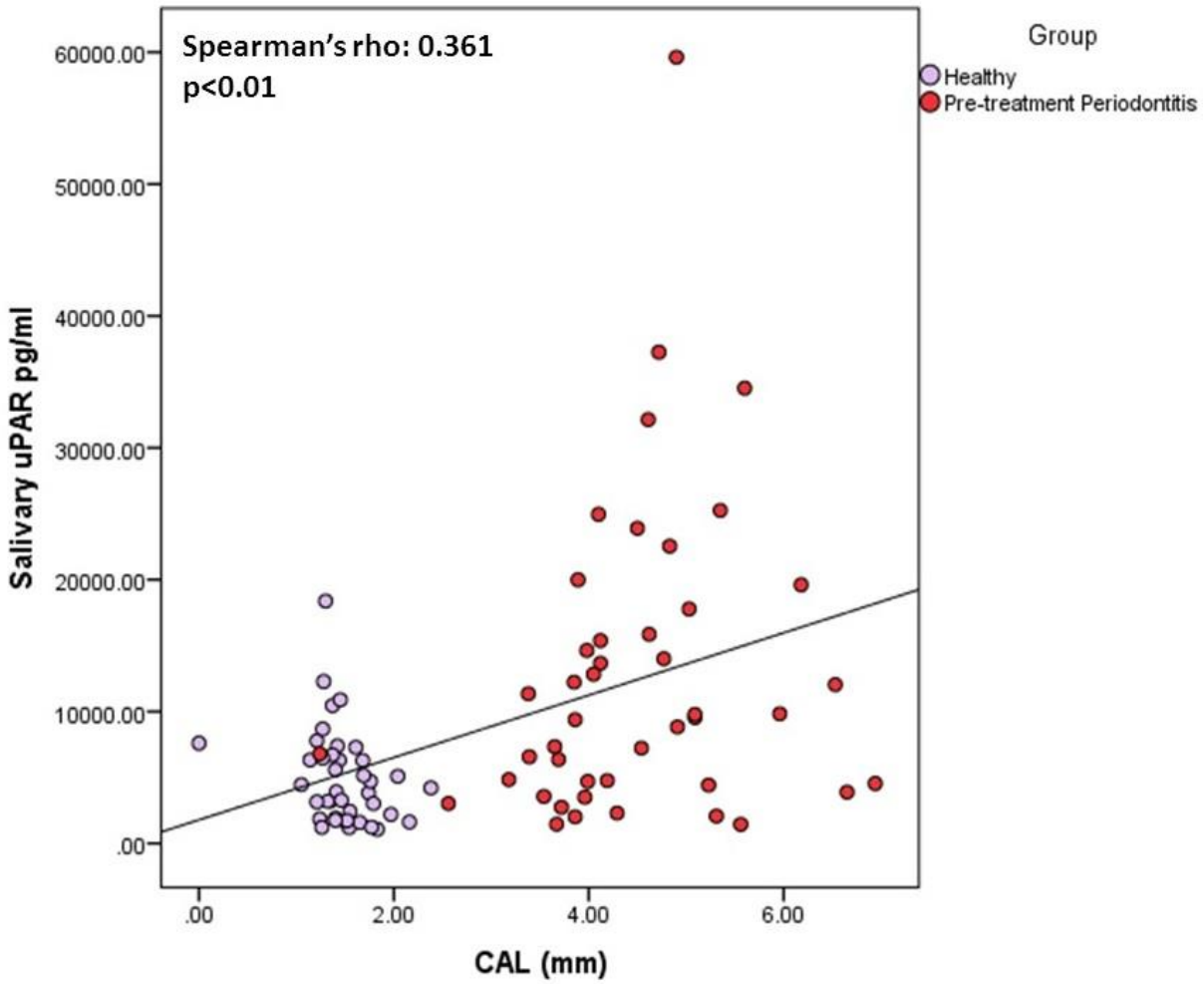

Figure 4.6: Relationship of salivary uPAR levels with CAL in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPAR levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the CAL index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

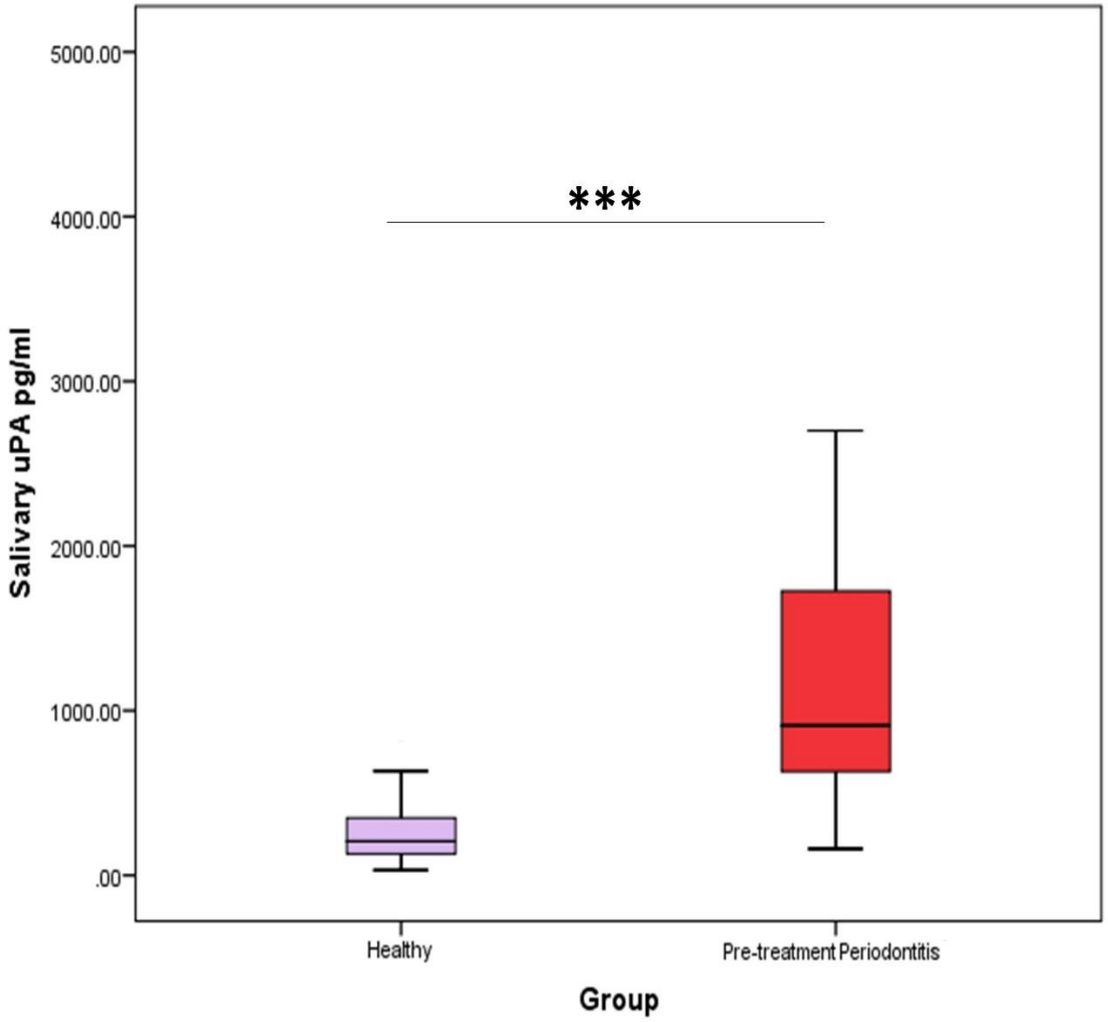

Figure 4.7: The pre-treatment salivary uPA levels in the periodontitis patients of study A.

The uPA levels were measured in saliva of 45 pre-treatment periodontitis patients in comparison to 40 healthy control subjects. The box plots represent the median and IQR for each group. ***=p<0.001 (Mann-Whitney U test).

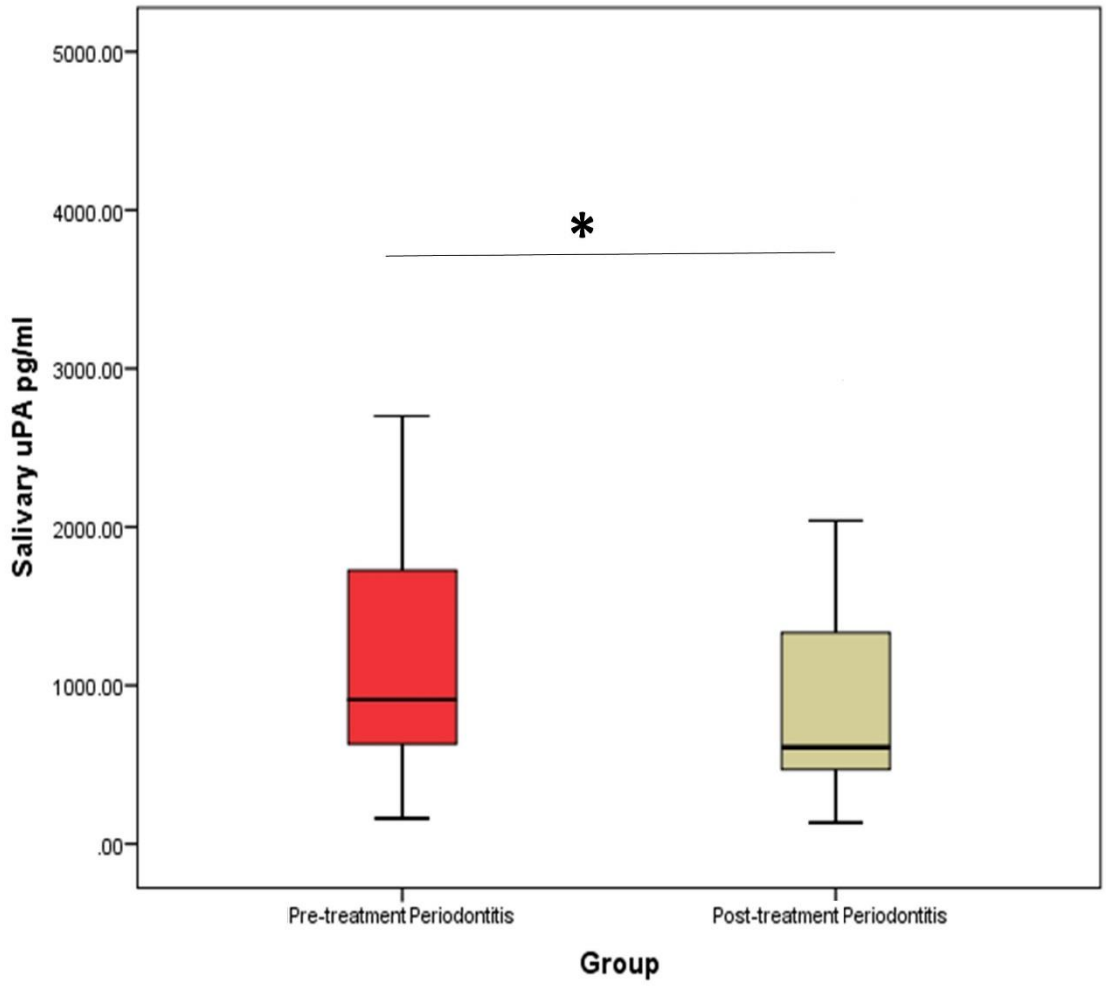

Figure 4.8: The post-treatment salivary uPA levels in the periodontitis patients of study A.

Salivary uPA levels were measured in 45 periodontitis patients after 6 months of non-surgical treatment in comparison to their pre-treatment status. Box plots represent the median and IQR for each group. *****=$p<0.05$ (Wilcoxon signed rank test).

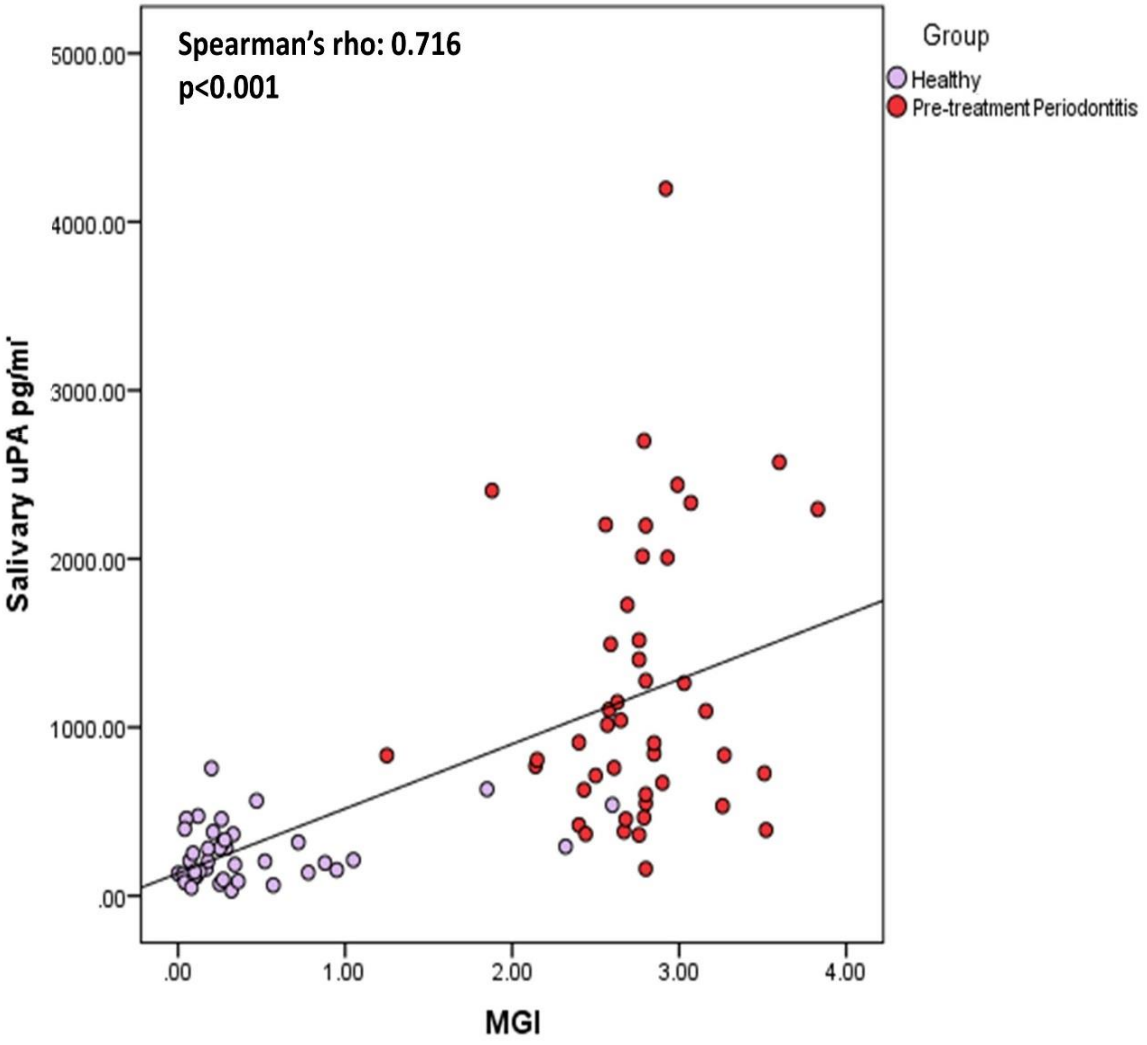

Figure 4.9: Relationship of salivary uPA levels with MGI in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the MGI index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

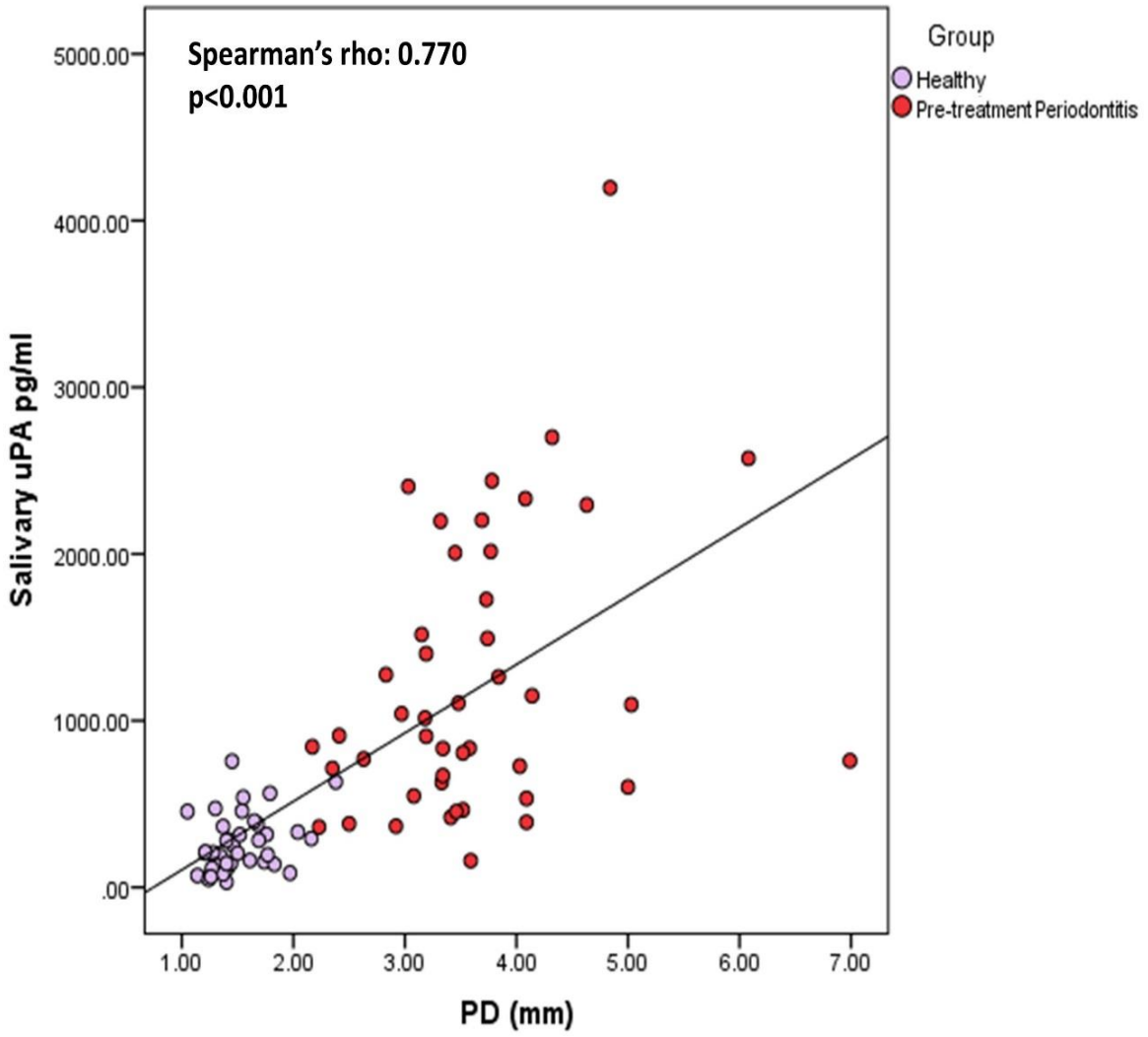

Figure 4.10: Relationship of salivary uPA levels with PD in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the PD index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

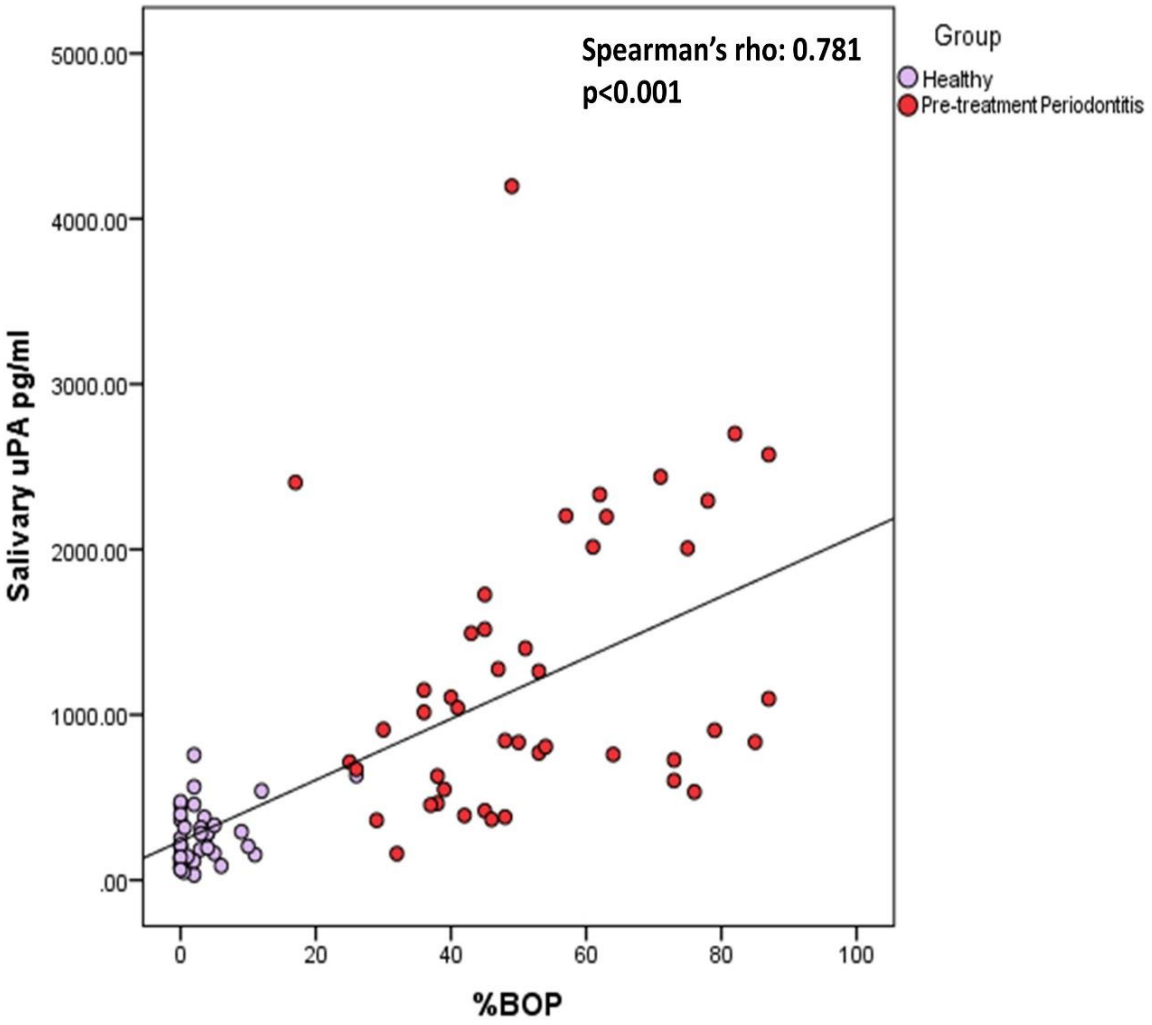

Figure 4.11: Relationship of salivary uPA levels with %BOP in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the %BOP index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

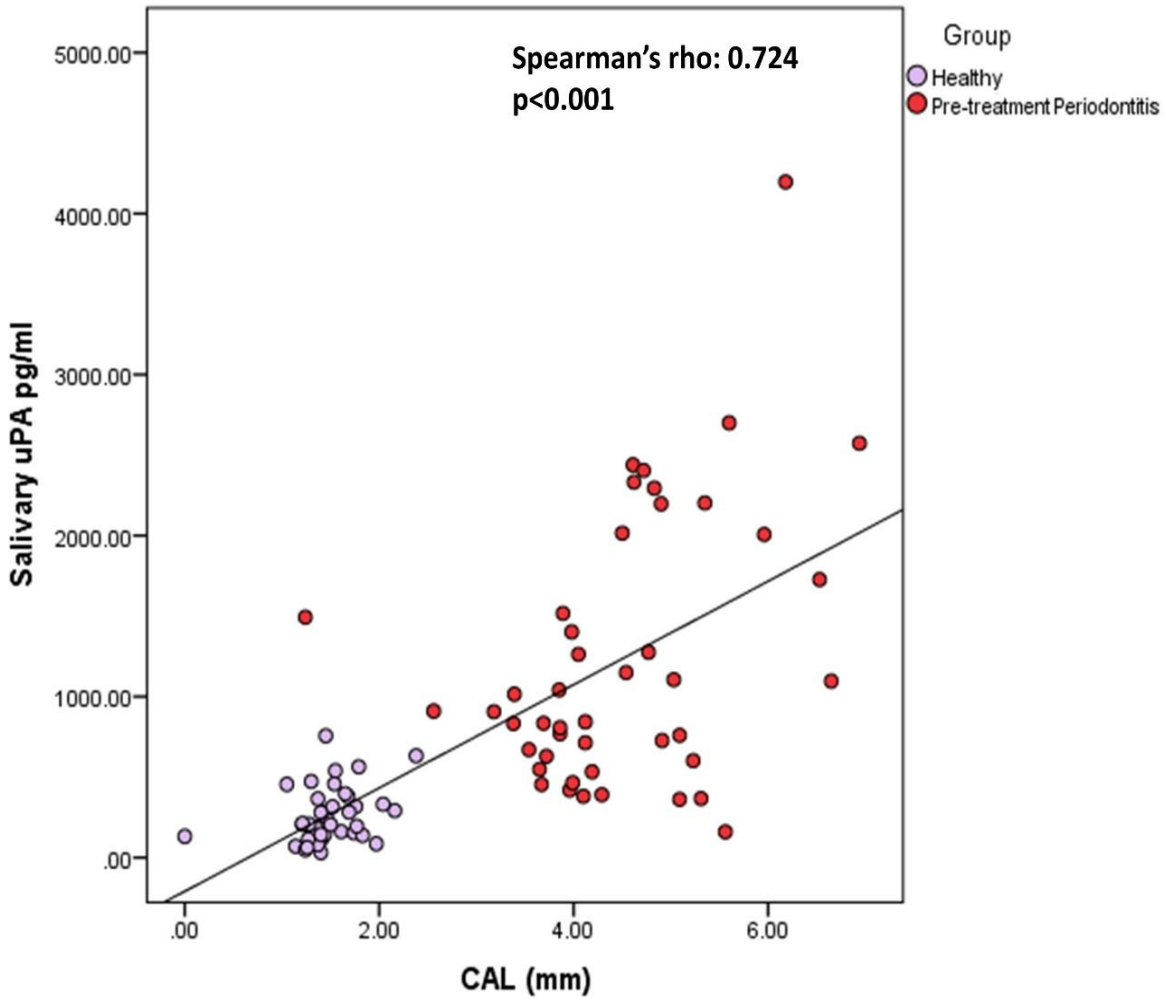

Figure 4.12: Relationship of salivary uPA levels with CAL in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 45 pre-treatment periodontitis patients and 40 healthy subjects with the CAL index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

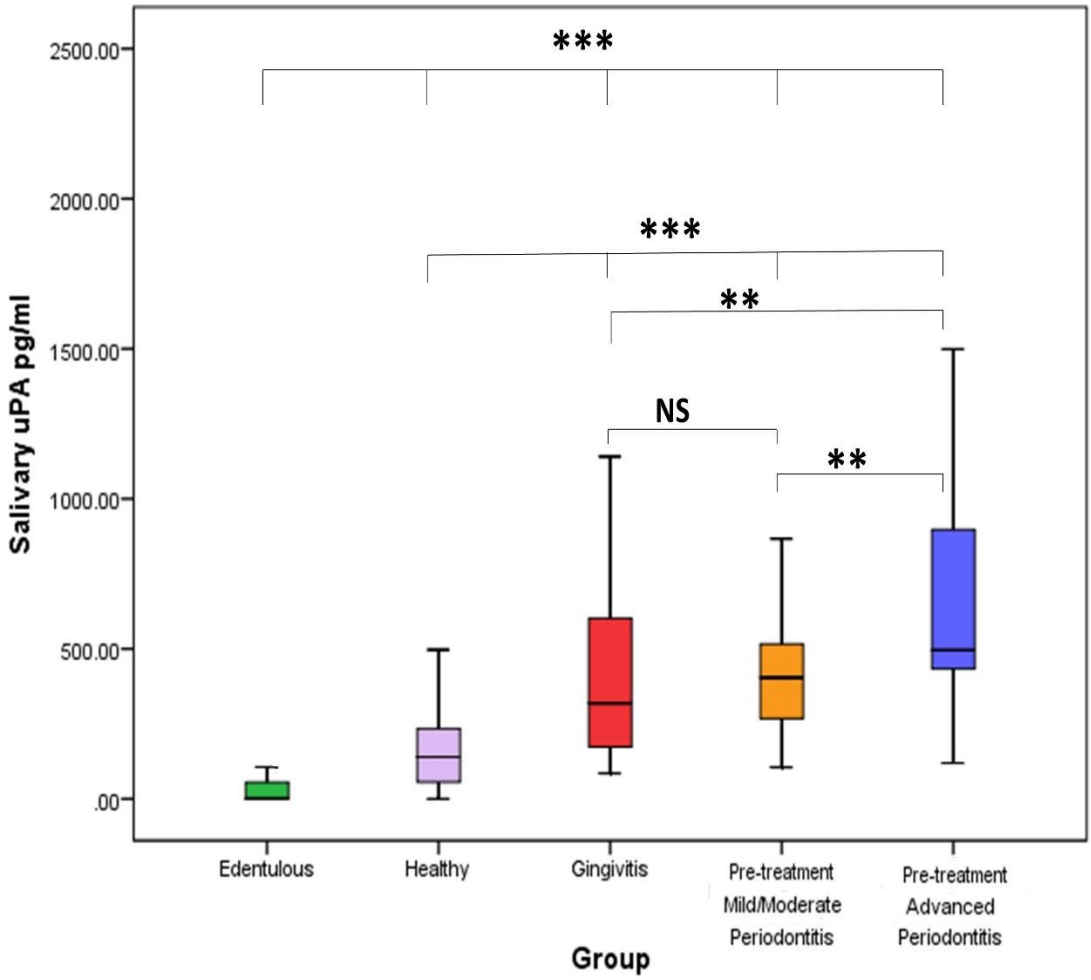

Figure 4.13: The pre-treatment salivary uPA levels in the periodontitis patients of study B.

The uPA levels were measured in saliva obtained from 31 pre-treatment mild/moderate periodontitis patients and 27 pre-treatment advanced periodontitis patients in comparison to 25 gingivitis patients, 26 edentulous subjects and 29 dentulous healthy subjects. The box plots represent the medians and IQRs for 4 groups, and the mean, minimum and maximum levels for the pre-treatment mild/moderate periodontitis patients. **=p<0.01, ***=p<0.001, and NS=non-significant (Kruskal-Wallis with Mann-Whitney U test and Bonferroni correction).

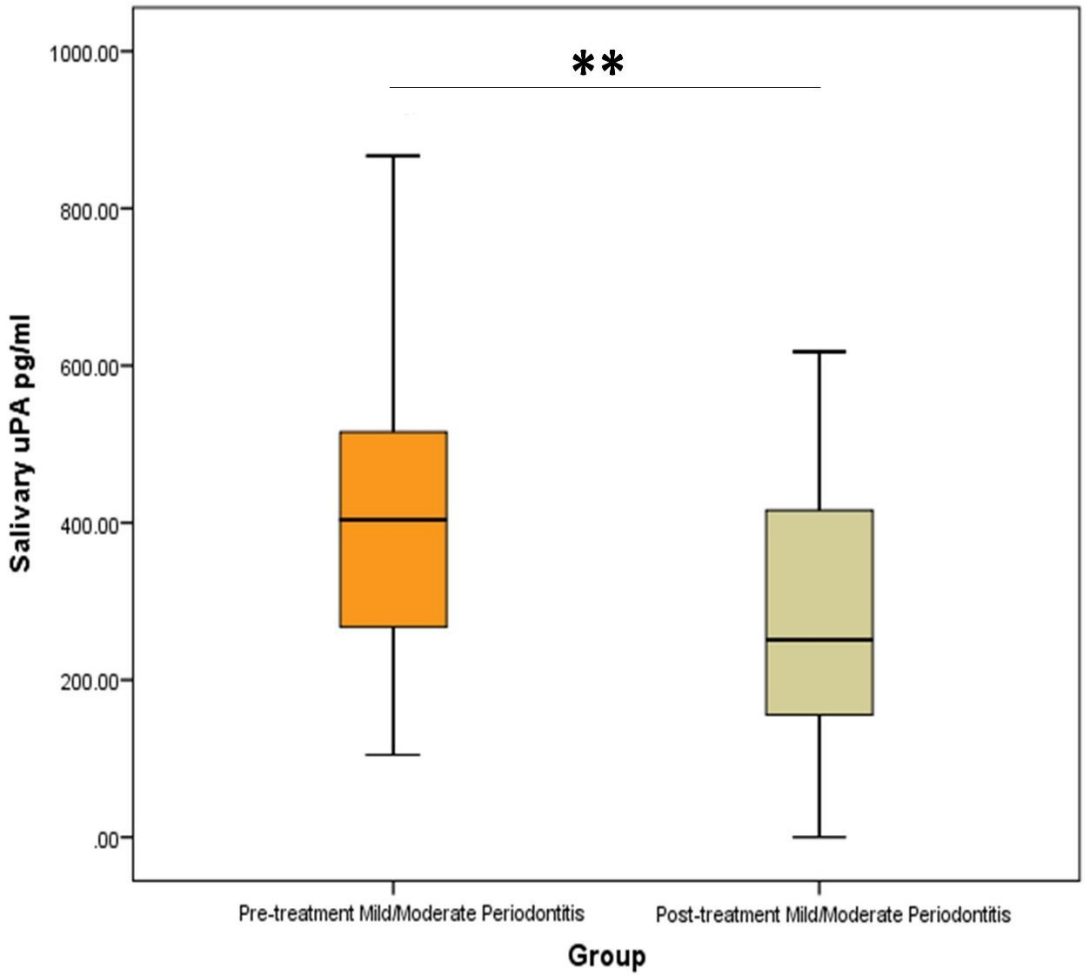

Figure 4.14: The post-treatment salivary uPA levels in the mild/moderate periodontitis patients of study B.

Salivary uPA levels were measured in 31 mild/moderate periodontitis patients in 12±2 weeks following non-surgical treatment in comparison to their pre-treatment levels. Data presented as mean, minimum and maximum levels. **=$p<0.01$ (Paired samples t-test).

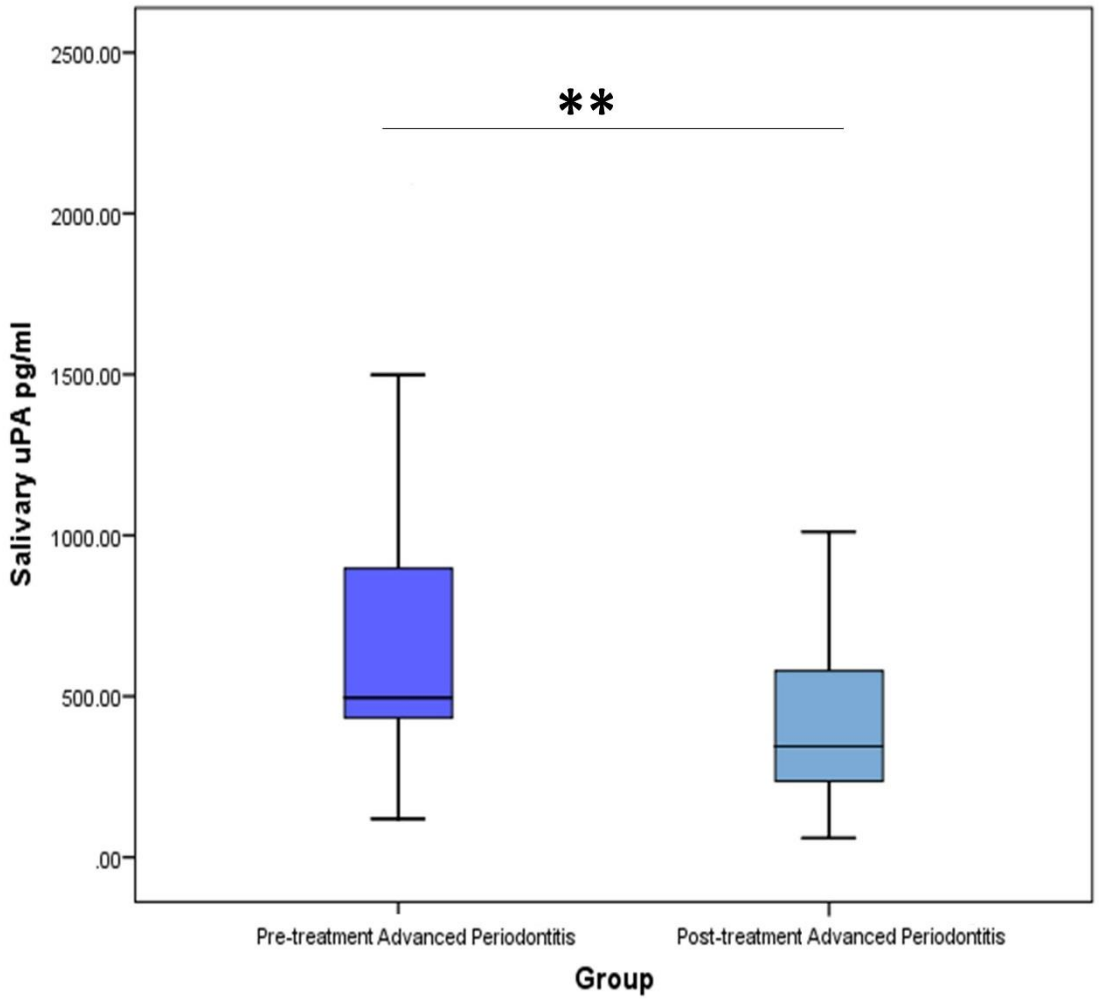

Figure 4.15: The post-treatment salivary uPA levels in the advanced periodontitis patients of study B.

Salivary uPA levels were measured in 27 advanced periodontitis patients in 12±2 weeks after treatment in comparison to their pre-treatment levels. The box plots represent the median and IQR for each group. **=p<0.01 (Wilcoxon singed rank test).

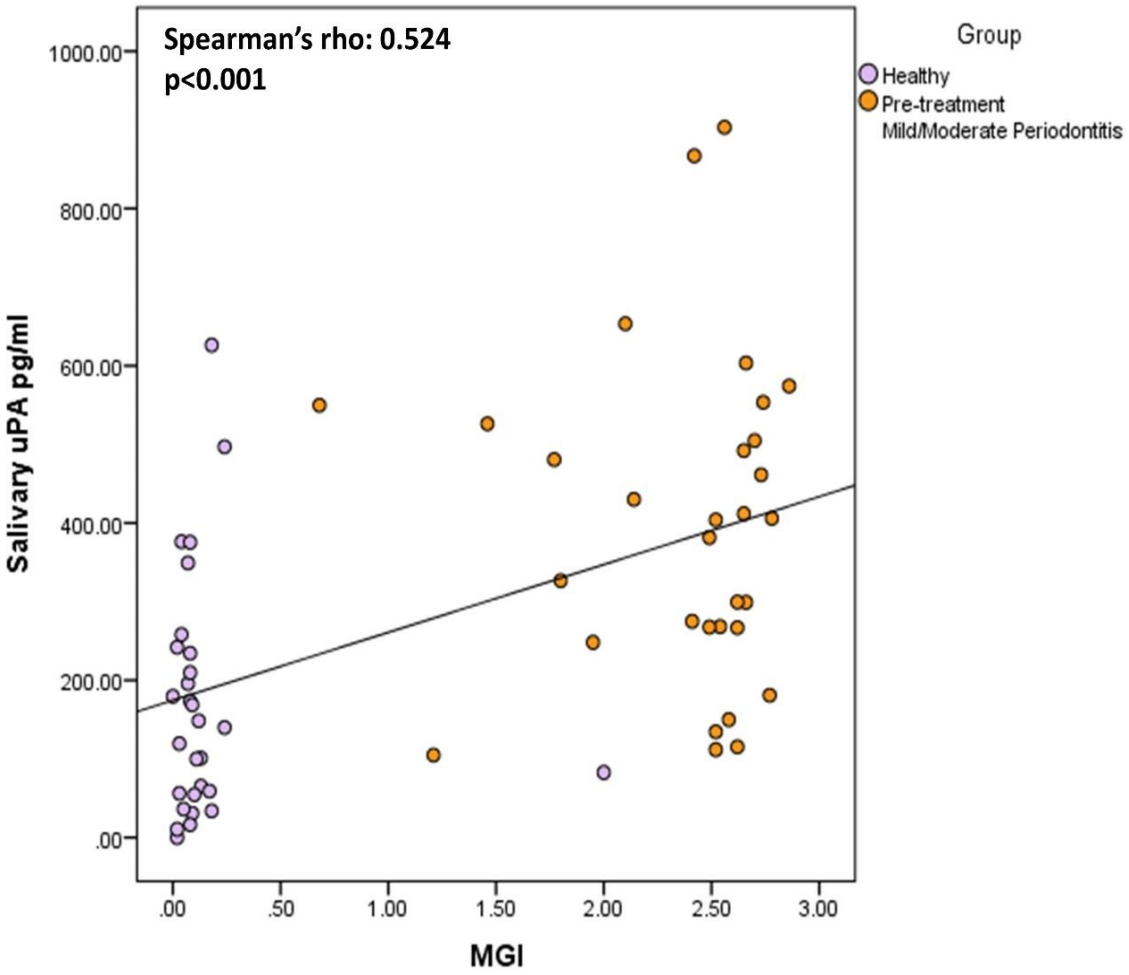

Figure 4.16: Relationship of salivary uPA levels with MGI in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the MGI index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

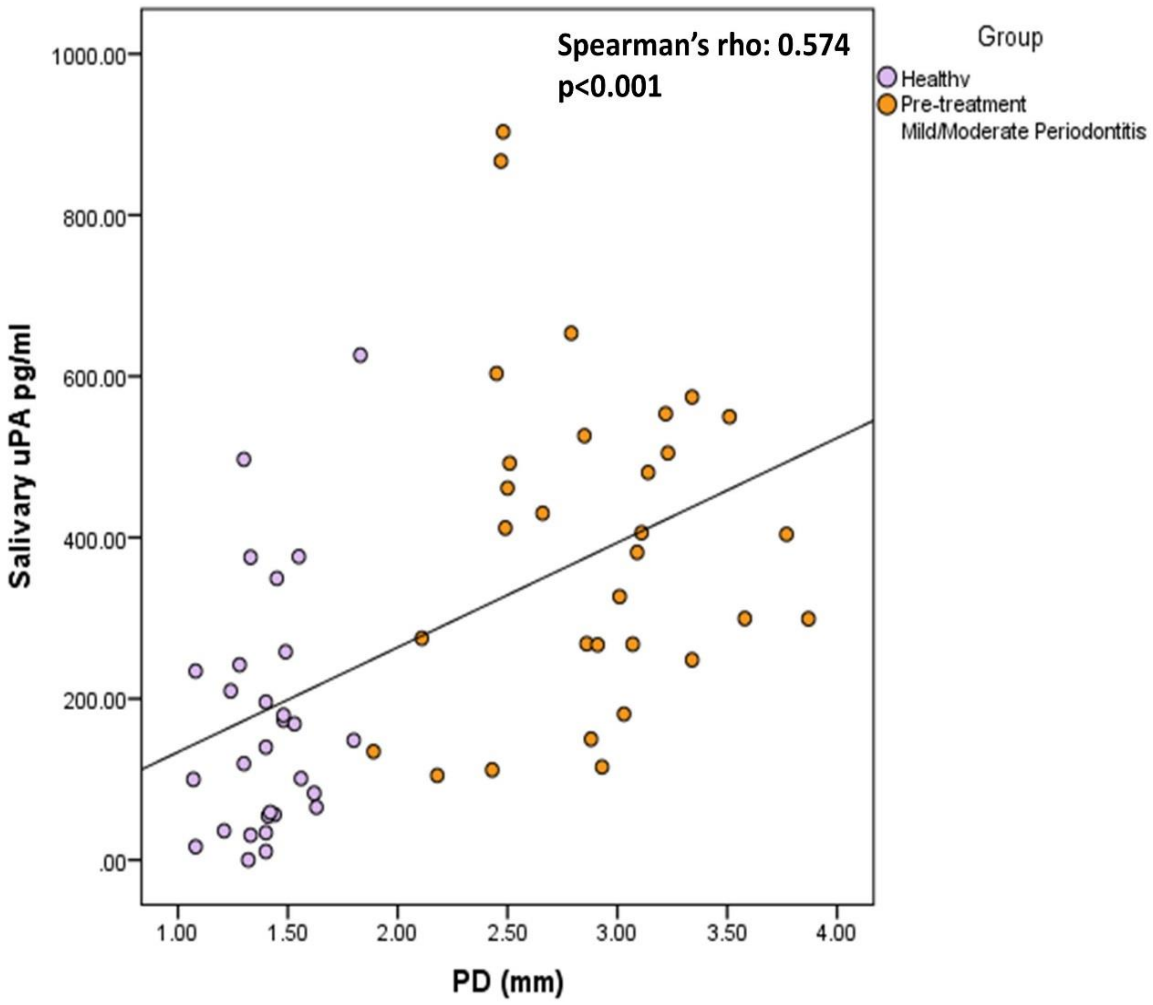

Figure 4.17: Relationship of salivary uPA levels with PD in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the PD index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

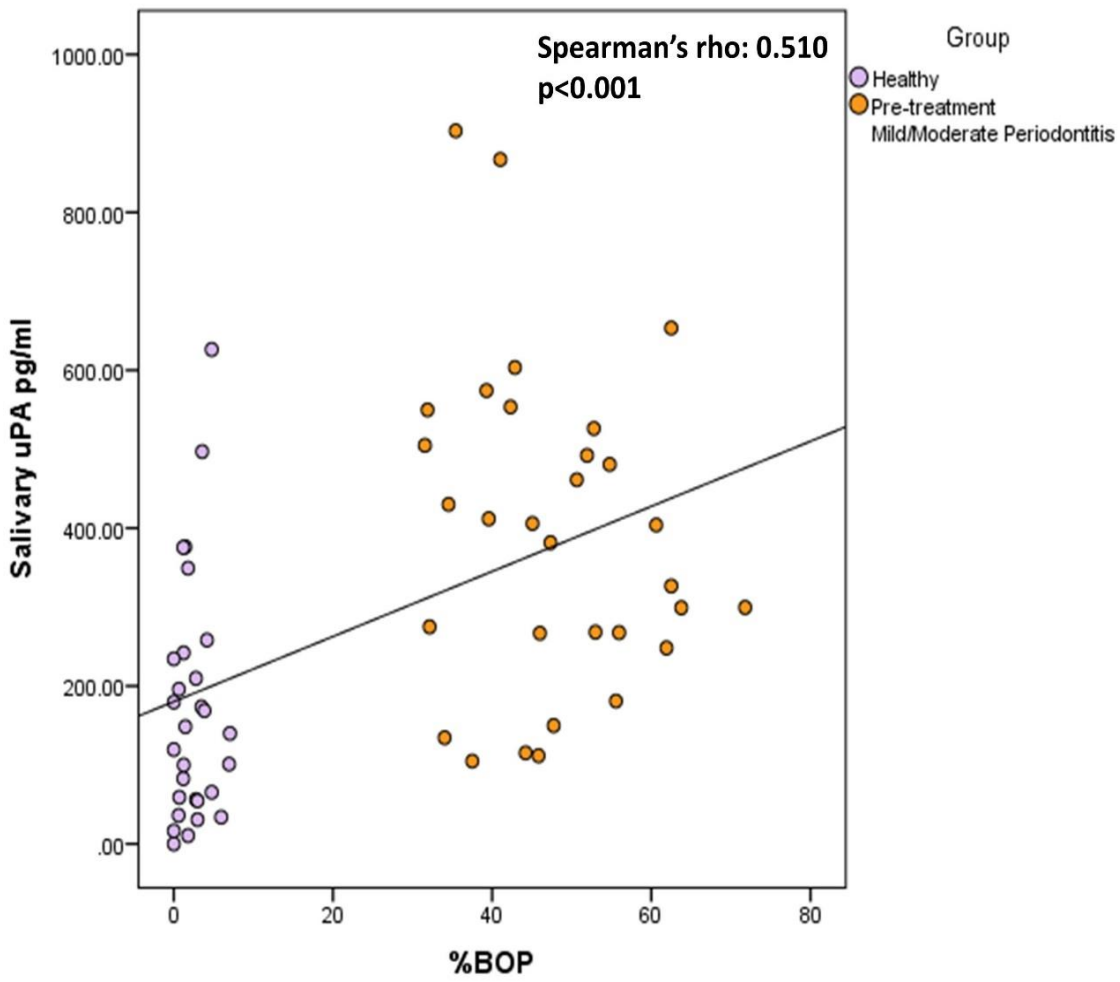

Figure 4.18: Relationship of salivary uPA levels with %BOP in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the %BOP index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

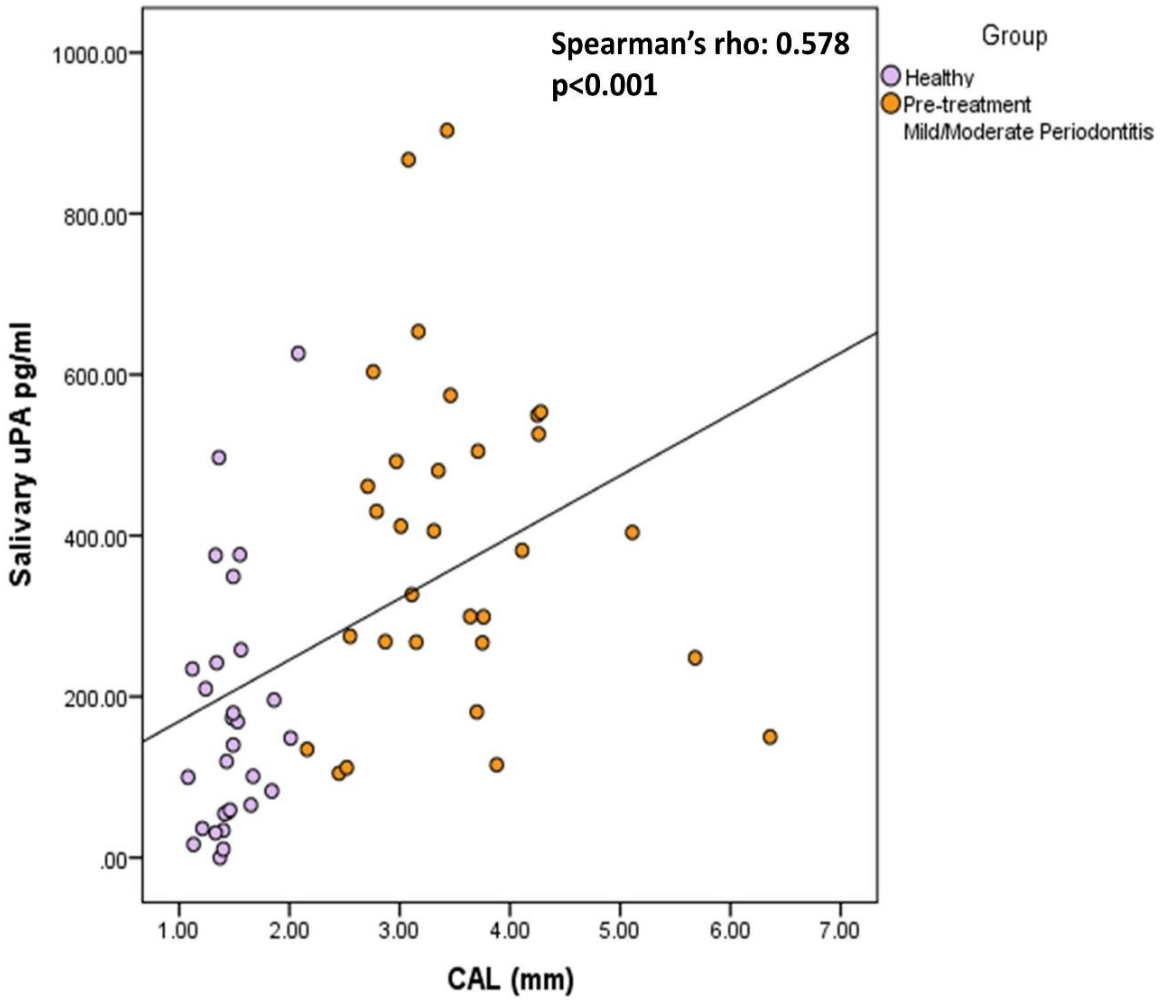

Figure 4.19: Relationship of salivary uPA levels with CAL in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the CAL index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

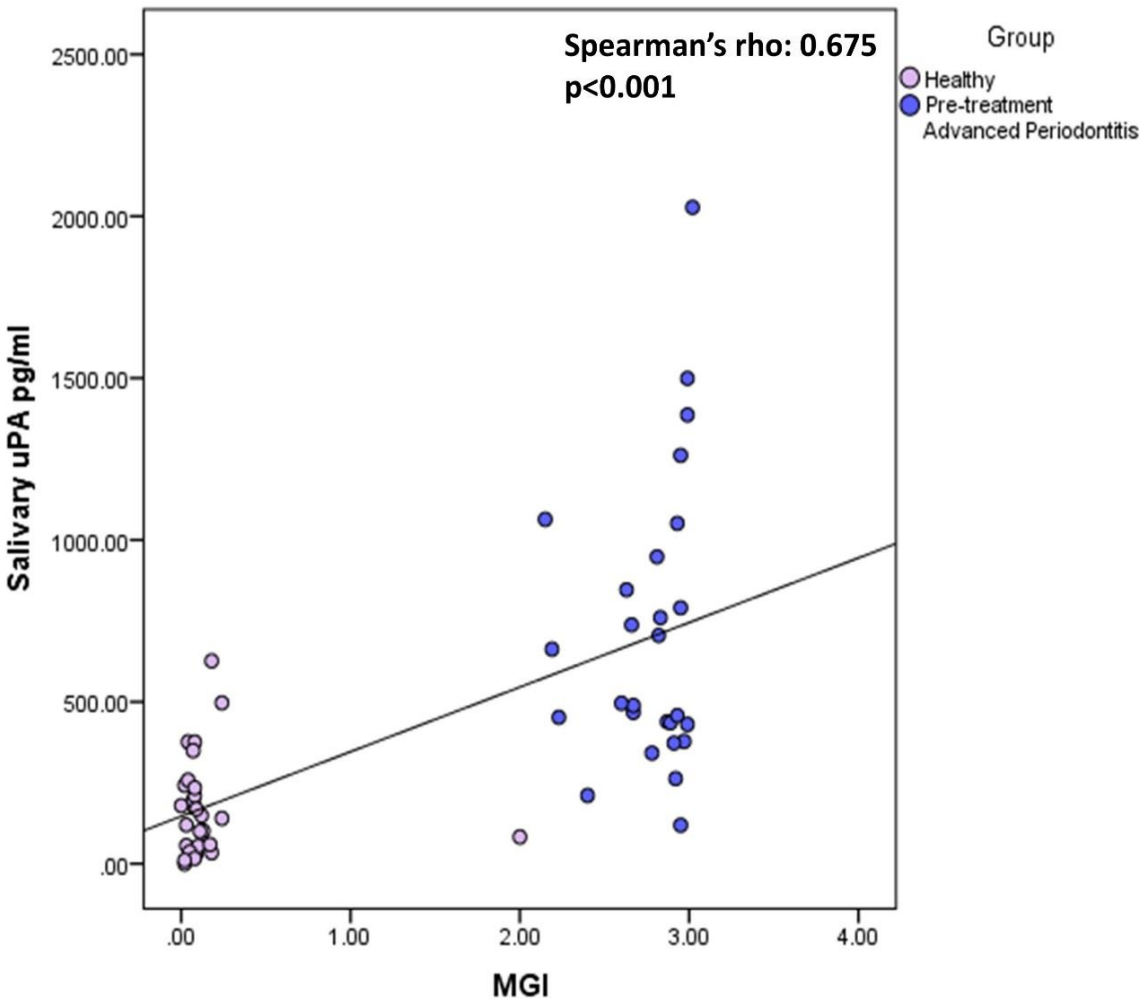

Figure 4.20: Relationship of salivary uPA levels with MGI in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the MGI index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

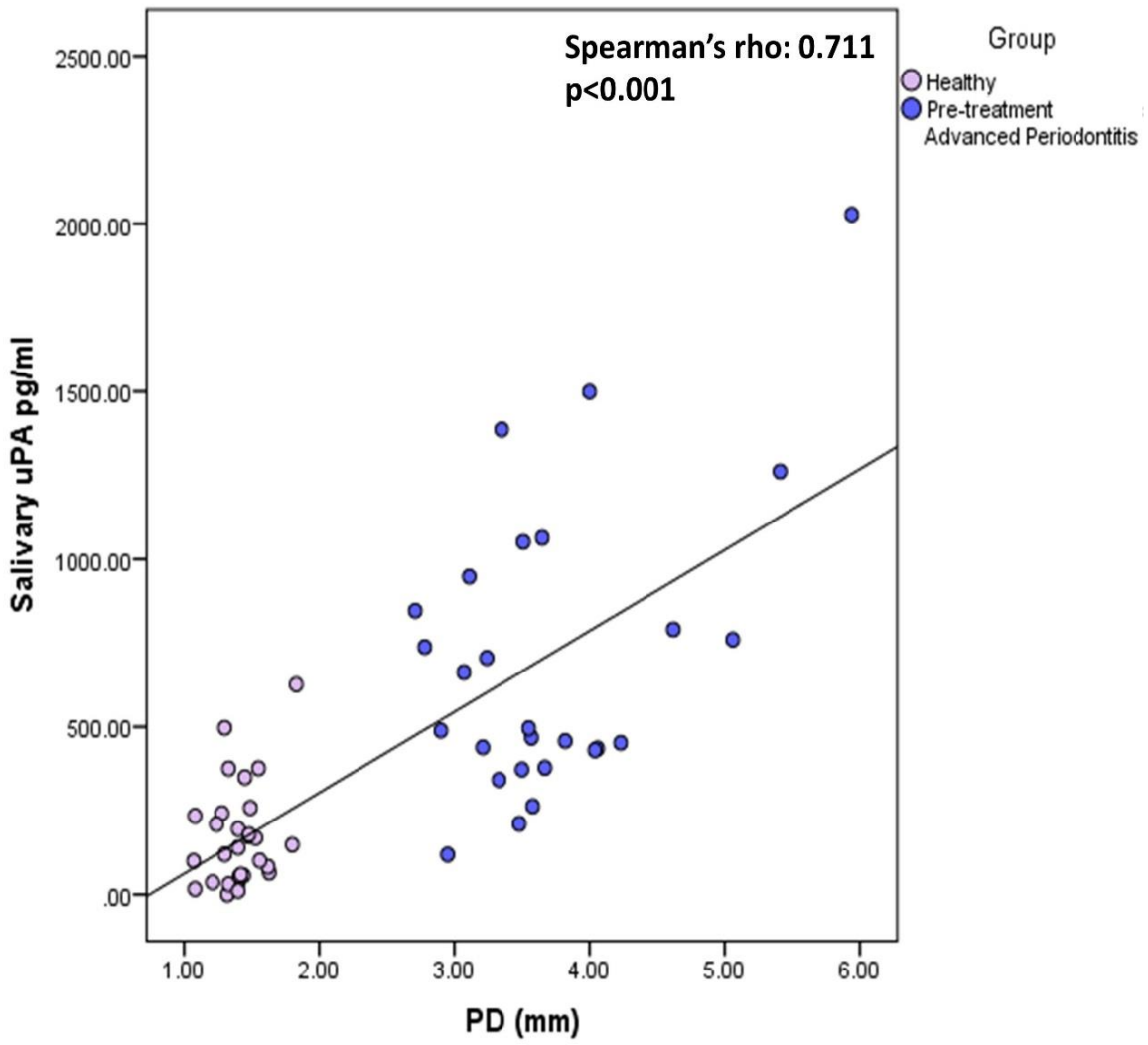

Figure 4.21: Relationship of salivary uPA levels with PD in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the PD index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

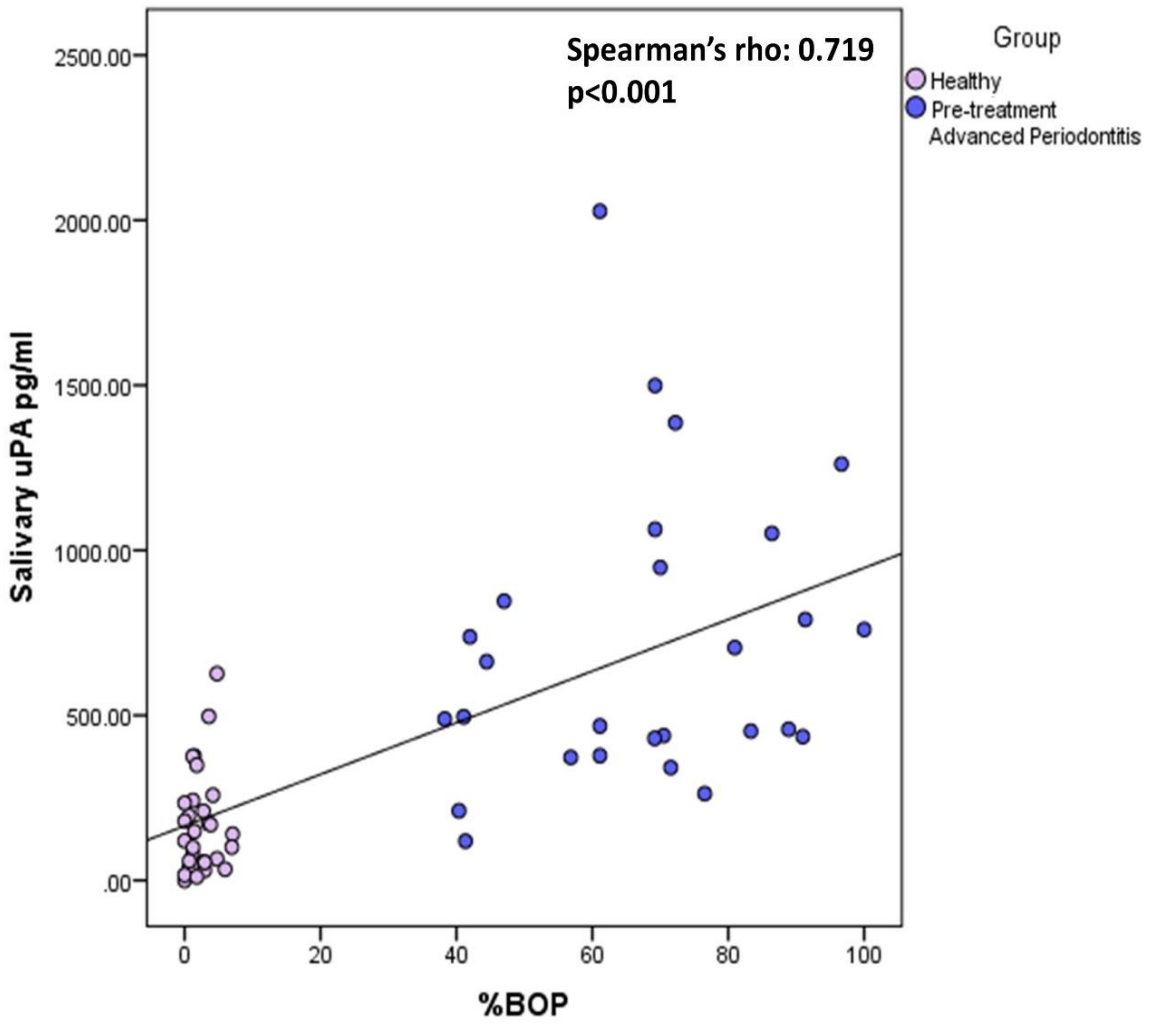

Figure 4.22: Relationship of salivary uPA levels with %BOP in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the %BOP index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

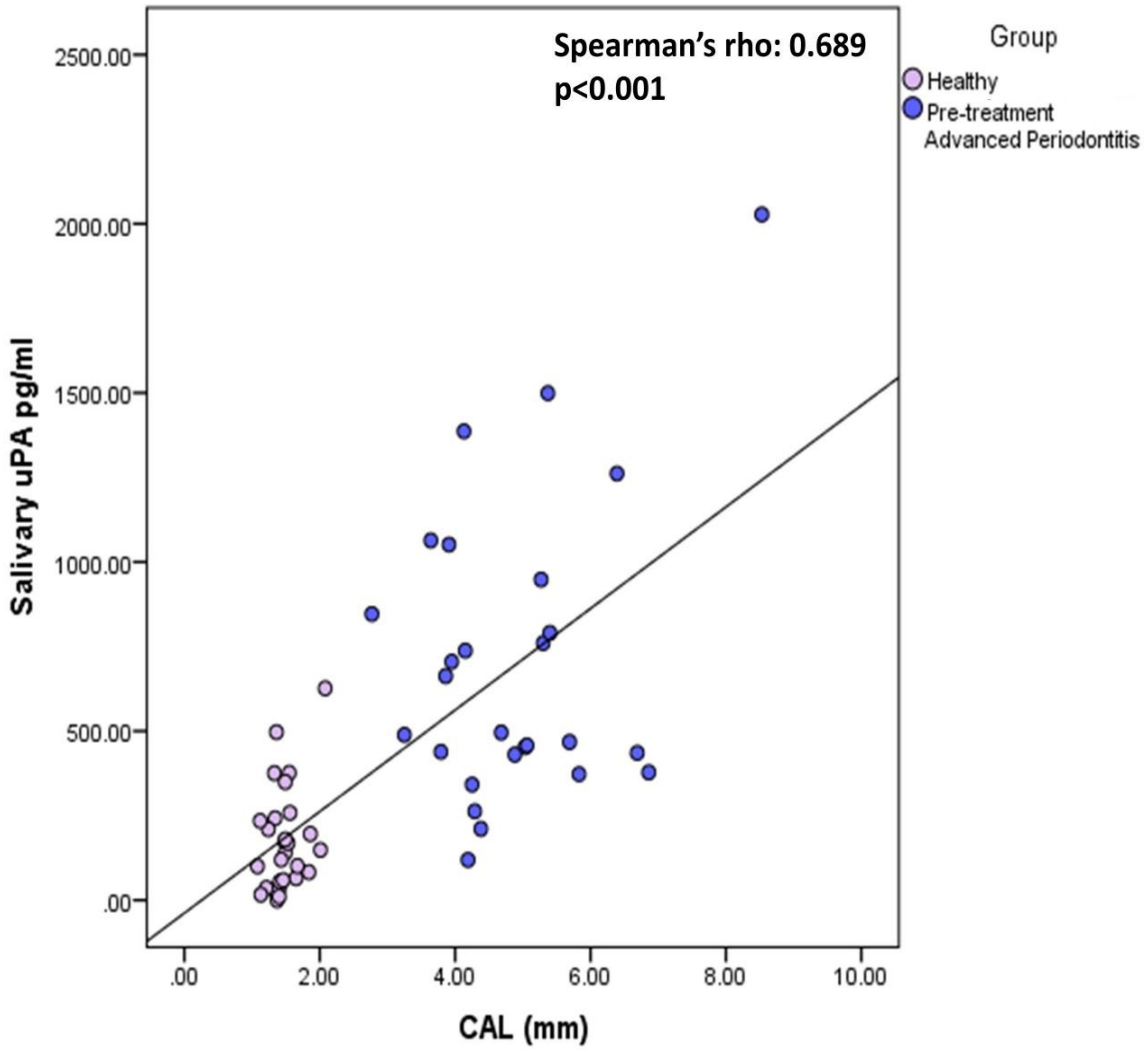

Figure 4.23: Relationship of salivary uPA levels with CAL in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA levels in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the CAL index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

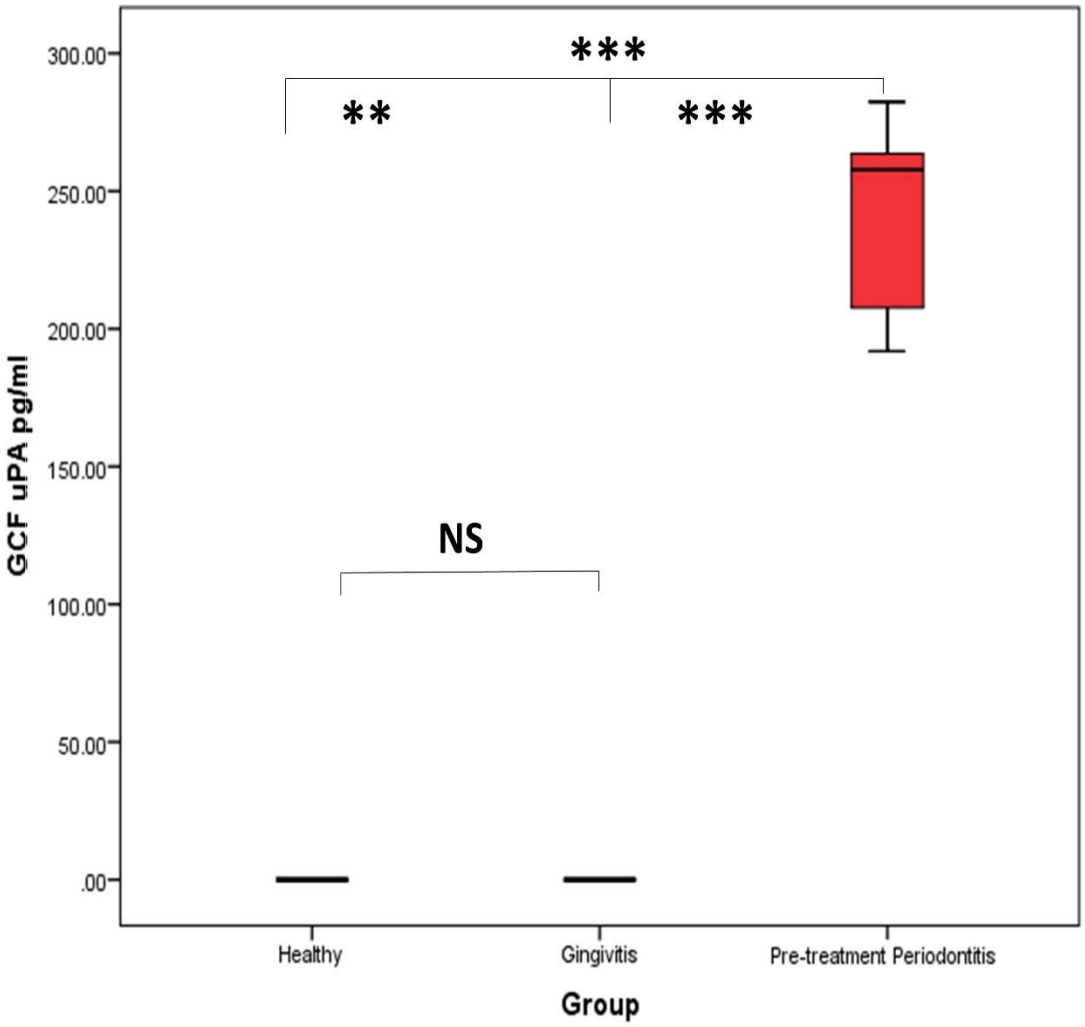

Figure 4.24: The pre-treatment GCF uPA levels in the periodontitis patients of study C.

The uPA levels were measured in GCF samples obtained from 9 pre-treatment periodontitis patients in comparison to 7 healthy control subjects and 13 gingivitis patients. Data presented as median and IQR. **=p<0.01, ***=p<0.001, and NS=non-significant (Kruskal-Wallis test with Mann-Whitney U test and Bonferroni correction).

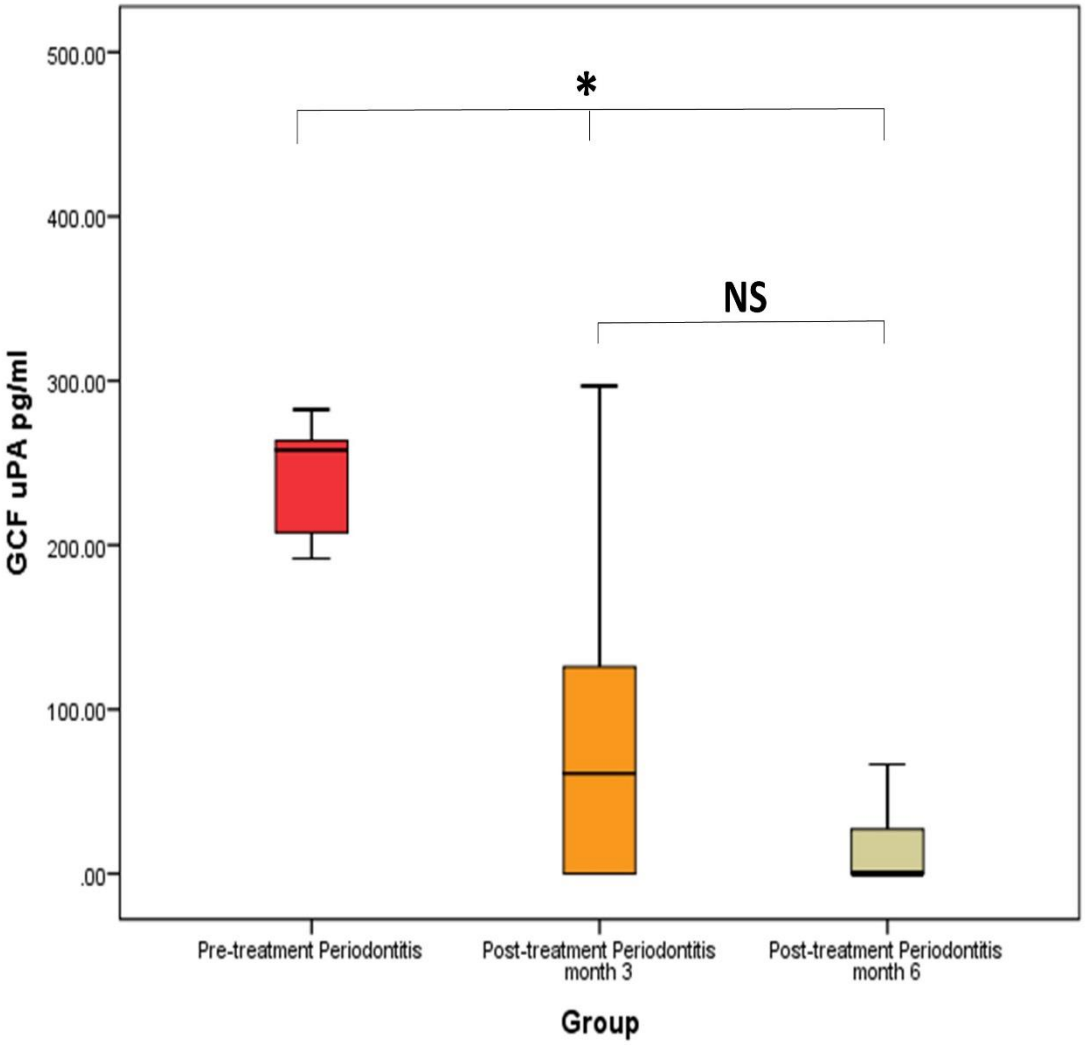

Figure 4.25: The post-treatment GCF uPA levels in the periodontitis patients of study C.

The uPA levels were measured in GCF samples of 9 periodontitis patients in 3 and 6 months following non-surgical treatment in comparison to their pre-treatment levels. The box plots represent the median and IQR for each group. *=$p<0.05$, and NS=non-significant (Friedman test with Wilcoxon signed rank test and Bonferroni correction).

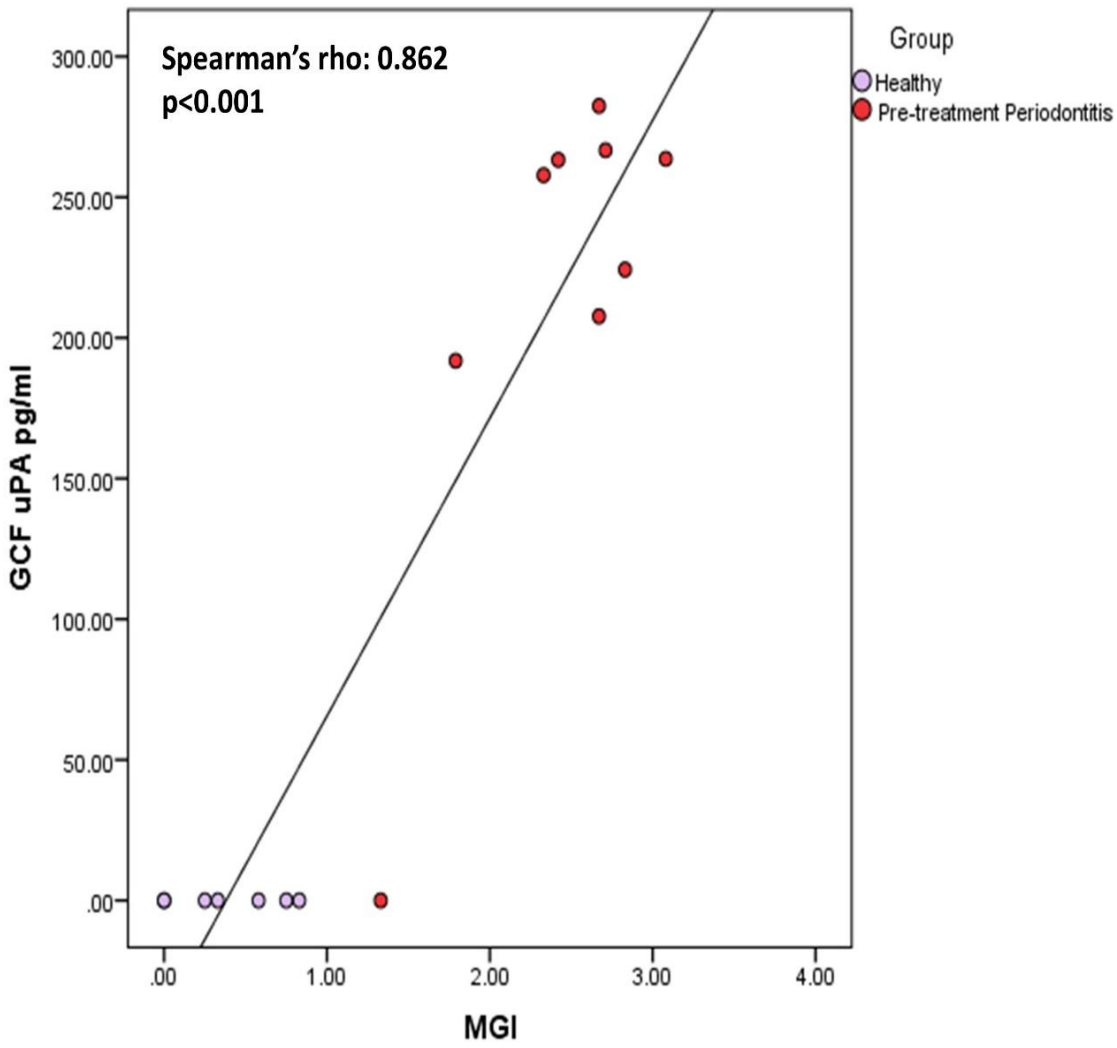

Figure 4.26: Relationship of GCF uPA levels with MGI in pre-treatment periodontitis patients.

The Spearman's correlation of the GCF uPA levels in 9 pre-treatment periodontitis patients and 7 healthy subjects with the MGI index in the clinical study C. The trend-line demonstrates the presence of a significant positive correlation.

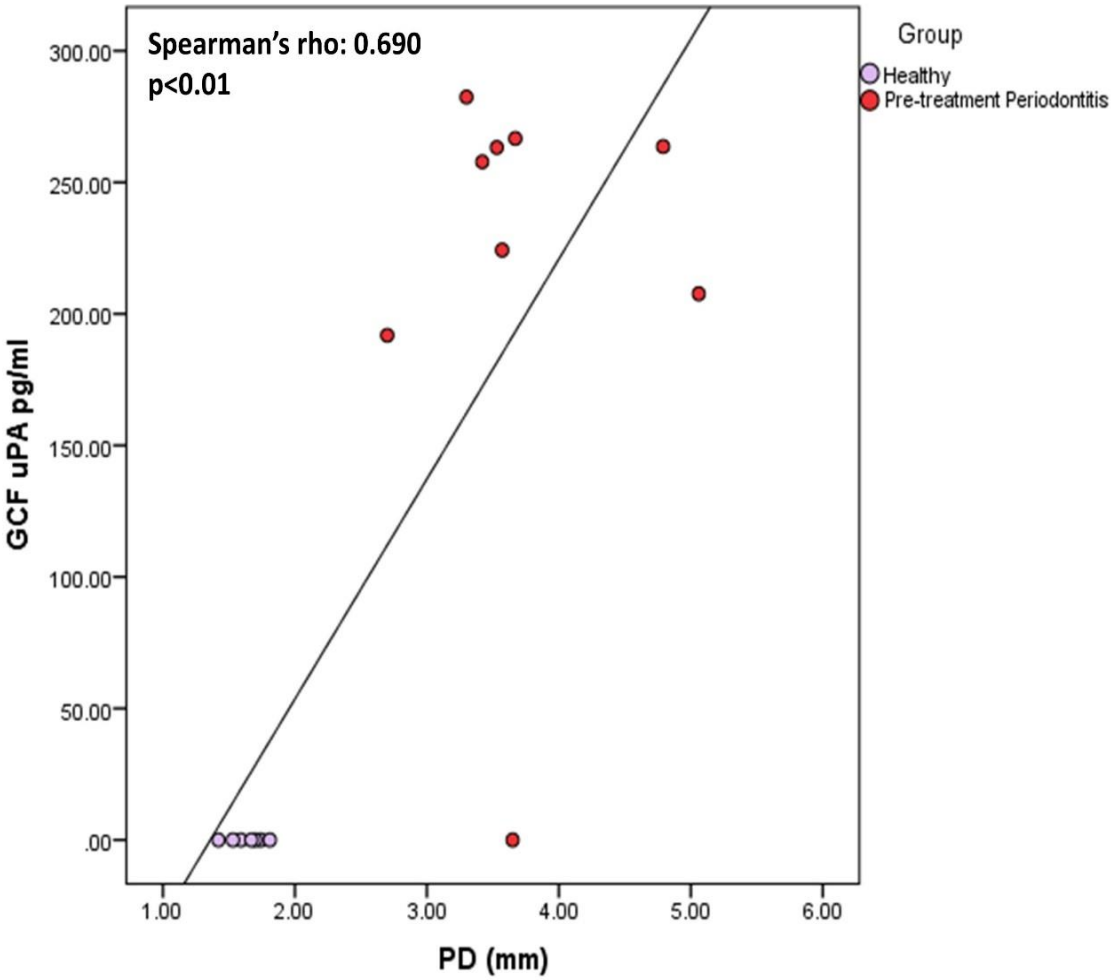

Figure 4.27: Relationship of GCF uPA levels with PD in pre-treatment periodontitis patients.

The Spearman's correlation of the GCF uPA levels in 9 pre-treatment periodontitis patients and 7 healthy subjects with the PD index in the clinical study C. The trend-line demonstrates the presence of a significant positive correlation.

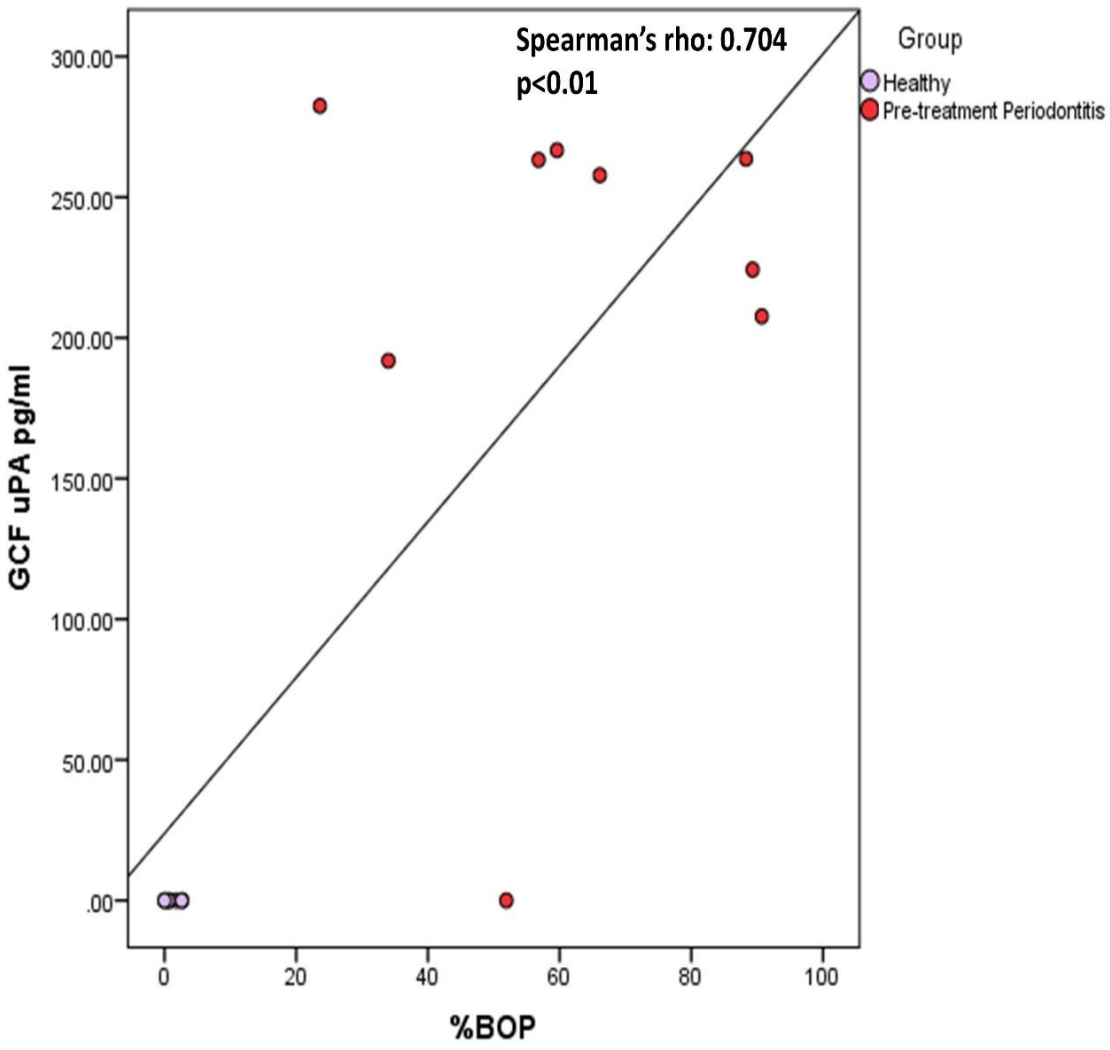

Figure 4.28: Relationship of GCF uPA levels with %BOP in pre-treatment periodontitis patients.

The Spearman's correlation of the GCF uPA levels in 9 pre-treatment periodontitis patients and 7 healthy subjects with the %BOP index in the clinical study C. The trend-line demonstrates the presence of a significant positive correlation.

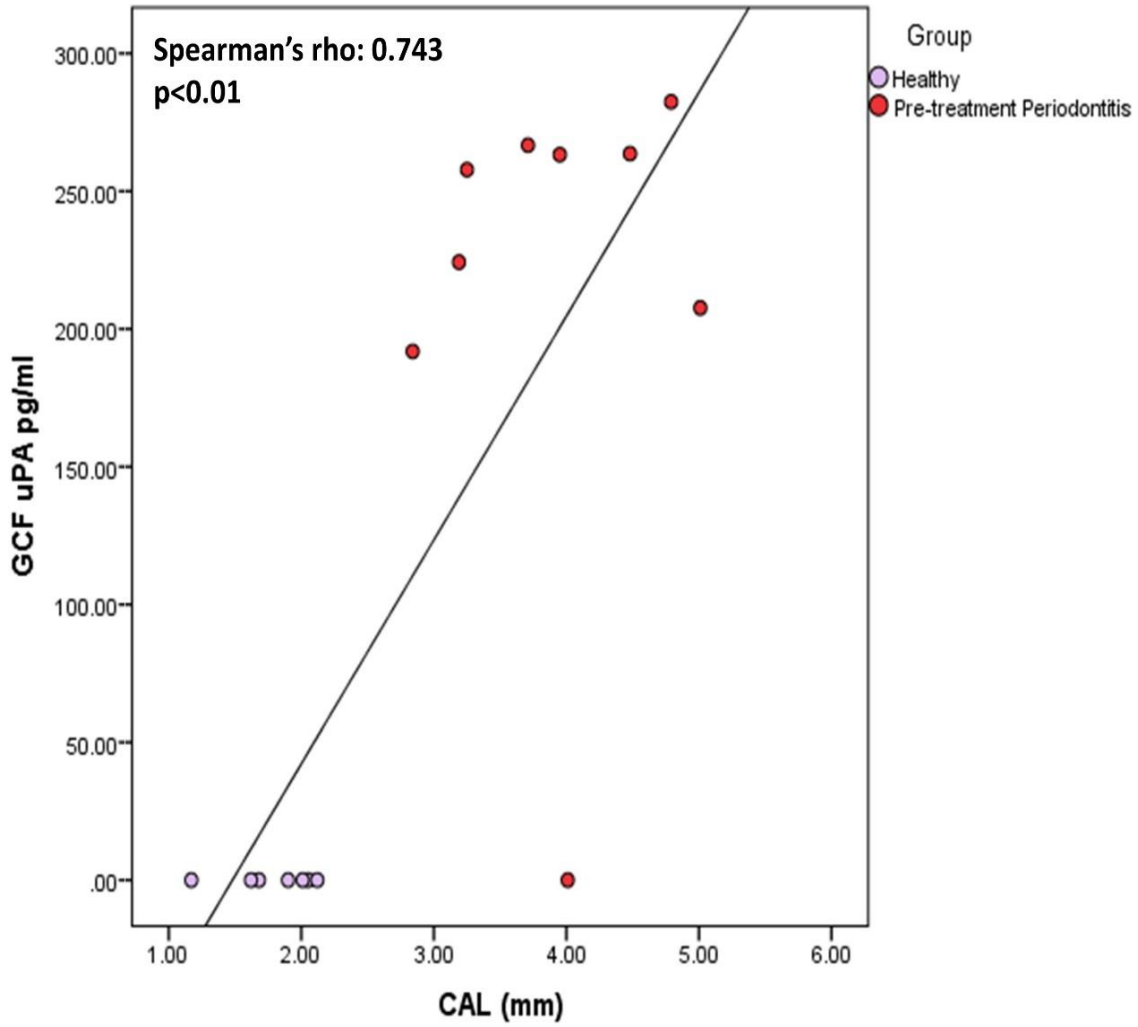

Figure 4.29: Relationship of GCF uPA levels with CAL in pre-treatment periodontitis patients.

The Spearman's correlation of the GCF uPA levels in 9 pre-treatment periodontitis patients and 7 healthy subjects with the CAL index in the clinical study C. The trend-line demonstrates the presence of a significant positive correlation.

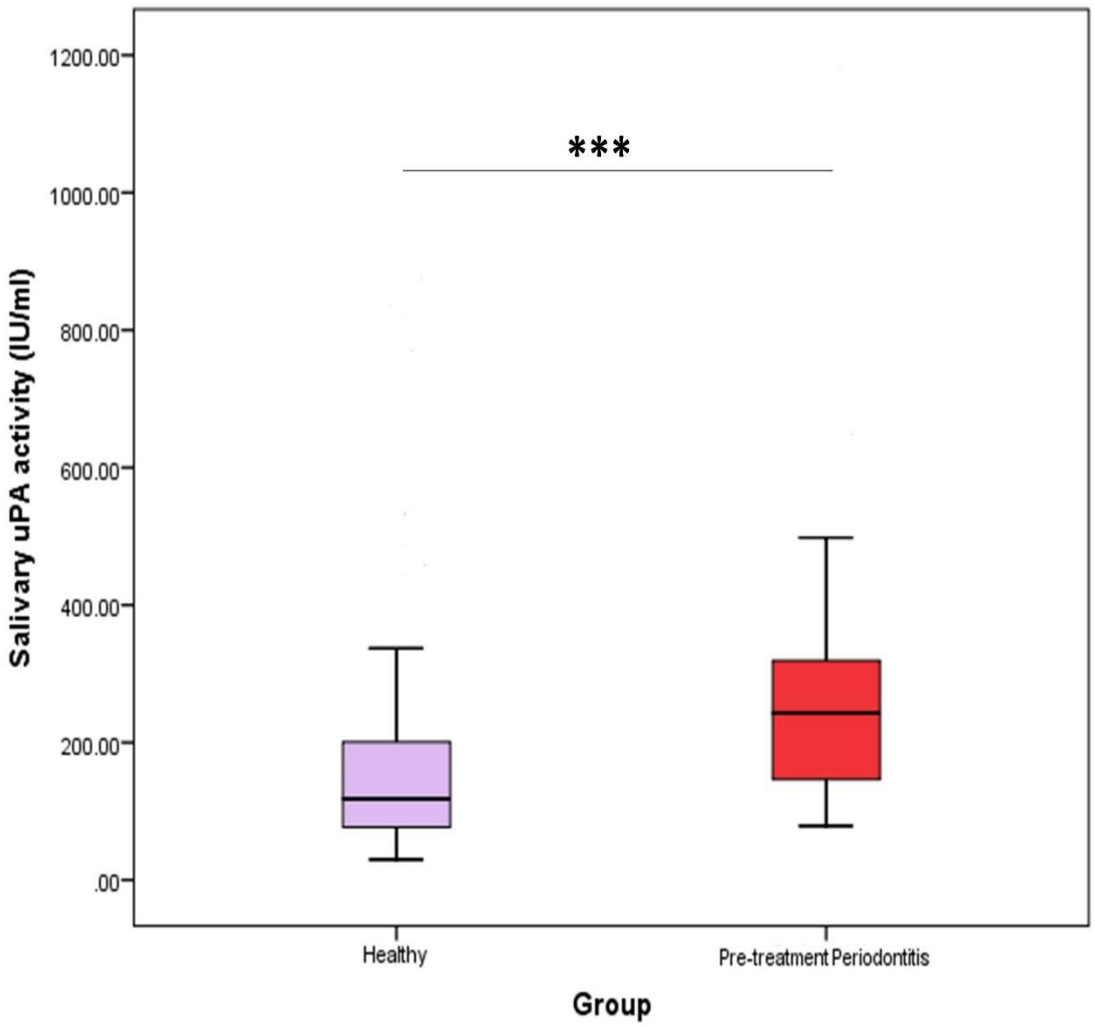

Figure 4.30: Salivary uPA activity in the periodontitis patients of study A.

The uPA activity was assayed in saliva of 45 pre-treatment periodontitis patients in comparison to 40 healthy subjects. Data presented as median and IQR for each group. ***=p<0.001 (Mann-Whitney U test).

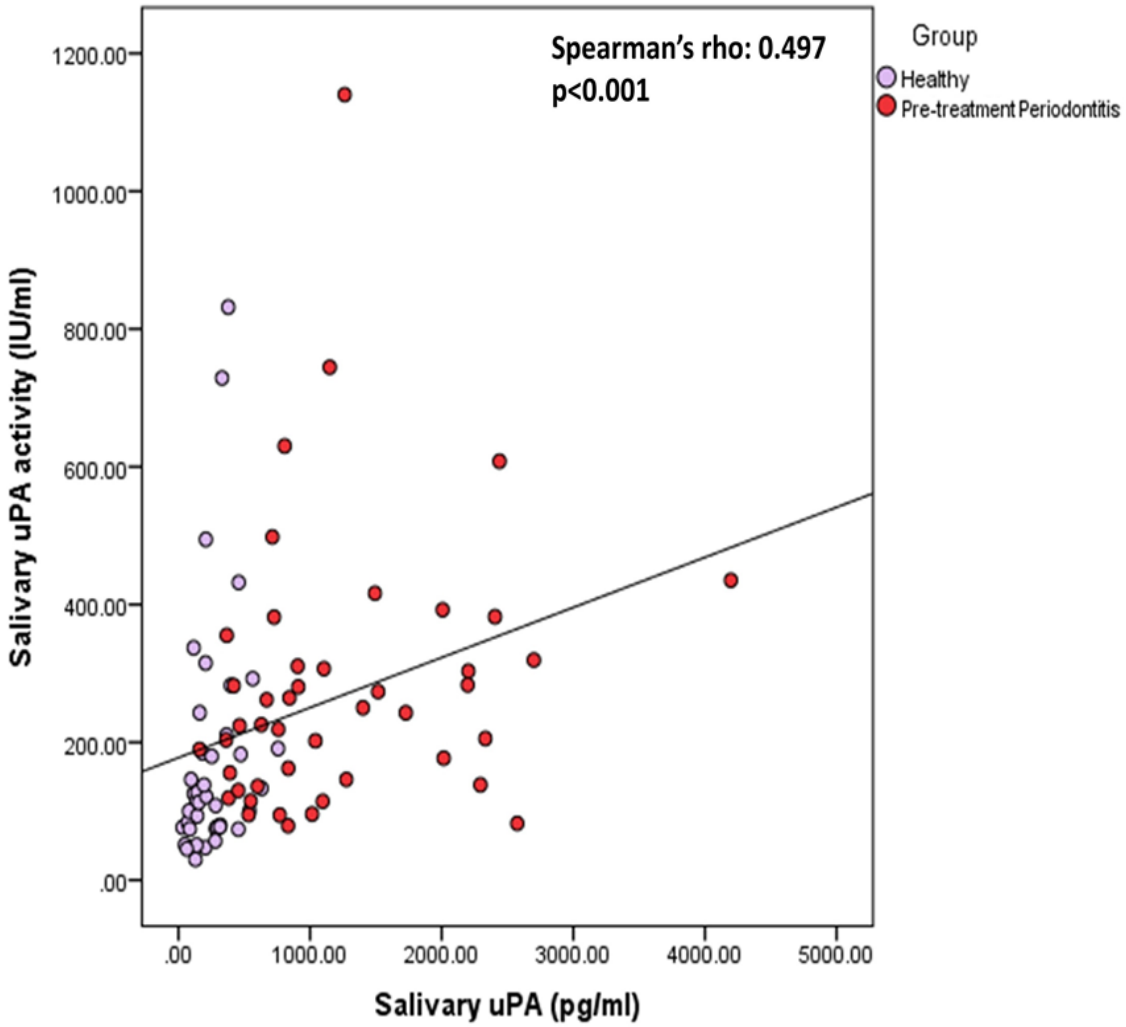

Figure 4.31: Relationship of salivary uPA activity with salivary uPA levels in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy with the salivary uPA levels in 45 pre-treatment periodontitis patients and 40 healthy subjects in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

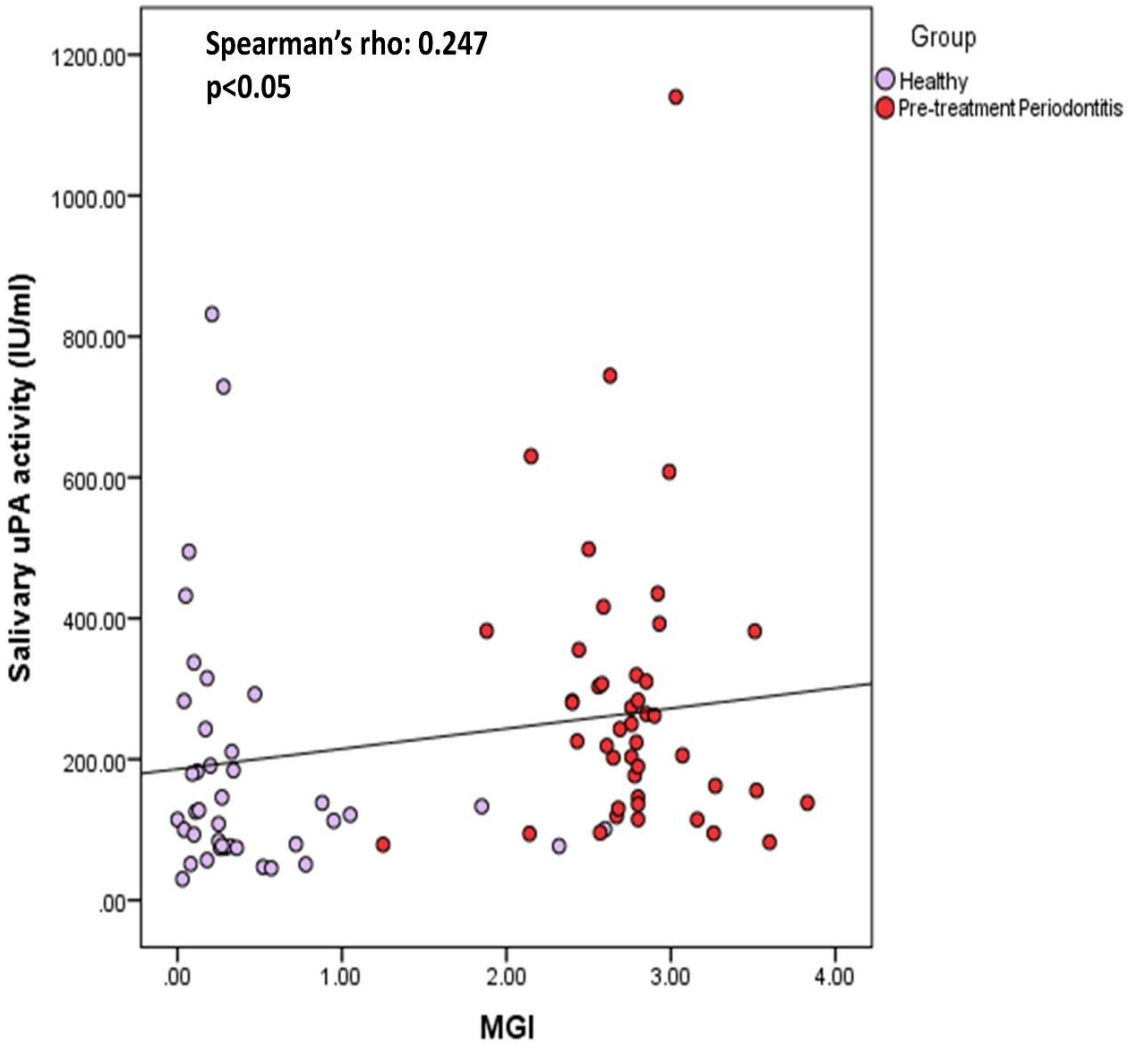

Figure 4.32: Relationship of salivary uPA activity with MGI in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 45 pre-treatment periodontitis patients and 40 healthy subjects with the MGI index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

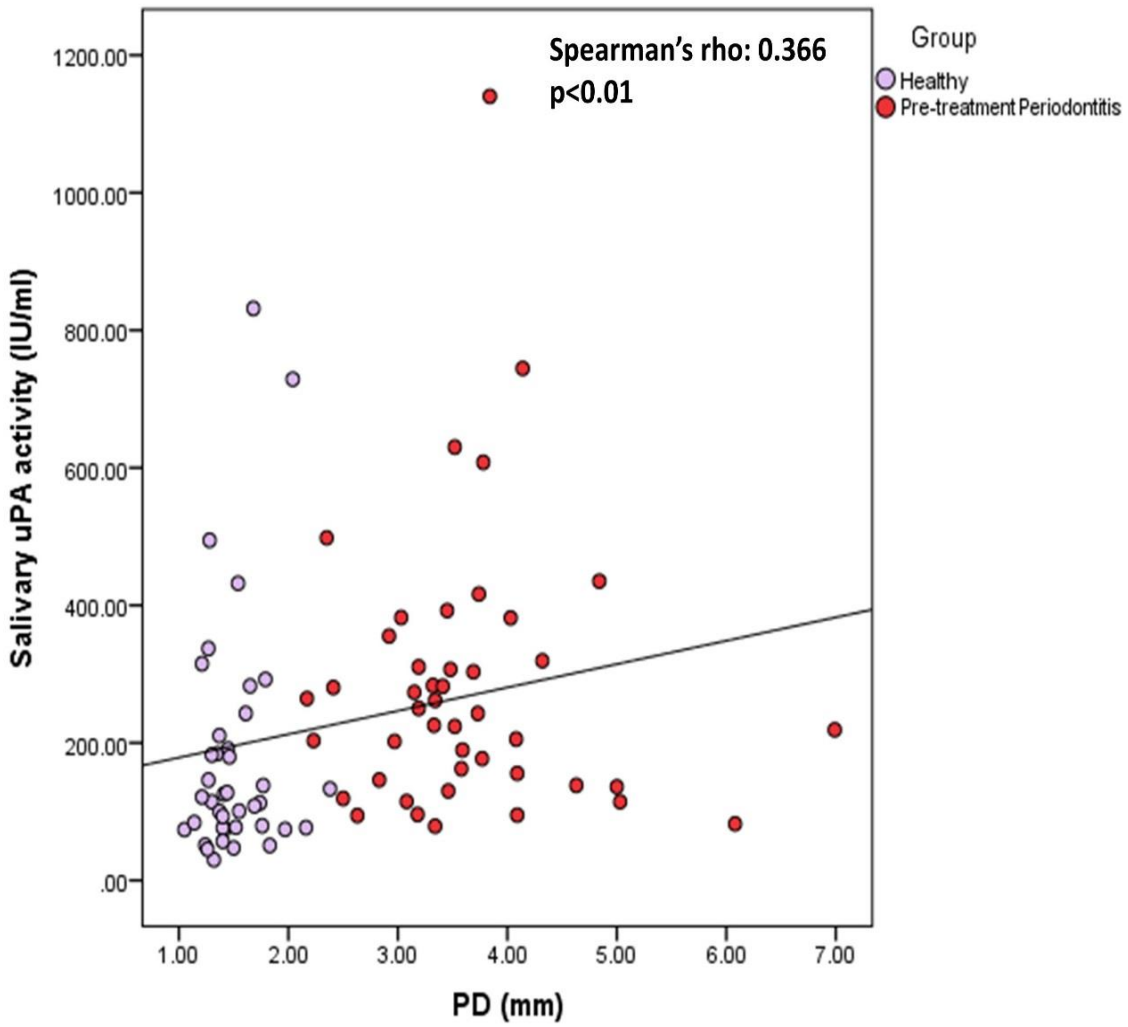

Figure 4.33: Relationship of salivary uPA activity with PD in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 45 pre-treatment periodontitis patients and 40 healthy subjects with the PD index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

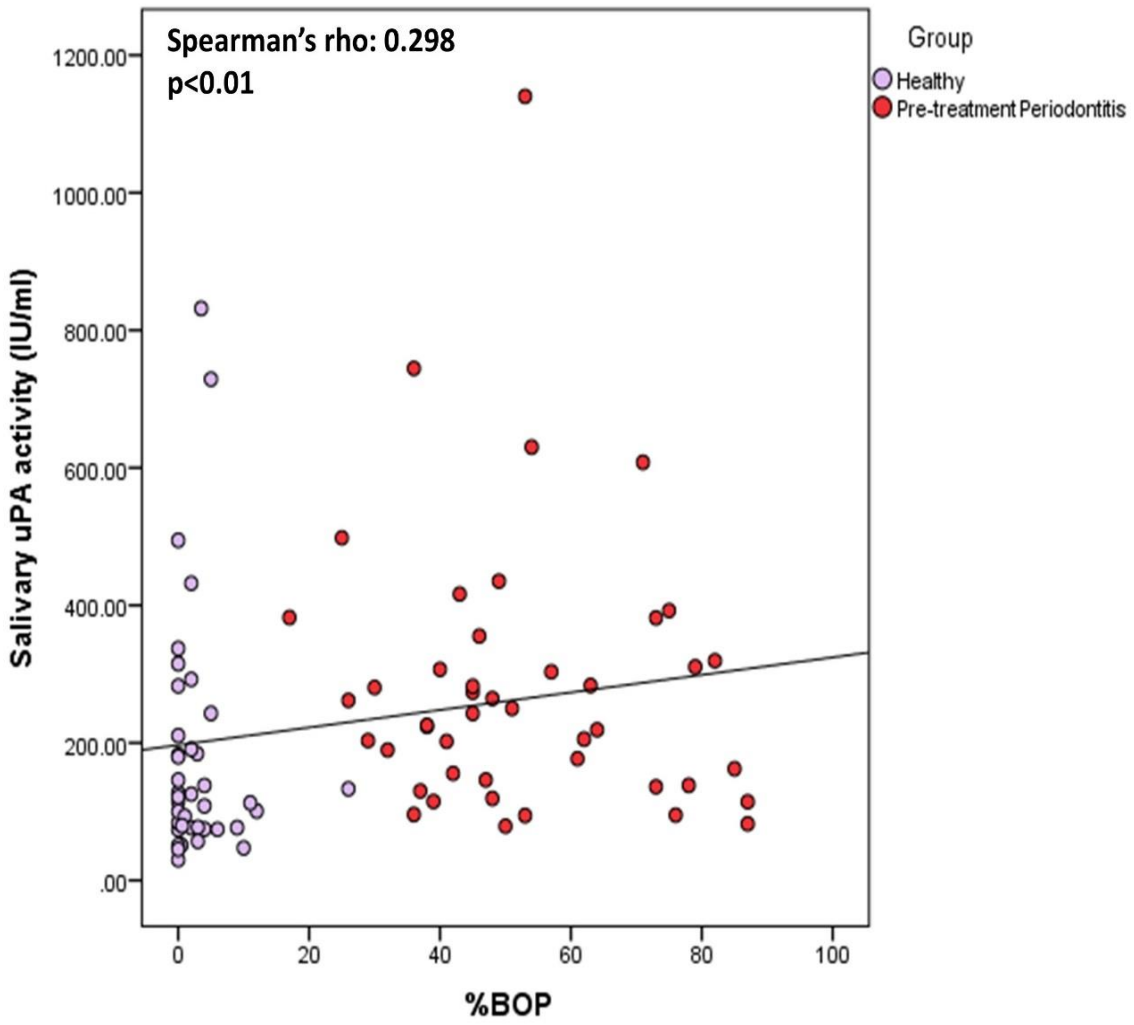

Figure 4.34: Relationship of salivary uPA activity with %BOP in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 45 pre-treatment periodontitis patients and 40 healthy subjects with the %BOP index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

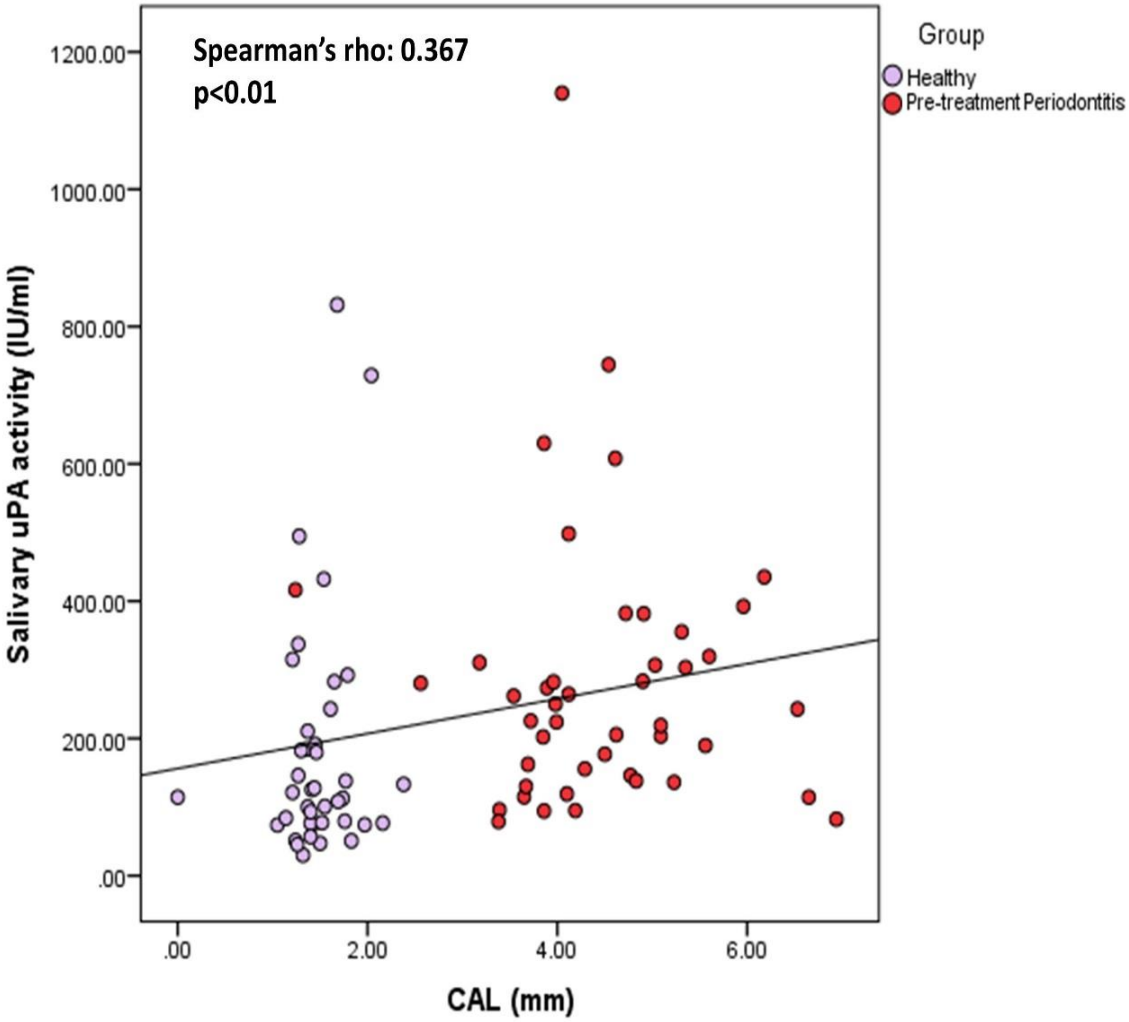

Figure 4.35: Relationship of salivary uPA activity with CAL in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 45 pre-treatment periodontitis patients and 40 healthy subjects with the CAL index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

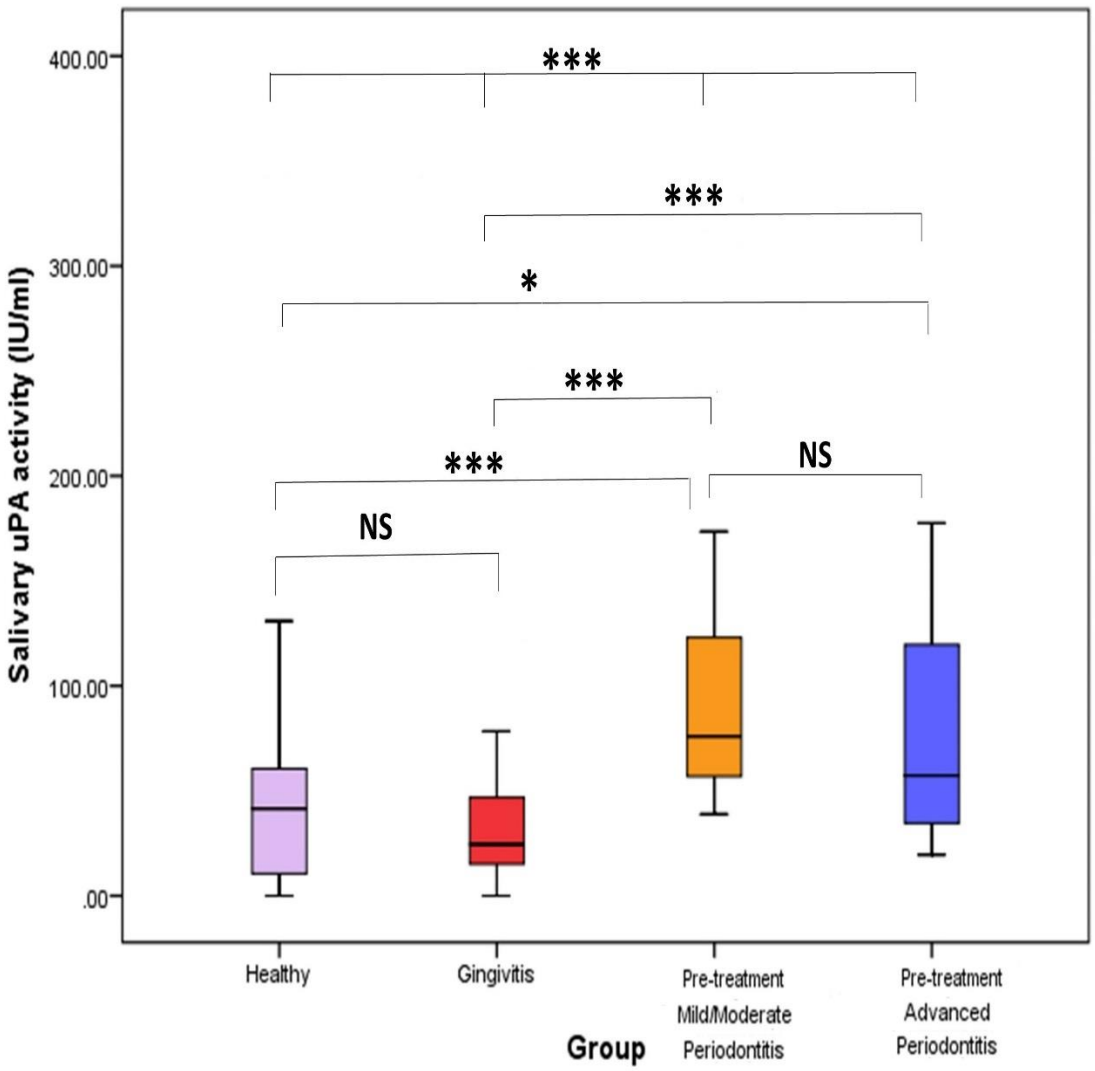

Figure 4.36: Salivary uPA activity in the periodontitis patients of study B.

Salivary uPA activity was assayed in 31 pre-treatment mild/moderate periodontitis patients and 27 pre-treatment advanced periodontitis patients in comparison to 25 gingivitis patients and 29 healthy subjects. The box plots represent the median and IQR for each group. *=p<0.05, ***=p<0.001, and NS=non-significant (Kruskal-Wallis with Mann-Whitney U test and Bonferroni correction).

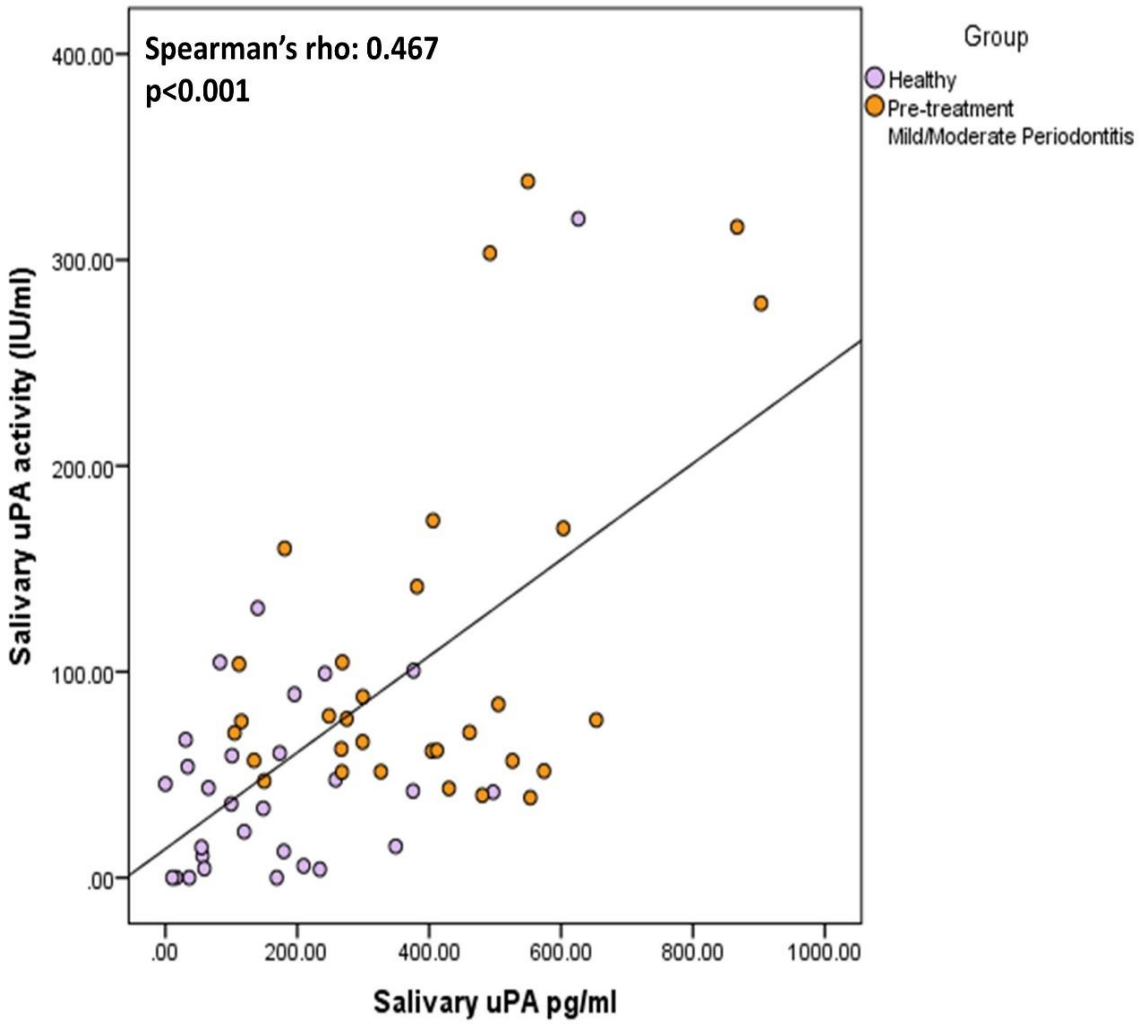

Figure 4.37: Relationship of salivary uPA activity with salivary uPA levels in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy with the salivary uPA levels in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

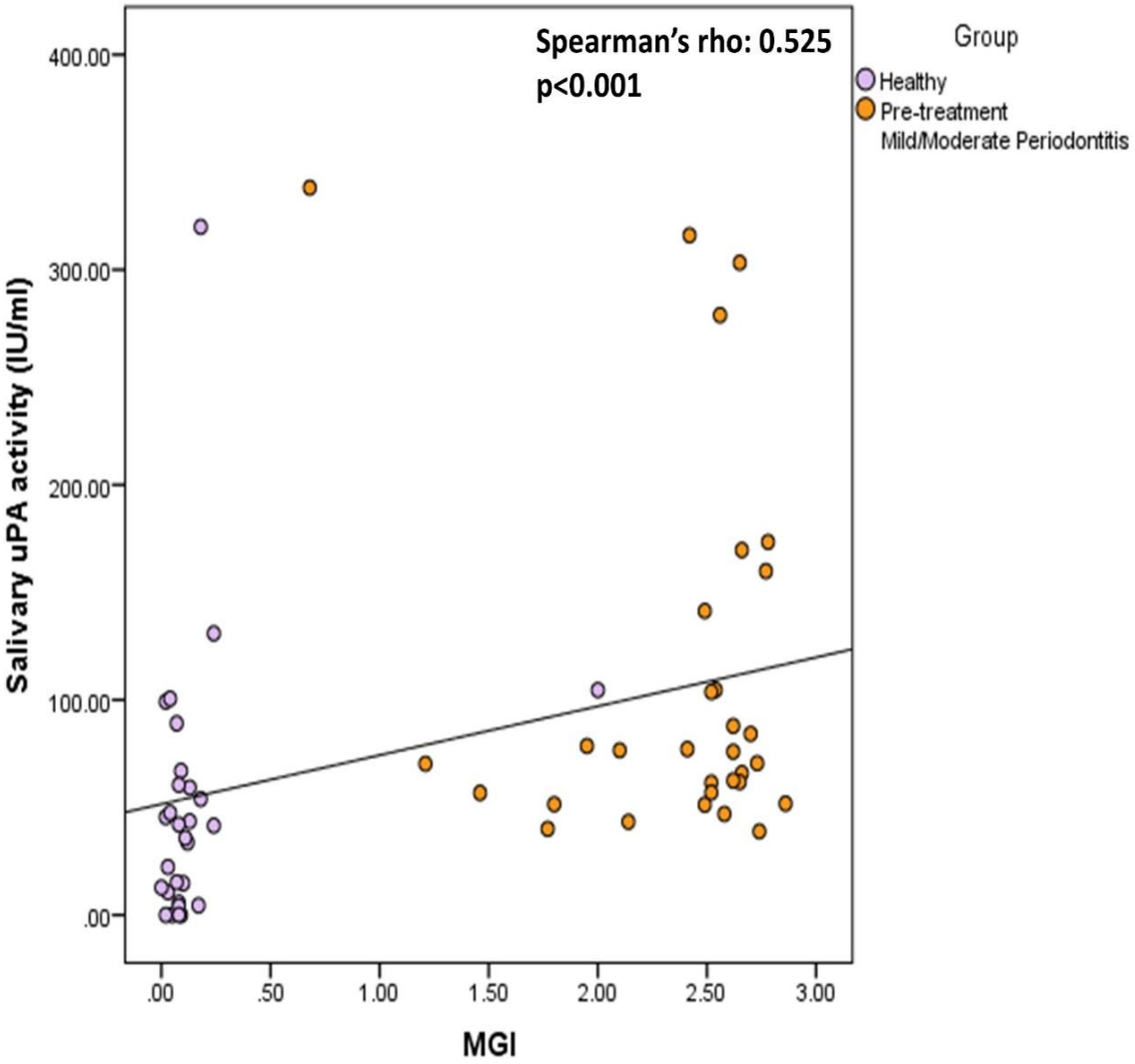

Figure 4.38: Relationship of salivary uPA activity with MGI in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the MGI index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

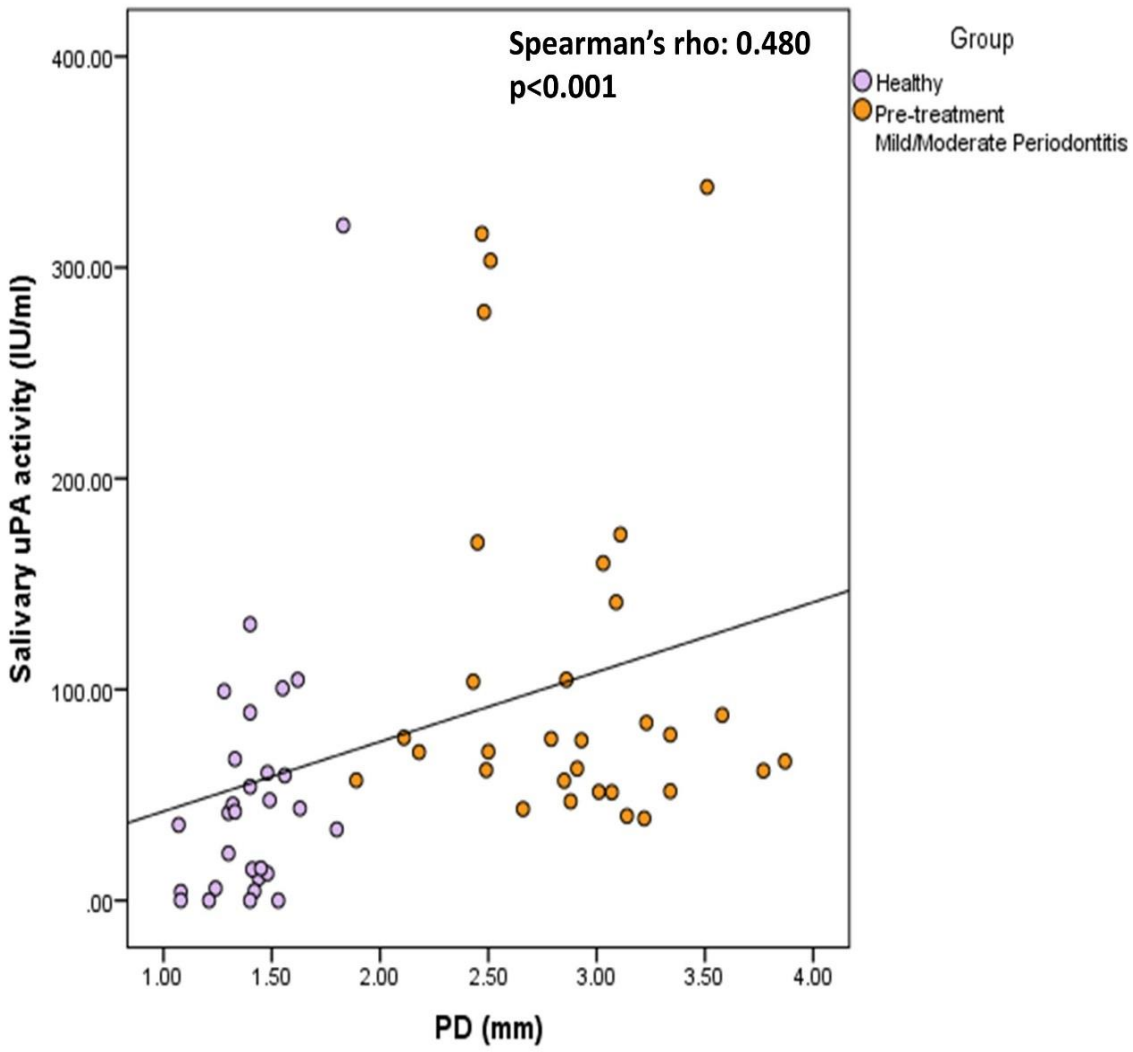

Figure 4.39: Relationship of salivary uPA activity with PD in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the PD index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

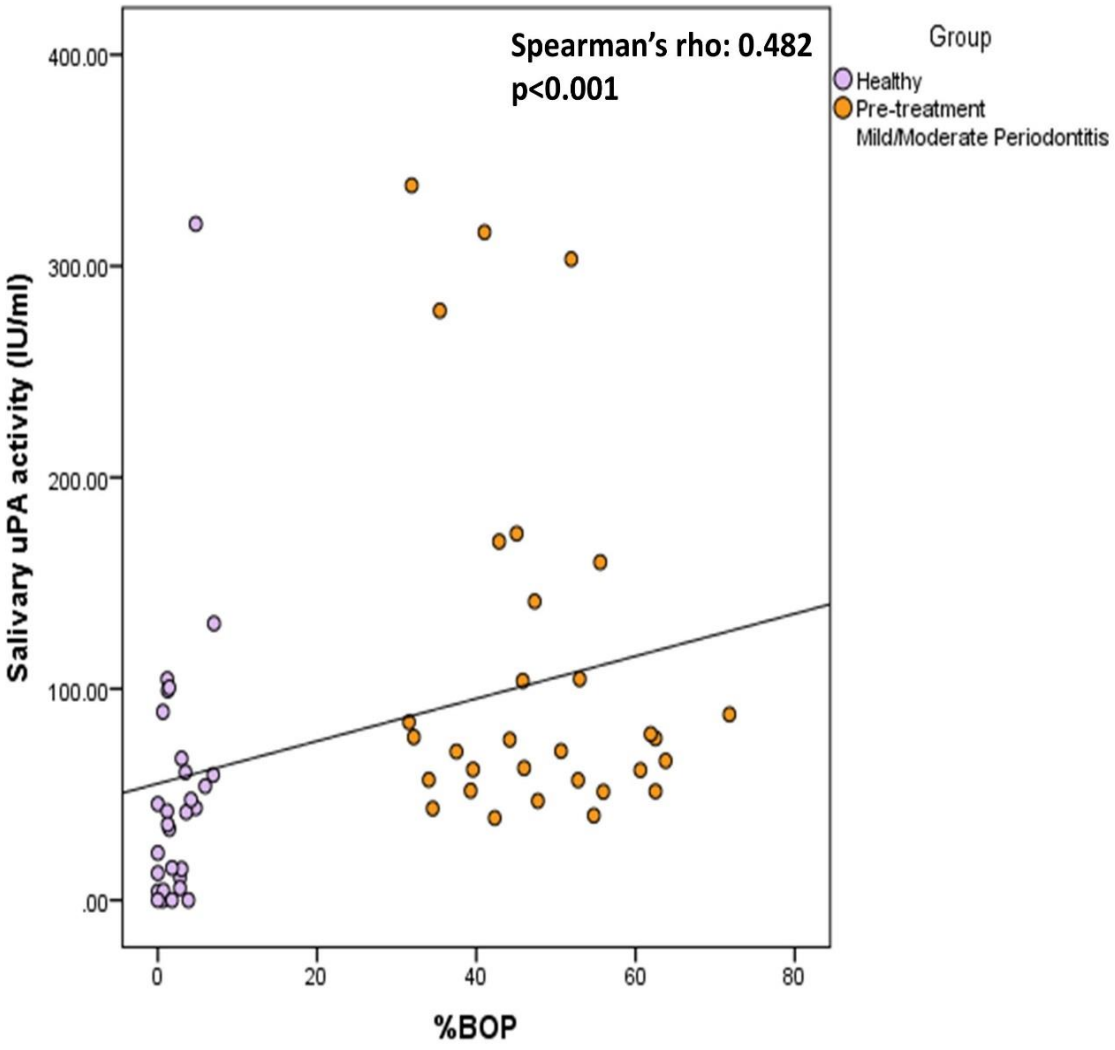

Figure 4.40: Relationship of salivary uPA activity with %BOP in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the %BOP index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

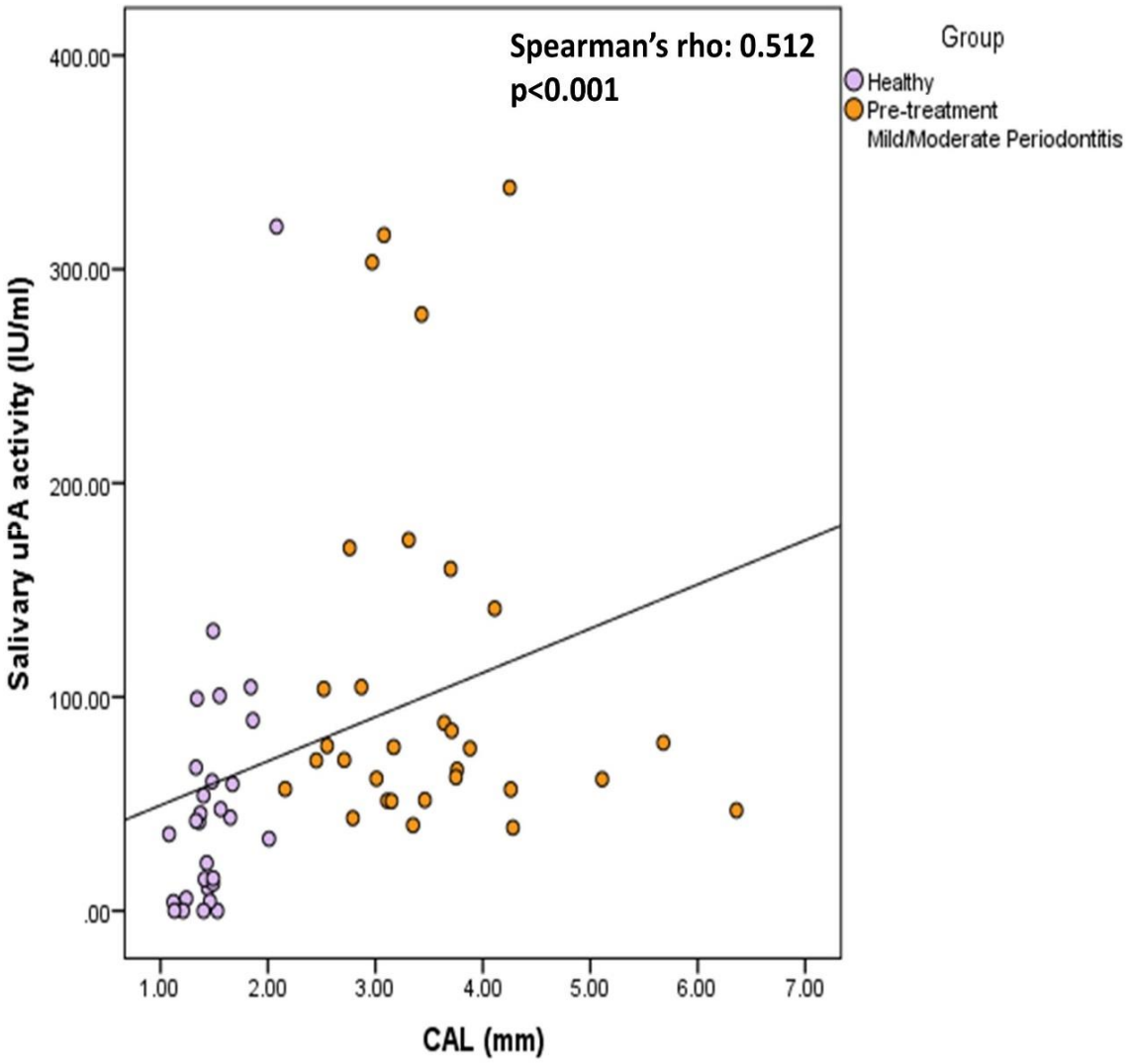

Figure 4.41: Relationship of salivary uPA activity with CAL in pre-treatment mild/moderate periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 31 pre-treatment mild/moderate periodontitis patients and 29 healthy subjects with the CAL index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

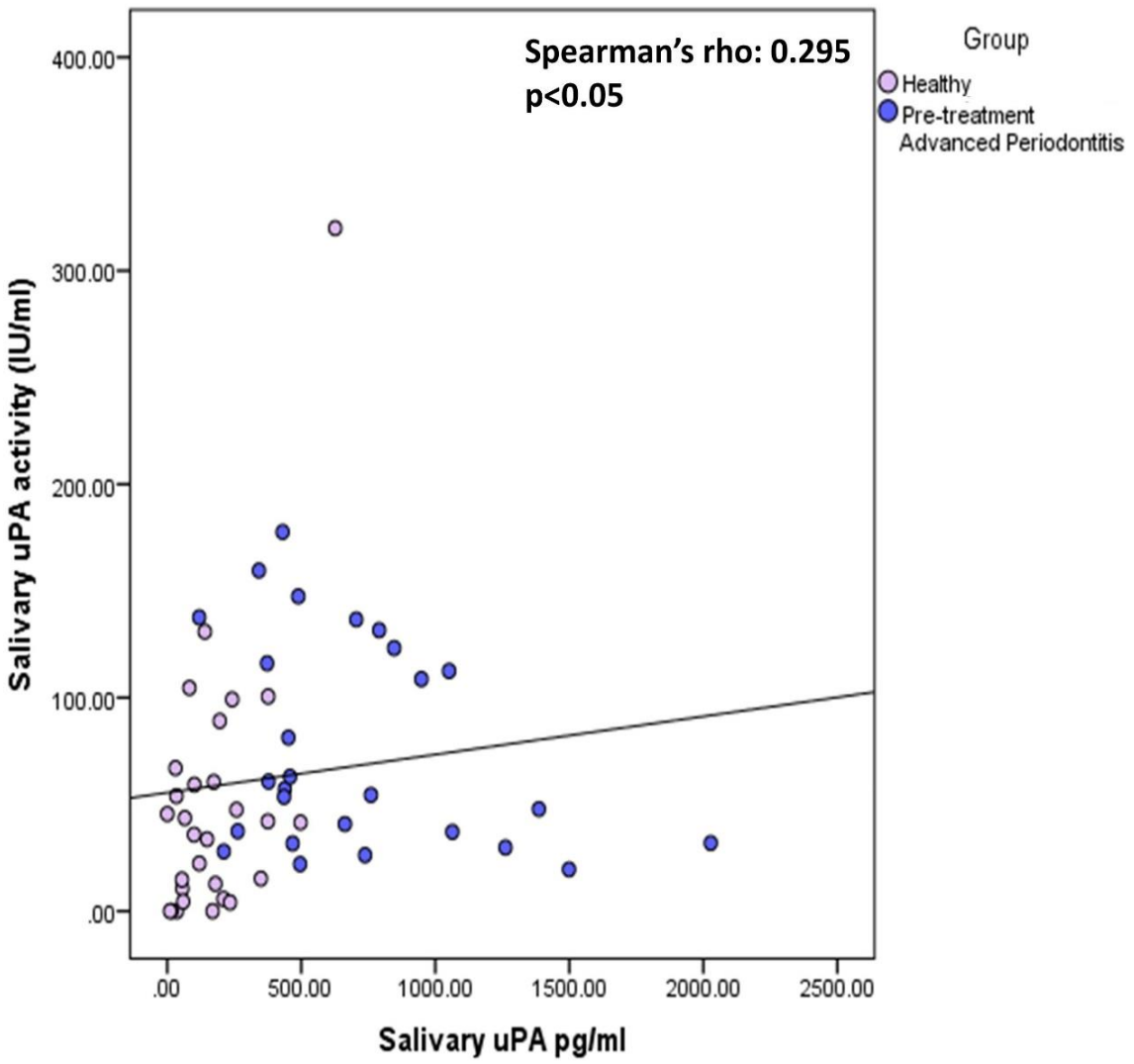

Figure 4.42: Relationship of salivary uPA activity with salivary uPA levels in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy with the salivary uPA levels in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

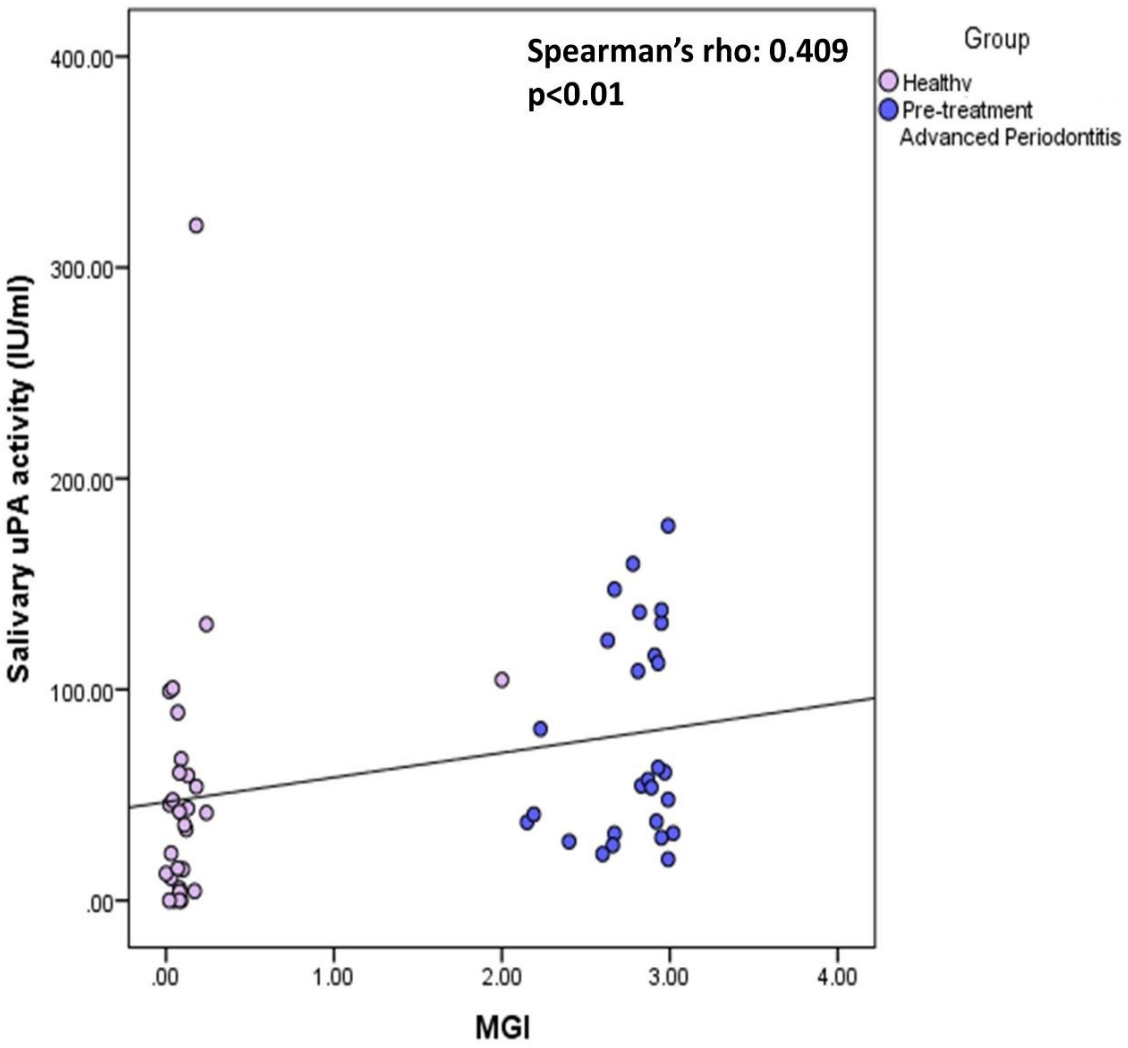

Figure 4.43: Relationship of salivary uPA activity with MGI in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the MGI index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

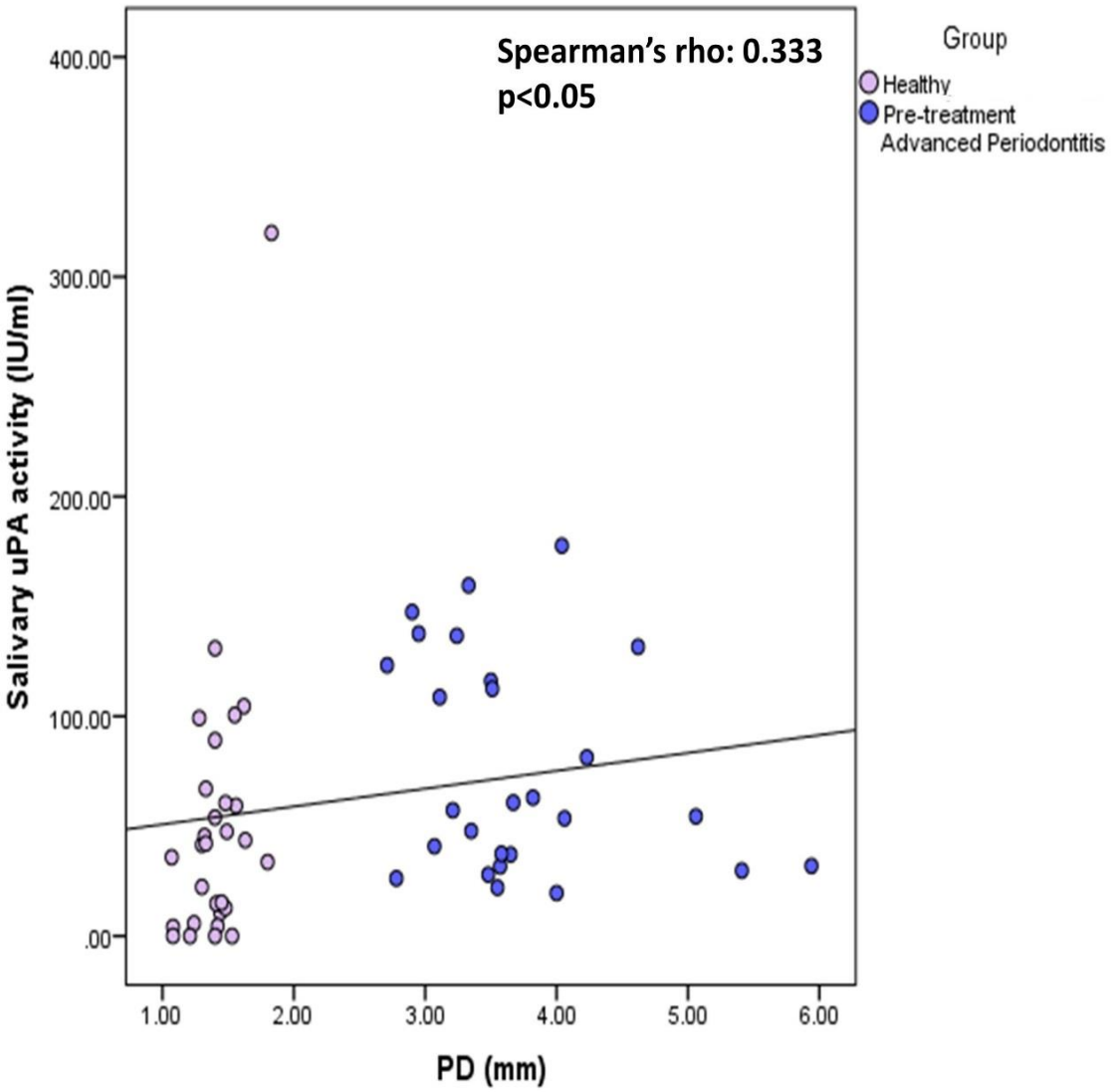

Figure 4.44: Relationship of salivary uPA activity with PD in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the PD index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

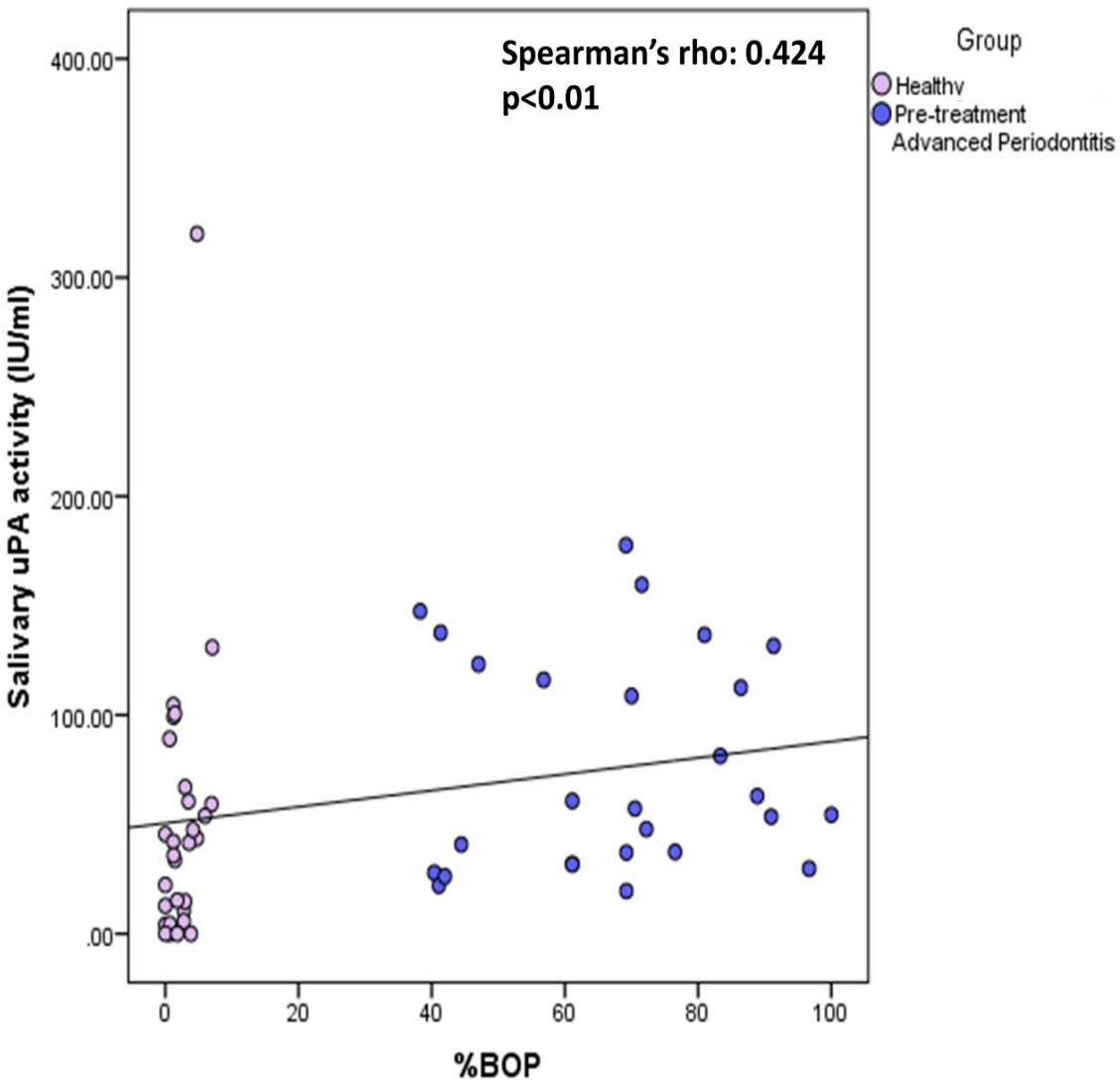

Figure 4.45: Relationship of salivary uPA activity with %BOP in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the %BOP index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

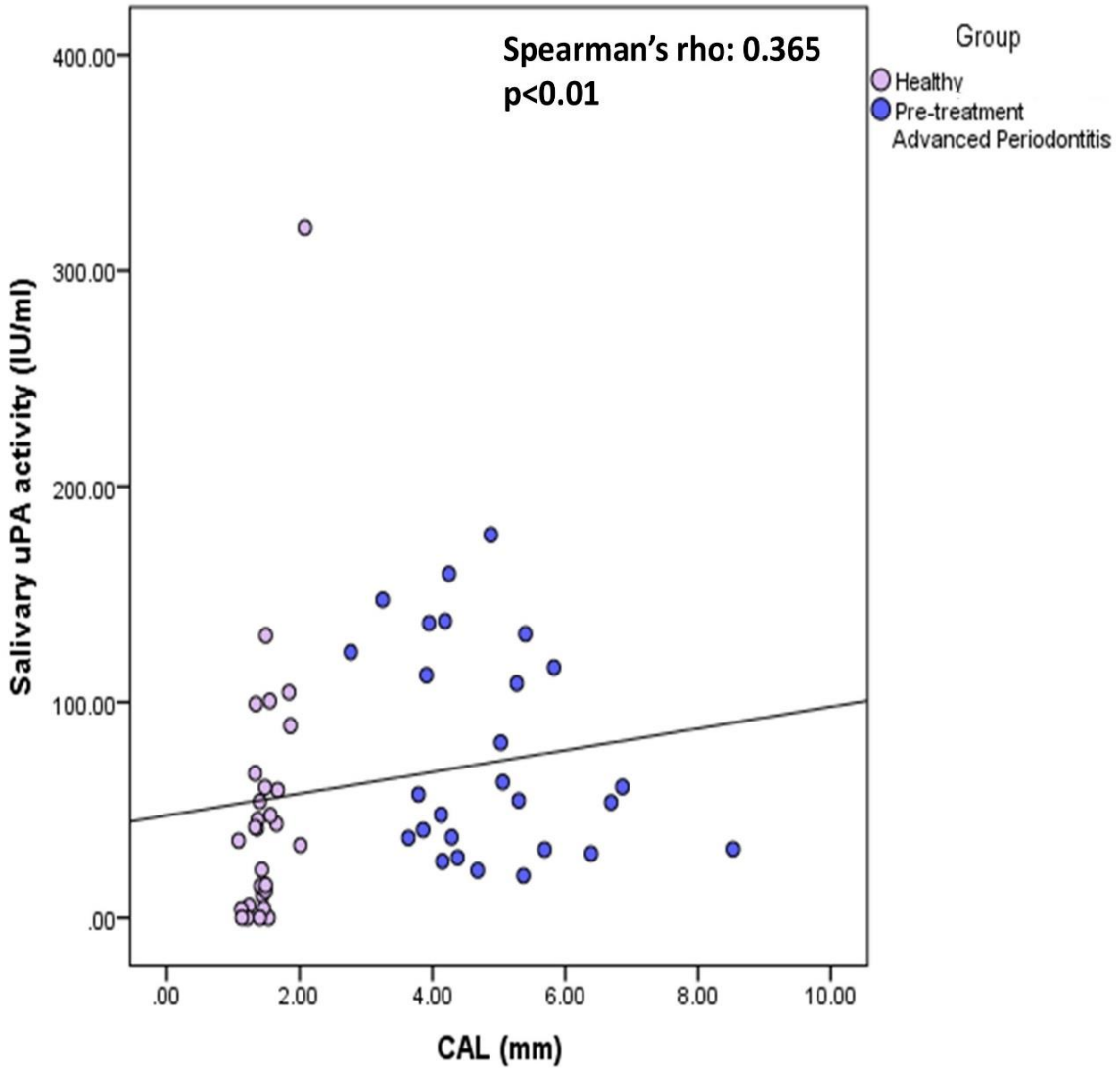

Figure 4.46: Relationship of salivary uPA activity with CAL in pre-treatment advanced periodontitis patients.

The Spearman's correlation of the salivary uPA activitiy in 27 pre-treatment advanced periodontitis patients and 29 healthy subjects with the CAL index in the clinical study B. The trend-line demonstrates the presence of a significant positive correlation.

Chapter 5 Investigation of the association between salivary VDBP and periodontitis

5.1 Introduction

Among the proteins which was found to be elevated in saliva of the chronic periodontitis patients in the experiments using PPA assays, was the vitamin D binding protein (VDBP) (See Chapter 3, section 3.2.2). The main function of VDBP is in the transportation of vitamin D (VD) and its metabolites in serum. Vitamin D plays a major role in maintaining normal serum levels of calcium and in electrolyte balance. VDBP is synthesized by the hepatocytes, a process regulated by oestrogen and pro-inflammatory cytokines such as IL-6. In comparison to other carrier proteins, VDBP has stable levels throughout life (300-600 µg/ml in plasma of healthy subjects) and always higher than its own ligand VD (Guha et al. 1995; White and Cooke 2000; Speeckaert et al. 2006; Malik et al. 2013; Zhang et al. 2013b; Bhan 2014). It has been reported that VD may have immune-modulating effects and VD has been proposed to be associated with periodontal tissue health (Van der Velden et al. 2011). In addition to VD transport, VDBP is involved in a number of functions such actin scavenging, fatty acids transport, macrophage activation and chemotaxis (Yamamoto and Kumashiro 1993; Kew et al. 1995; Yamamoto and Naraparaju 1996; White and Cooke 2000; Speeckaert et al. 2006; Malik et al. 2013; Bhan 2014; Zhang et al. 2014; Fu et al. 2016; Herrmann et al. 2016). The few studies that have been carried out to investigate the role of VDBP in the pathogenesis of periodontal diseases have investigated VDBP in plasma or in GCF of periodontitis patients (Wu et al. 2009; Zhang et al. 2013b; Zhang et al. 2014). One study carried out by Krayer et al. (1987) identified VDBP in stimulated saliva of periodontitis patients; however, this study did not investigate salivary VDBP levels in periodontitis patients following treatment. Therefore, the present study was the first to carry out substantial investigations on this candidate salivary biomarker of periodontitis. The research presented in this chapter aimed to investigate the VDBP levels in the whole unstimulated saliva of patients with chronic periodontitis in an attempt to study the relationship between salivary VDBP and periodontitis, and therefore assess the validity of salivary VDBP as a candidate biomarker.

5.2 Results

Following the identification of VDBP as a candidate salivary biomarker for periodontitis, ELISA assays were carried out to confirm the results from the cytokine PPA assays and to thereby quantify the VDBP levels in saliva of periodontitis patients. Experiments were carried out using human VDBP Quantikine ELISA kit. ELISA validation was carried out on the VDBP ELISA assay prior to use on the study samples. The ELISA results were compared between the patients and control groups, and in the patients before and after treatment. The results were correlated with the periodontal disease indices used for the clinical assessment of periodontitis.

The results of the ELISA assays (concentrations ng/ml) are presented as median and interquartile ranges (IQR) [median (upper quartile-lower quartile)] because data were not normally distributed.

5.2.1 The VDBP Quantikine ELISA validation

Though the human VDBP Quantikine ELISA was valid for assaying VDBP in saliva samples according to the manufacturer (R & D systems); as mentioned before in the ELISA validation assays (see Chapter 2, section 2.3.2) and (Jaedicke et al. 2012), it is necessary to perform at least one of the validation assays before using a new ELISA assay on study samples. Therefore the determination of optimal dilution was performed on the VDBP ELISA. The rest of the validation assays values were obtained from R & D systems.

Determination of optimal dilution

Though the manufacturer recommendations were to dilute saliva samples in a 1:2 ratio using the calibrator diluent supplied; two more detailed dilution assays were carried out on 3 saliva samples obtained from 3 healthy volunteers for each assay. Thus, the samples were assayed in 5 dilutions for each sample (neat, 1:2, 1:3, 1:4 & 1:8) in duplicates. The assays revealed that 1:4 and 1:8 were the suitable dilution factors at which VDBP was detected within the range of the assay standard curve (15.6-250 ng/ml), because at the 1:2 dilution factor recommended by the manufacturer, the VDBP levels in most of the samples were higher than the range of the assay standard curve. The 1:4 dilution factor was used for most of the healthy and periodontitis saliva samples, 1:8 dilution factor was used with some of the

periodontitis samples in which the VDBP levels were higher than the standard curve range at the 1:4 dilution.

Recovery and linearity measurements

The values of the recovery and linearity measurements were obtained from the manufacturer. The recovery values for VDBP in saliva were quoted as 90-103%. Linearity values were quoted as: 90-103% for the 1:2 dilution in 47 saliva samples, and 96-103% for the 1:4 dilution in 47 saliva samples. All recovery and linearity values were within the accepted range (80-120%) (R & D systems) and (Jaedicke et al. 2012).

Intra- and inter-assay variations

The intra-assay variation (precision) values were obtained from R & D systems. The CV% values were (5.7, 5.8, and 6.2) all less than 10-15% (R & D systems) and (Hanneman et al. 2011). The inter-assay variation values were also obtained from the manufacturer. The CV% values were (5.1, 6, and 7.4) all were within the acceptable range.

Assay sensitivity

The assay sensitivity value for the VDBP Quantikine ELISA, was obtained from R & D systems. The minimum detectable concentration was 0.65 ng/ml. All periodontitis and healthy samples measured by VDBP ELISA in the present research were above this minimum concentration (minimum concentration detected was 45.96 ng/ml in one of the healthy samples).

Summary of ELISA validation

The optimal dilution assay results together with the values obtained from R & D systems, confirmed that the VDBP Quantikine ELISA assay can measure VDBP in saliva samples within the standard curve range of the assay.

5.2.2 Investigation of salivary VDBP levels in periodontitis

The VDBP Quantikine ELISA kits were used to measure the VDBP levels in whole unstimulated saliva samples obtained from the clinical study A. Investigations were carried out to study the relationships of VDBP levels in saliva samples with the periodontal disease indices used for the clinical assessment of periodontitis.

Salivary VDBP levels in pre- and post-treatment samples of periodontitis patients

The VDBP levels were measured in saliva samples of 40 patients with untreated chronic periodontitis (pre-treatment samples) and 40 healthy control subjects (healthy samples) (cross-sectional investigation). All samples were obtained at visit 2 of the clinical study A (see Chapter 2, section 2.1.3, study A). Salivary VDBP levels were higher in the pre-treatment periodontitis patients [682 (331-930) ng/ml] than in the healthy subjects [215 (146-392) ng/ml] and the difference was highly significant (Mann-Whitney U test, $p<0.001$), (Figure 5.1).

The VDBP levels were measured again in saliva samples of the same 40 periodontitis patients following non-surgical periodontal treatment (longitudinal investigation). The post treatment periodontitis samples were obtained at the visit 6 which was 6 months from the visit 2 of clinical study A (i.e. 6 months after obtaining the pre-treatment samples) (see Chapter 2, section 2.1.3, study A). There was no statistically significant difference in the salivary VDBP levels between the pre-treatment visit [682 (331-930) ng/ml] and the post-treatment visit [635 (419-959) ng/ml], (Wilcoxon signed rank test, $p>0.05$).

Salivary VDBP correlations with periodontal disease indices

Determination of the Spearman's rank correlation coefficient (rho) was used to study the relationships of the salivary VDBP levels in 40 pre-treatment periodontitis patients and 40 healthy controls with the periodontal disease indices used for the clinical assessment of the periodontitis in the clinical study A. These correlations gave information about the potential of salivary VDBP as a biomarker that might indicate the severity of chronic periodontitis. The statistical analysis of the salivary VDBP correlations revealed that there was a significantly positive correlation between the salivary VDBP levels and each of the indices: MGI ($rho=0.494$, $p<0.001$), PD ($rho=0.616$, $p<0.001$), %BOP ($rho=0.560$, $p<0.001$), and CAL ($rho=0.563$, $p<0.001$), (Figure 5.2, Figure 5.3, Figure 5.4, & Figure 5.5).

5.3 Discussion

5.3.1 Salivary VDBP levels and their relationship with periodontitis

VDBP was first identified in saliva of periodontitis patients by Krayer et al. (1987), but their study was carried out on stimulated saliva and did not investigate salivary VDBP

levels in response to treatment. Hence, the present study was the first to investigate the VDBP levels in whole unstimulated saliva of chronic periodontitis patients before and after non-surgical treatment, and to study the salivary VDBP relationship with chronic periodontitis. The results of the ELISA assays carried out on the saliva samples obtained from the patients and healthy controls revealed that, the VDBP levels were higher in saliva of the pre-treatment chronic periodontitis patients than in the healthy subjects and the difference was highly significant. These high levels of salivary VDBP in the periodontitis patients were positively correlated with the periodontal indices used for the clinical assessment of periodontitis.

The high levels of VDBP measured in saliva of the periodontitis patients may be explained by the roles of this multi-functional protein during inflammation. Aside from its main role as a carrier and transporter of vitamin D and its' metabolites, VDBP play vital roles in the inflammatory cells chemotaxis especially neutrophils and macrophages, the activation of macrophages, and the synthesis of macrophage activating factor (VDBP-MAF) complex which induces bone morphogenesis and remodelling via the activation of macrophages and osteoclasts differentiation (Kew and Webster 1988; Perez et al. 1988; Yamamoto and Kumashiro 1993; Perez 1994; Piquette et al. 1994; Yamamoto et al. 1994; Kew et al. 1995; Schneider et al. 1995; Yamamoto and Naraparaju 1996; DiMartino and Kew 1999; White and Cooke 2000; Gumireddy et al. 2003; Schneider et al. 2003; Speeckaert et al. 2006; Malik et al. 2013; Zhang et al. 2013b; Zhang et al. 2014). Such inflammatory events mediated by VDBP may take place during the pathogenesis of periodontitis, which may explain the significantly elevated levels of VDBP in saliva of the patients.

The Krayer et al. (1987) study was carried out on 13 patients with periodontitis in comparison to control subjects comprised 10 dentulous periodontally healthy subjects and 9 edentulous subjects. The patients and subjects were clinically assessed using pocket depth, bleeding on probing, radiography of bone loss, and the (Loe 1967) gingival index (GI). Whole stimulated saliva and stimulated parotid saliva samples were collected from the patients and the control subjects. In their investigations for salivary VDBP, Krayer et al. (1987) used a double radial immunodiffusion assay and found that the VDBP was detected in the whole stimulated saliva of 10 patients, and in the parotid stimulated saliva of one patient only. Though the precipitin lines were weaker than those of the periodontitis patients;

VDBP was also detected by the double radial immunodiffusion assay in the whole stimulated saliva of the dentulous control subjects but not detected in their parotid stimulated saliva. VDBP was detected in the whole stimulated saliva of only one edentulous subject, and in the parotid stimulated saliva of 2 edentulous subjects only. Despite the fact that they used a different technique, the findings of Krayer et al. (1987) were in agreement with the findings of the present study in regard to the identification of VDBP in whole unstimulated saliva of untreated chronic periodontitis patients using the PPA assays. Krayer et al. (1987), also carried out ELISA assays on their study samples and reported that, in the stimulated parotid saliva the VDBP levels were relatively low and constant (8-80 ng/ml) in all groups and there was no distinct relationship between the parotid VDBP levels and any of the periodontal diseases measures. In the whole stimulated saliva, Krayer et al. (1987) found that there was no statistically significant difference in the VDBP levels between the dentulous and the edentulous control subjects, whereas the VDBP levels were higher in the periodontitis patients as compared to both the dentulous and edentulous control subjects and the difference was statistically significant ($p<0.01$). In a similar manner, the present study detected higher levels of VDBP in the whole unstimulated saliva of the pre-treatment periodontitis patients [635 (419-959) ng/ml] as compared to the healthy subjects [215 (146-392) ng/ml] and the difference was highly significant ($p<0.001$). Therefore, the present study results were in agreement with (Krayer et al. 1987). Krayer et al. (1987), reported positive correlations between the VDBP levels in the whole stimulated saliva of the periodontitis patients and the periodontal disease measures from which the most prominent relationship was with the GI index, a finding that was in agreement with the present study which demonstrated significantly positive correlations between the salivary VDBP levels and the periodontal disease indices.

In their study carried out on patients with generalized aggressive periodontitis (GAgP), Wu et al. (2009) identified VDBP in whole unstimulated saliva of the patients. Two-dimensional gel electrophoresis (2DE) was used to investigate the proteome profile of whole unstimulated saliva obtained from 5 GAgP patients and 5 healthy control subjects. The Wu et al. (2009) 2DE proteomic assays revealed that, VDBP was among the 11 highly expressed proteins detected in saliva of the GAgP

patients in comparison to the control subjects, the findings of the present study are in agreement with this.

Zhang et al. (2014), investigated the VDBP levels in plasma of 59 GAgP patients in comparison to 58 healthy control subjects, and in the GCF of 22 GAgP patients in comparison to 23 healthy controls. Zhang et al. (2014), found that the patients had lower levels of VDBP in the GCF but higher levels in plasma as compared to the healthy subjects. The study also reported that the GCF VDBP levels were negatively correlated with both the periodontal pocket depth and the attachment loss periodontal measures. Zhang et al. (2014), concluded that the GAgP is associated with lower levels of GCF VDBP but higher levels of plasma VDBP. However, Zhang et al. (2014) suggested that the presence of high VDBP levels in the GCF of healthy subjects indicated that this protein might have a role in the periodontal health, and that in addition to serum, the periodontal tissues might represent another source for VDBP in the human GCF. In addition to their own findings, Zhang et al. (2014) proposed that the GCF VDBP might have contributed to the high levels of salivary VDBP detected by the Krayer et al. (1987) study, as both the volume and flow rate of GCF are increased in periodontal diseases. This may explain the high salivary VDBP levels in the periodontitis patients found by the present study. Nevertheless, its GCF levels were lower in the GAgP periodontitis patients than in the controls; Zhang et al. (2014) proposed that the presence of VDBP in the GCF of the patients might be an indication that this protein participates in the inflammatory process of periodontal diseases, which may support the present study for the identification and measure of VDBP in high levels in saliva of the periodontitis patients. Another study carried out by Zhang et al. (2013b), investigated VDBP along with IL-6, procalcitonin, and calcidiol (a vitamin D metabolite) in the plasma of 44 patients with GAgP and 32 healthy control subjects. The Zhang et al. (2013b) ELISA results revealed that, the VDBP levels were significantly elevated in plasma of the GAgP patients in comparison to the healthy subjects, and the plasma VDBP levels were associated with the disease severity as they were significantly positively correlated with the periodontal disease measures in the GAgP patients (PD, LOA, and bleeding index). Despite the fact that the study was carried out on plasma samples but not saliva or GCF, the Zhang et al. (2013b) study was a further evidence for the association of VDBP with periodontal diseases.

Zhang et al. (2014), recommended a longitudinal study to investigate the influence of periodontal treatment on the local and systemic VDBP levels and to confirm the proposed relationship between VDBP and periodontal diseases. The present study was the first to carry out such a longitudinal investigation, in which salivary VDBP levels were measured in 40 periodontitis patients before and after non-surgical treatment. The ELISA results revealed that, there was no statistically significant difference in the salivary VDBP levels of the patients between the pre-and post-treatment visits. As previously explained for the salivary uPAR levels in response to treatment (see Chapter 4, section 4.3.1), the persistent levels of salivary VDBP following treatment could be explained by the fact that though the periodontal health status was improved in the periodontitis patients which was obvious by the improvement in the periodontal disease indices (such as %BOP reduced from 52 to 18, and CAL reduced from 4.5 mm to 3.6 mm); when the periodontal supporting tissues are destroyed and pockets formed during periodontitis, they will never return back to the ideal healthy status (Pihlstrom et al. 2005), this means that sites of mild inflammation may still exist in the periodontal supporting tissues from which VDBP may be expressed into the GCF to reach the whole saliva (Zhang et al. 2014). Moreover, VDBP possess stable high levels throughout life in the human serum which may be increased both locally and systemically during an inflammation (White and Cooke 2000; Speeckaert et al. 2006), this may give another explanation for the persistent considerable levels of VDBP following treatment, as serum together with the local periodontal tissue exudate are sources for the GCF contents which in turn flow into saliva (Zhang et al. 2014). Furthermore, as previously mentioned in respect to the roles of VDBP during inflammatory events (see section 5.1 Introduction), VDBP is involved in the synthesis of the VDBP-MAF factor. Aside from its' roles in the macrophages activation, and in bone morphogenesis and remodelling, the VDBP-MAF factor induces macrophage apoptosis via the stimulation of pro-apoptotic enzymes such as caspase-3, -8, and -9, which occurs when activated macrophages are no longer needed at the site of inflammation (Gumireddy et al. 2003), this may also explain the persistent high levels of VDBP in saliva of the periodontitis patients following treatment and improvement in their periodontal health status.

5.3.2 Summary of findings

The pre-treatment ELISA results revealed that, the salivary VDBP levels were significantly higher in the chronic periodontitis patients as compared to the healthy control subjects, and there were significantly positive correlations between these high salivary VDBP levels and each of the periodontal disease indices used for the clinical assessment of periodontitis. Accordingly, salivary VDBP is suggested as a good biomarker for the diagnosis of periodontitis, indication of the disease severity, and distinguishing between periodontitis patients and healthy subjects.

The post-treatment ELISA results revealed that, the salivary VDBP levels were not reduced in the patients following non-surgical periodontal treatment. Therefore, salivary VDBP may not be a good biomarker for following the clinical course of periodontitis in response to treatment.

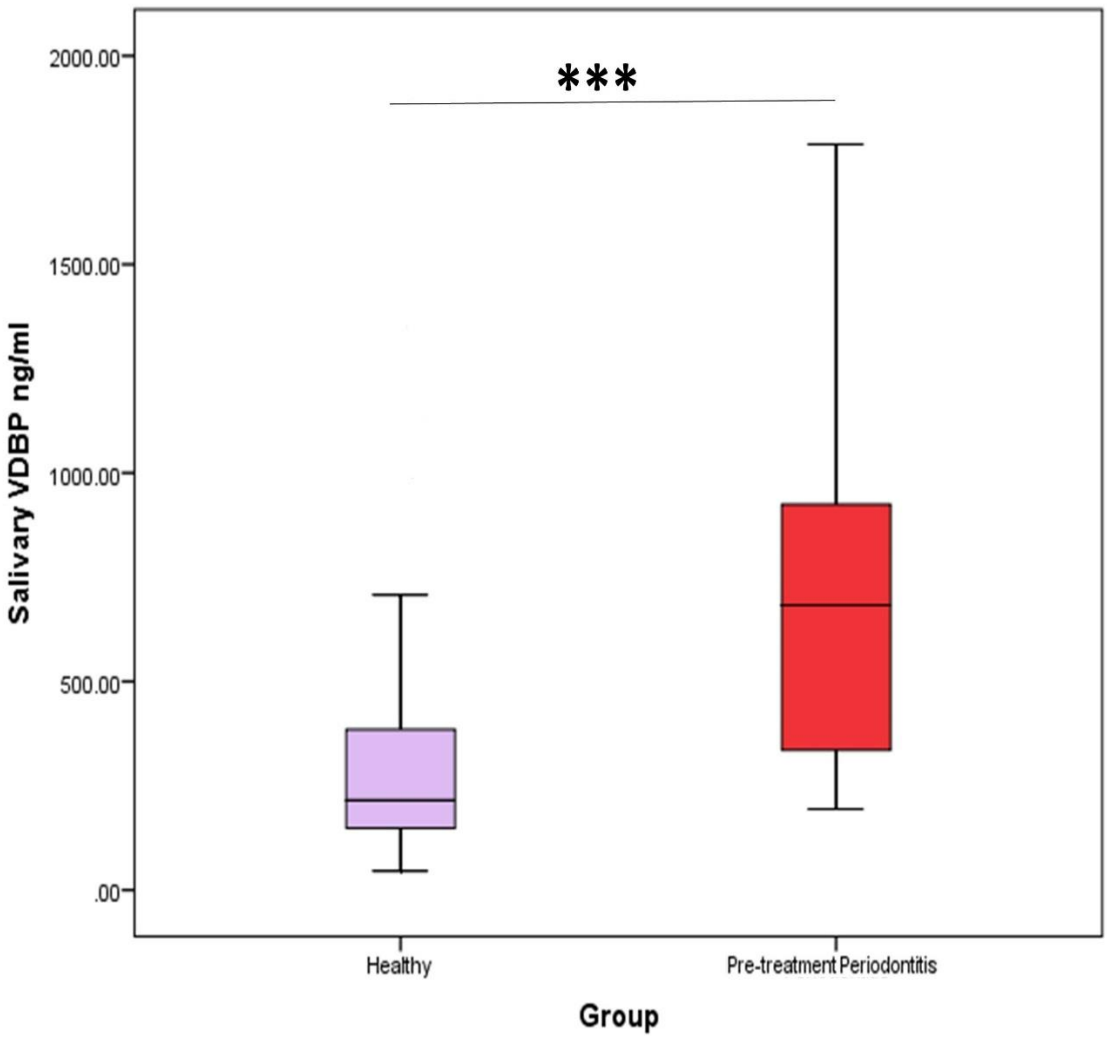

Figure 5.1: The pre-treatment salivary VDBP levels in periodontitis patients as compared to salivary VDBP in healthy volunteers.

Salivary VDBP levels were measured by ELISA in samples obtained from 40 pre-treatment periodontitis patients in comparison to 40 healthy subjects. The box plots represent the median and IQR for each group. ***=p< 0.001 (Mann-Whitney U test).

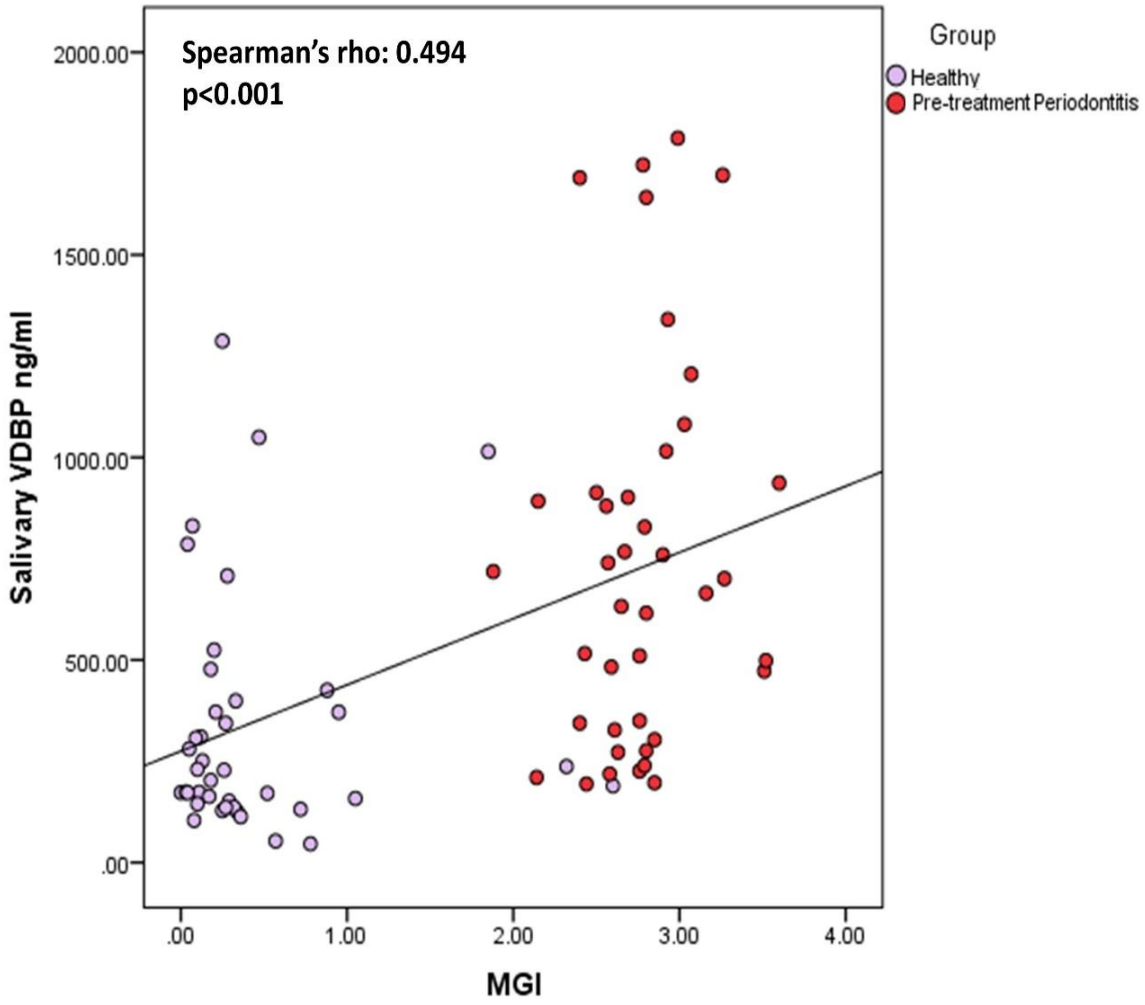

Figure 5.2: Relationship of salivary VDBP levels with MGI in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary VDBP levels in 40 pre-treatment periodontitis patients and 40 healthy subjects with the MGI index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

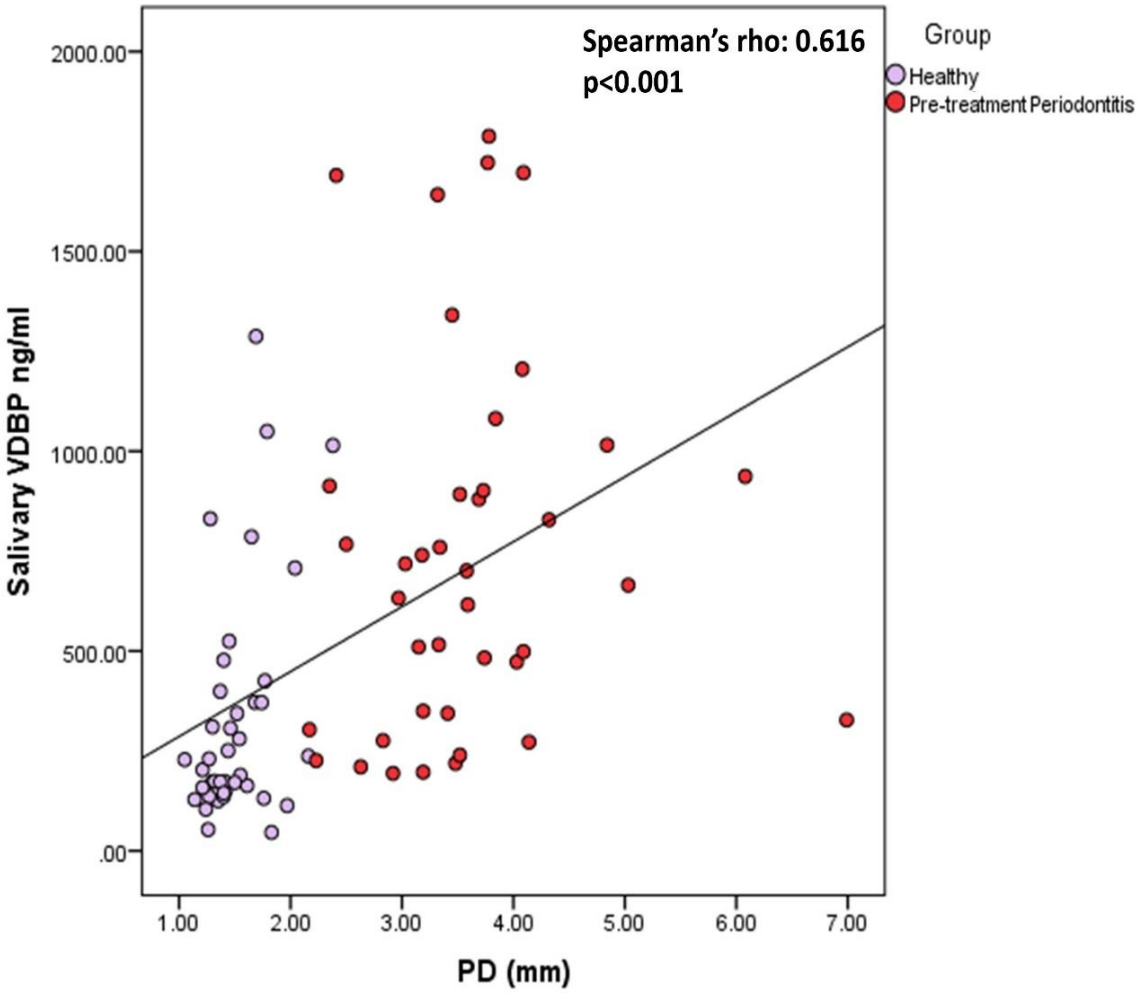

Figure 5.3: Relationship of salivary VDBP levels with PD in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary VDBP levels in 40 pre-treatment periodontitis patients and 40 healthy subjects with the PD index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

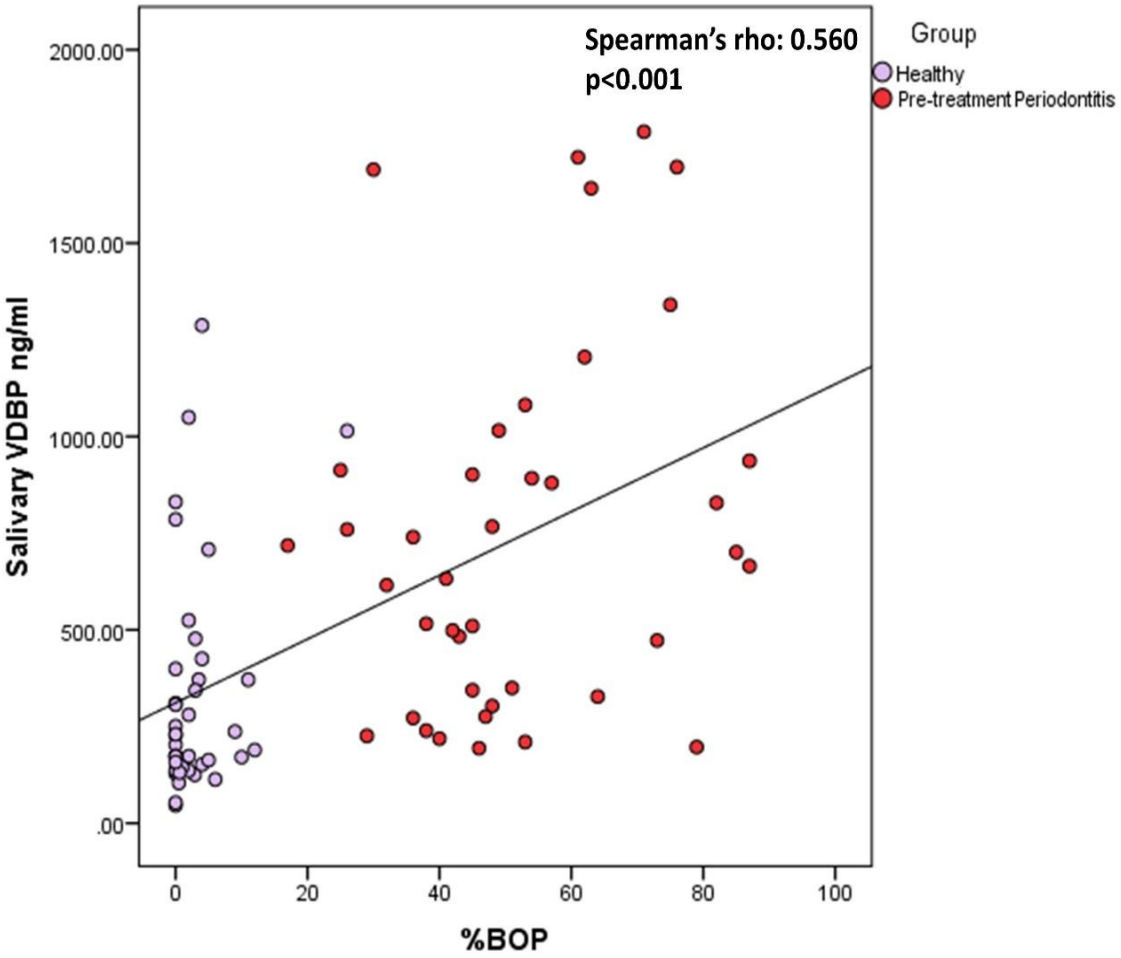

Figure 5.4: Relationship of salivary VDBP levels with %BOP in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary VDBP levels in 40 pre-treatment periodontitis patients and 40 healthy subjects with the %BOP index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

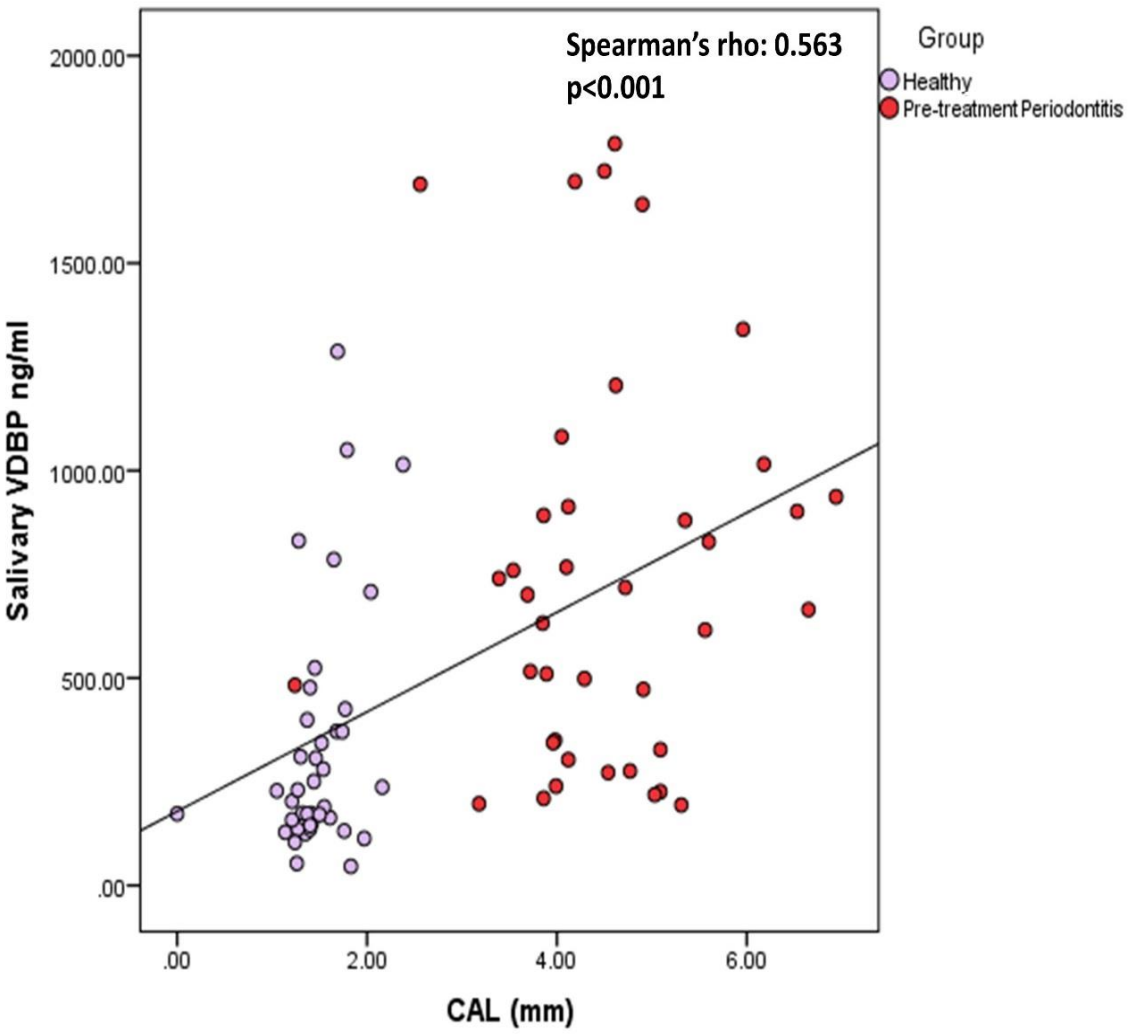

Figure 5.5: Relationship of salivary VDBP levels with CAL in pre-treatment periodontitis patients.

The Spearman's correlation of the salivary VDBP levels in 40 pre-treatment periodontitis patients and 40 healthy subjects with the CAL index in the clinical study A. The trend-line demonstrates the presence of a significant positive correlation.

Chapter 6 Investigation of the in vitro production of uPA and uPAR by stimulated hGFs

6.1 Introduction

As described in Chapters 3 and 4, uPA and its receptor uPAR were identified by the PPA assays as candidate salivary biomarkers for periodontitis, then they were investigated for their levels in both saliva and GCF samples of patients with gingivitis and periodontitis in comparison to healthy control subjects, and their relationships with the periodontal disease indices were determined. Furthermore, the two biomarkers were investigated in both saliva and GCF samples of the periodontitis patients before and after non-surgical periodontal treatment. Finally, the uPA enzymatic activity was assayed in saliva of the periodontitis patients, and its relationships with both the salivary uPA levels and the periodontal disease indices were investigated. uPA and uPAR are among the most important proteins of the plasminogen activating (PA) system, and they act together to initiate the PA system activities especially proteolysis. During inflammation and in the presence of a stimulus such bacterial toxin or host inflammatory mediator, uPA binds to its receptor uPAR which will in turn stimulate and localize the uPA activity on the cell surface, where the active uPA activates the pro-enzyme plasminogen into the active plasmin (Crippa 2007; Blasi and Sidenius 2010; Smith and Marshall 2010). A number of cell culture studies investigated the production or activity of different PA system proteins in gingival epithelium and in gingival fibroblasts. Thus, Tanaka et al. (1997), reported the production of uPA by hGFs in response to exogenous oxygen radicals. It has been found that uPA along with tPA, PAI-1, and PAI-2 proteins were produced by the fibroblasts of healing periodontal wounds generated in rat models (Xiao et al. 2001b). Smith and Martinez (2006), reported the production of uPA from hGFs in response to stimulation with the transforming growth factor-β1 (TGF-β1).

Though they were both investigated in a number of cell culture studies, it was necessary to carry out cell culture experiments to investigate the local production of uPA and uPAR proteins by hGFs in response to an in vitro stimulation to validate these mediators as biomarkers for periodontal diseases. Therefore, this chapter aimed to: investigate the production of both uPA and uPAR by the hGFs in response to the in vitro stimulation with different doses of interleukin -1β (IL-1β), and both the

E. coli and *P. gingivalis* LPS, and to investigate the uPA enzymatic activity in the supernatants of the stimulated hGFs.

6.2 Results

Experiments were carried out on primary hGF cells previously prepared by Dr Rachel Williams from gingival tissues obtained from patients undergoing canine exposure surgery (Williams et al. 2016) (see Chapter 2 section 2.2.2). All stimulation experiments were carried out independently on cells obtained from 8 donors in total (see Chapter 2 section 2.3.3). Before carrying out any stimulating experiment, the primary hGF cells were brought out their storage in liquid nitrogen, allowed to thaw, cultured in DMEM+ medium in T75 flasks till they reach 80-90% confluent growth. The cells were then seeded into plastic-ware including T25 flasks (at density of 2-4x10^5 cells/flask) and 6 multi-well plates (at density of 2x10^5 cells/well), and sub-cultured to allow the cells to grow till 80-90% confluency. At this point, the hGF cells were serum starved by replacing the DMEM+ medium with the SFM medium for 24 hours. Following serum-starvation, stimulating experiments were carried out on the hGF cells including: stimulation with the IL-1β, *E. coli* LPS, and *P. gingivalis LPS* for 24 hours in separate experiments (see Chapter 2 section 2.3.3). After 24-hour stimulation, the hGF cell culture supernatants were aliquoted into cryovials and stored at -80°C till use. The levels of uPAR, uPA, and MMP-1 were measured in the hGF cell culture supernatants using the human uPAR DuoSet, human uPA DuoSet, and human MMP-1 DuoSet ELISA assays (R & D systems), (see Chapter 2 section 2.3.2). The uPA enzymatic activity in the stimulated hGF cell culture supernatants was assayed using the uPA activity fluorometric assay kit (Sigma-Aldrich), (see Chapter 2 section 2.3.4).

The results of the ELISA assays (concentrations pg/ml) and the uPA enzymatic activity assays (IU/ml), presented either as median and IQR [median (upper quartile-lower quartile)] when data were not normally distributed, or as mean and SEM (mean ±SEM) when data were normally distributed.

6.2.1 The in vitro stimulation of the hGFs with IL-1β

Following their primary confluent growth in the T75 flasks, hGFs were stimulated with IL-1β in two sets of experiments. The first was a preliminary stimulation experiment carried out using T25 flasks in which the hGFs were seeded at density of (2-4x10^5

cells/flask), sub-cultured and allowed to grow till 80-90% confluency then stimulated. The second was a set of stimulation experiments carried out using the 6-multi well plates in which the cells were seeded at density of ($2x10^5$ cells/well), sub-cultured and allowed to grow till 80-90% confluency then stimulated.

The preliminary IL-1β stimulation experiment

This preliminary stimulation experiment was carried out using three T25 cell culture flasks, in which hGFs from 1 donor were stimulated for 24 hours with 5 ml/flask of 2 doses of IL-1β in SFM (5 ng/ml and 0.05 ng/ml) as compared to control (0 ng/ml of SFM). The aim of this experiment was to investigate if the IL-1β will stimulate the hGFs to produce MMP-1, which is a measure of pro-inflammatory response by such cells. MMP-1 levels were measured by ELISA, the results revealed that the 5 ng/ml stimulated the hGFs to produce an MMP-1 concentration of 55031± 872 pg/ml, and the 0.05 ng/ml stimulated the hGFs to produce an MMP-1 concentration of 38326± 2668 pg/ml, whereas the control (unstimulated) hGFs produced MMP-1 concentration of 6908±144 pg/ml, no statistical analysis was carried out as the n=1 for each dose (Figure 6.1).

The IL-1β stimulation experiments

Following the preliminary experiment, three independent IL-1β stimulation experiments were carried out, in which the hGFs were cultured in the 6 multi-well plates. Once their growth reached 80-90% confluency, the hGFs were stimulated with 2 ml/well of 2 doses of IL-1β in SFM (5 ng/ml and 0.05 ng/ml) in comparison to control (0 ng/ml of SFM) for 24 hours. The IL-1β stimulation experiments were carried out as 10 replicates for each of the stimulating doses and control using hGFs from 3 donors. The aims of these experiments were to investigate if the hGFs will produce the biomarkers uPA and uPAR in response to the IL-1β stimulation, and to investigate the uPA activity in the supernatants of the stimulated cells.

MMP-1 production by hGFs in response to IL-1β stimulation

The MMP-1 levels were measured first in the supernatants of the stimulated cells to investigate the pro-inflammatory response of the hGFs to the IL-1β stimulation. The ELISA results revealed that there was a statistically significant difference among the stimulating and control doses (One-Way ANOVA test with Bonferroni correction, $P<0.001$). Both the 5 ng/ml and 0.05 ng/ml of IL-1β stimulated the hGFs to produce

higher levels of MMP-1 (33365±3784 pg/ml, and 28024±3196 pg/ml respectively) than the control hGFs (5856±402 pg/ml) and the difference was highly significant (Independent samples t-test, $p<0.001$ respectively). There was no statistically significant difference in the MMP-1 levels produced by the 5 ng/ml and the 0.05 ng/ml stimulated hGFs (Independent samples t-test, $p>0.05$), (Figure 6.2).

uPA production by hGFs in response to IL-1β stimulation

Following the 24-hour stimulation with IL-1β, uPA DuoSet ELISA experiments were carried out on the supernatants of the stimulated cells. The results revealed that there was no statistically significant difference among the stimulating and control doses (Kruskal-Wallis test, $p>0.05$). Though the hGFs stimulated with 5 ng/ml and 0.05 ng/ml of IL-1β produced higher levels of uPA [138 (109-516) pg/ml and 70 (60-463) pg/ml respectively] in comparison to the hGFs incubated with the control dose [48 (42-297) pg/ml], the difference was not statistically significant (Mann-Whitney U test, $p>0.05$). There was no statistically significant difference in the supernatants uPA levels between the 5 ng/ml and the 0.05 ng/ml stimulated cells (Mann-Whitney U test, $p>0.05$).

uPA activity in supernatants of the IL-1β stimulated hGFs

The uPA activity fluorometric assay was used to assay the uPA activity in the supernatants of the hGFs stimulated with IL-1β. The results revealed that there was no statistically significant difference in the uPA activity among the stimulating and control doses (0.4647 (0.4628-0.4781) IU/ml, 0.4638 (0.4620-0.4766) IU/ml, and 0.4626 (0.4604- 0.4751) IU/ml respectively] (Kruskal-Wallis and Mann-Whitney U tests, $p>0.05$).

uPAR production by hGFs in response to IL-1β stimulation

The uPAR levels were measured in the supernatants of the stimulated and control hGFs by uPAR ELISA. The results revealed that, there was a statistically significant difference among the stimulating and control doses (One-Way ANOVA with Bonferroni correction, $p<0.01$). The hGFs stimulated with the 5 ng/ml of IL-1β produced higher levels of uPAR (138±11 pg/ml) than the control cells (85±9 pg/ml) and the difference was highly significant (Independent samples t-test, $p<0.01$). The hGFs stimulated with the 0.05 ng/ml of IL-1β also produced higher levels of uPAR (116±9 pg/ml) as compared to the control cells (85±9 pg/ml) and the difference was

statistically significant (Independent samples t-test, $p<0.05$). There was no statistically significant difference between the 5 ng/ml and 0.05 ng/ml stimulating doses (Independent samples t-test, $p>0.05$), (Figure 6.3).

Summary of the IL-1β stimulation experiments

The 24-hour incubation of the hGFs with 5 ng/ml and 0.05 ng/ml of IL-1β stimulated the cells to produce significantly higher levels of MMP-1 and uPAR as compared to the control cells. IL-1β stimulated the hGFs to produce uPA in numerically higher levels as compared to the control cells; however, the difference was not statistically significant. There was no statistically significant difference in the uPA activity measured in the supernatants of the IL-1β stimulated and control hGFs.

6.2.2 The in vitro stimulation of the hGFs with E. coli LPS

After their primary confluent growth in the T75 flasks, the hGFs were seeded into the 6-multi well plates at density of ($2x10^5$ cells/well), cultured till the cells reached 80-90% confluent growth. Then stimulated in two independent experiments for 24 hours with 2 ml/well of 100 ng/ml of *E. coli* LPS in SFM as compared to control (0 ng/ml of SFM). The *E. coli* LPS stimulation experiments were carried out as 6 replicates for each of the stimulating dose and control using hGFs from 2 donors. The aims of these stimulation experiments were to investigate the uPA and uPAR production by the hGFs in response to the *E. coli* LPS stimulation, along with investigating the uPA activity in the supernatants of the stimulated hGFs.

MMP-1 production by hGFs in response to E. coli LPS stimulation

The MMP-1 levels were measured first in the supernatants of the stimulated cells to investigate the hGFs pro-inflammatory response to the *E. coli* LPS stimulation. The ELISA results revealed that, the hGFs stimulated with 100 ng/ml of *E. coli* LPS produced higher levels of MMP-1(16706±4787 pg/ml) in comparison to the control cells (7581±1283 pg/ml); however, the difference was not statistically significant (Independent samples t-test, $p>0.05$).

uPA production by hGFs in response to E. coli LPS stimulation

Following the 24-hour stimulation with *E. coli* LPS, ELISA assays were carried out to measure the uPA in the supernatants of the stimulated hGFs. Results revealed that, though the hGFs stimulated with 100 ng/ml of *E. coli* LPS produced higher levels of

uPA [223 (128-320) pg/ml] than the cells incubated with the control dose [110 (88-162) pg/ml], the difference was not statistically significant (Mann-Whitney U test, p>0.05).

uPA activity in supernatants of the E. coli LPS stimulated hGFs

The uPA enzymatic activity was measured in the supernatants of the hGFs stimulated with *E. coli* LPS. Results revealed that, there was no statistically significant difference between the stimulated and control hGFs [0.4785 (0.4777-0.4796) IU/ml and 0.4779 (0.4748 - 0.4783) IU/ml respectively], (Mann-Whitney U test, p>0.05).

uPAR production by hGFs in response to E. coli LPS stimulation

The uPAR levels produced by the hGFs stimulated with *E. coli* LPS for 24 hours were measured by ELISA. Results showed that, though the hGFs stimulated with 100 ng/ml of *E. coli* LPS produced higher levels of uPAR (176±25 pg/ml) than the control cells (123±10 pg/ml), the difference was not statistically significant (Independent samples t-test, p>0.05).

Summary of the E. coli LPS stimulation experiments

The 24-hour incubation of the hGFs with 100 ng/ml of *E. coli* LPS stimulated the cells to produce MMP-1, uPA and uPAR in numerically higher levels than the control cells; however, the difference was not statistically significant. Furthermore, no difference was found in the supernatants uPA activity between the stimulated and control cells.

6.2.3 The in vitro stimulation of the hGFs with P. gingivalis LPS

The *P. gingivalis* LPS stimulation experiments were carried out by seeding the hGFs into the 6-multi well plates at density of ($2x10^5$ cells/well) in two independent experiments, and cultured till the cells reached 80-90% confluent growth At this point the cells were stimulated for 24 hours with 2 ml/well of 100 ng/ml of *P. gingivalis* LPS in SFM in comparison to control (0 ng/ml of SFM). The *P. gingivalis* LPS stimulation experiments were carried out as 6 replicates for each of the stimulating dose and control using hGFs from 2 donors. These stimulation experiments aimed to investigate the uPA and uPAR production by the hGFs, and to investigate the uPA activity in the supernatants of the cells in response to the *P. gingivalis* LPS stimulation.

MMP-1 production by hGFs in response to P. gingivalis LPS stimulation

Using ELISA, the MMP-1 levels were measured in the supernatants of the stimulated cells to investigate their pro-inflammatory response to stimulation with *P. gingivalis* LPS. Results revealed that, the hGFs stimulated with 100 ng/ml of *P. gingivalis* LPS produced higher levels of MMP-1 (8449±1030 pg/ml) in comparison to the control cells (5179±1002 pg/ml) and the difference was statistically significant (Independent samples t-test, $p<0.05$), (Figure 6.4).

uPA production by hGFs in response to P. gingivalis LPS stimulation

After the 24-hour stimulation with *P. gingivalis* LPS, the uPA levels in the supernatants of the stimulated hGFs were measured by uPA ELISA. Results demonstrated that the cells stimulated with 100 ng/ml of *P. gingivalis* LPS produced higher levels of uPA (112±8 pg/ml) than the hGFs incubated with the control dose (33±14 pg/ml), and the difference was highly significant (Independent samples t-test, $p<0.01$), (Figure 6.5).

uPA activity in supernatants of the P. gingivalis LPS stimulated hGFs

The uPA activity was measured in the supernatants of the hGFs stimulated with *P. gingivalis* LPS for 24 hours. Results revealed that, there was a significant difference in uPA activity between the stimulated hGFs (0.4871±0.00033 IU/ml) and the control cells (0.4861±0.0002 IU/ml) (Independent samples t-test, $p<0.05$), (Figure 6.6). However, the uPA activity levels measured in the supernatants of the stimulated hGFs were very low as compared to the uPA activity levels measured in the saliva samples of the periodontitis patients and healthy controls (see Chapter 4, section 4.2.4).

uPAR production by hGFs in response to P. gingivalis LPS stimulation

The uPAR ELISA was used to measure the uPAR levels in the supernatants of the hGFs stimulated with *P. gingivalis* LPS for 24 hours. The stimulated hGFs produced higher levels of uPAR (86±3 pg/ml) than the cells incubated with the control dose (60±4 pg/ml), and the difference was highly significant (Independent samples t-test, $p<0.01$), (Figure 6.7).

Summary of the P. gingivalis LPS stimulation experiments

The hGFs incubated with 100 ng/ml of *P. gingivalis* LPS for 24 hours, were stimulated to produce significantly higher levels of MMP-1, uPA and uPAR in comparison to the control cells. There was a statistically significant difference in the supernatants uPA activity between the stimulated and control cells.

6.3 Discussion

6.3.1 Regulation of uPA and uPAR by IL-1β

IL-1β is a well-known inflammatory cytokine that executes a number of important functions that regulate the inflammation, inflammatory response to pathogens such as microbial invasion, and healing of injured tissues. As an inflammatory mediator, the IL-1β is involved in the pathogenesis of periodontal diseases by its roles in the tissue destruction, stimulation of chemokines, recruiting inflammatory cells to the inflamed site, and inducing other inflammatory mediators potentiating the inflammatory response (Barksby et al. 2007; Buduneli and Kinane 2011; Taylor 2014; Jaedicke et al. 2016). Hence, studies have reported higher levels of IL-1β in gingival tissues and GCF from inflamed sites, and saliva of periodontitis patients in comparison to healthy tissues and healthy subjects (Figueredo et al. 1999; Suwatanapongched et al. 2000; Ogura et al. 2001; Dinarello 2009; Salminen et al. 2014; Javed et al. 2015; de Lima et al. 2016; Gomes et al. 2016; Jaedicke et al. 2016). IL-1β stimulates the production and activity of the matrix metallo-proteinases such as MMP-1 in human gingival fibroblasts, therefore IL-1β is an important mediator of connective tissue and matrix degradation associated with periodontal diseases (Gogly et al. 1998; Kida et al. 2005; Liu et al. 2010). On this basis, IL-1β was used in the stimulation experiments of the primary hGFs by the present study to investigate its role as a stimulant for the candidate biomarkers uPA and uPAR production, along with investigating the IL-1β stimulatory effects on the uPA activity in the hGFs.

The results of the cell culture experiments carried out by the present study showed that, the incubation of the hGFs with 5 ng/ml and 0.05 ng/ml of IL-1β for 24 hours stimulated the cells to produce higher levels of MMP-1 in comparison to the controls, which was an indication for the pro-inflammatory response by the hGFs to the IL-1β stimulation. These results were in agreement with Williams et al. (2016) who also

found that the 24-hour incubation of hGFs with 0.05 ng/ml of IL-1β stimulated the hGFs to produce higher levels of MMP-1 in comparison to the unstimulated cells. Such results may be explained by the ability of IL-1β to stimulate the production of other inflammatory mediators, proteins, and pro-enzymes such as MMP-1 and PGE_2 in the hGFs (Kida et al. 2005). Following 3 days incubation of hGFs with 1-20 ng/ml of IL-1β, active MMP-1 (collagenase enzyme) was detected in the stimulated hGFs by western blotting in comparison to the pro-enzymatic MMP-1 detected in the unstimulated cells (Cox et al. 2006). This finding by Cox et al. (2006), may explain the high levels of MMP-1 measured in the supernatants of the hGFs stimulated with IL-1β found by the present study.

Though the hGFs incubated with 5 ng/ml and 0.05 ng/ml of IL-1β for 24 hours were stimulated to produce numerically higher levels of uPA in comparison to the controls; the difference was not statistically significant. This finding was in contrast to the results of Ogura et al. (2001) study, which was the only available study (before conducting the present project) that investigated the role of IL-1β in the stimulation of hGFs to express both uPA and uPAR. Ogura et al. (2001), found that the 24-hour incubation with 1 unit/ml of IL-1β stimulated the hGFs to express higher mRNA and protein levels of uPA in comparison to the unstimulated cells.

The present study investigated the uPA activity in the supernatants of hGFs following 24-hour stimulation with IL-1β using the uPA fluorometric activity assay. The assay results indicated that there was no statistically significant difference in the uPA activity between the stimulated and control cells, a finding that may contradict the Ogura et al. (2001) results. Using a modified activity assay described by Pfeilschifter et al. (1990) which measures the release of chromogenic plasmin substrate, Ogura et al. (2001), found that IL-1β stimulated the hGFs to demonstrate higher plasminogen (PA) activity in their lysates in a time dependent manner (1 unit/ml of IL-1β for 4, 8, and 24 hours) which was greater at each time point as compared to the unstimulated cells ($p<0.01$), and in a dose dependent manner (0.01, 0.1, 1, and 10 unit/ml of IL-1β for 7 hours) with the highest PA activity at 1 and 10 unit/ml ($p<0.01$) then 0.1 unit/ml ($p<0.05$) and no difference was found with the 0.01 unit/ml of IL-1β as compared to the unstimulated cells. Furthermore, the releasable PA activity from the phosphatidylinositol-specific phospholipase C (PI-PLC) treated hGFs lysates was

higher in the hGFs stimulated with 1 unit/ml of IL-1β for 7 hours in comparison to the control cells.

On the other hand, the present study found that the 24-hour incubation with 5 ng/ml and 0.05 ng/ml of IL-1β, stimulated the hGFs to produce significantly higher levels of uPAR in comparison to the unstimulated cells, a finding that was in harmony with Ogura et al. (2001) study which reported higher protein levels and mRNA expression of uPAR by the hGFs in response to 24-hour stimulation with 1 unit/ml of IL-1β. Despite the fact that there was no significant difference in the uPA levels and activity between the stimulated and control hGFs; the production of uPA and uPAR by the stimulated hGFs, indicated that the present study was a further evidence along with the Ogura et al. (2001) study for the role of the inflammatory cytokine IL-1β in stimulating the local production of both uPA and uPAR by the hGFs.

Along with the IL-1β, Williams et al. (2016) investigated the effects of IL-1α alone and in synergism with the leptin hormone on hGFs. Williams et al. (2016), found that the 24-hour incubation of hGFs with 0.05 ng/ml of IL-1α or 10 µg/ml of leptin stimulated the hGFs to produce higher levels of MMP-1 in comparison to the unstimulated cells, and this stimulation was maximized when the hGFs were incubated with the both stimulants (i.e. a synergistic effect). As IL-1α and IL-1β both belong to the same interleukin-1 superfamily, the production of MMP-1 by hGFs stimulated with IL-1α reported by Williams et al. (2016), is consistent with the findings of the present study regarding the production of MMP-1 by the IL-1β stimulated hGFs.

Two previous studies investigated the stimulatory effects of IL-1 on the hGFs production of MMP-1 and plasminogen activators (Mochan et al. 1988; Tewari et al. 1994). Mochan et al. (1988), carried out the first study to investigate the role of IL-1 in the stimulation of the plasminogen activator production by the hGFs. The PA activity was determined by measuring the hydrolysis of a chromogenic substrate. The 24-hour incubation of the hGFs with different concentrations of IL-1 (0-10 U/ml), stimulated the cells to express higher PA activity with the maximum effect at the 3 U/ml dose for the cell culture supernatants and at the 5 U/ml dose for the cell lysates. In addition to that, the cells were stimulated in a time dependent manner by 10 U/ml of IL-1 for (0-24 hours), the maximum stimulation of the PA activity was at the 24-hour time point for the cell culture supernatants and at the 4-hour time point for the

cell lysates. Moreover, the immuno-peroxidase staining of the cells demonstrated higher expression of the PA molecules in the stimulated hGFs. Nevertheless, they did not specify if the IL-1 they used was IL-1α or IL-1β, the Mochan et al. (1988) results were in contrast to the present study results in regard to the uPA activity which was not significant in the supernatants of the hGFs stimulated with the IL-1β. On the other hand, though they didn't define which PA protein was highly expressed, the Mochan et al. (1988) study may support the present study in respect to the higher production of uPAR by the hGFs stimulated with IL-1β for 24 hours.

The second study was by Tewari et al. (1994), who investigated the relative expression of the MMPs (1 and 3) and the plasminogen activator in response to IL-1 stimulation. The 24-hour incubation of the hGFs with 100 nM of IL-1 significantly stimulated the mRNA expression of MMP-1, MMP-3, and PA from the hGFs. Furthermore, Tewari et al. (1994) found that the IL-1 stimulated the expression of the 3 proteins from the hGFs in a time dependent manner by the incubation of the cells for (0-24 hours), the maximum stimulation for the MMP-1 was at the 2-hour time point and this remained until the 24-hour time point, whereas the maximum stimulation for both the MMP-3 and PA was at the 12-hour time point and this remained until the 24-hour time point. Though they didn't assign which PA protein production was stimulated, the Tewari et al. (1994) study may support the present study for the higher production of both the MMP-1 and uPAR by the IL-1β stimulated hGFs in comparison to the control cells.

6.3.2 Regulation of uPA and uPAR by LPS

Lipopolysaccharides are endotoxins produced by various gram-negative bacteria. Bacterial LPS have been implicated in the pathogenesis of periodontal diseases, they were detected in the dental plaque of periodontitis patients and positively correlated with the clinical measures of the disease. LPS can induce diverse responses in the periodontal tissues which will result in inflammation and degradation of these supporting tissues, in addition to the alveolar bone resorption leading to periodontitis (Gupta 2013; Hasan and Palmer 2014; Kang et al. 2016). LPS may exert direct toxic effects on the hGFs and other periodontal tissues, stimulate the release and activity of host inflammatory mediators and enzymes such as collagenases, PGE_2, and interleukins (such as IL-1β), and may stimulate the osteoclasts formation in the periodontal tissues (Ogura et al. 1995; Mochizuki et al. 1999; Xiao et al. 2001a;

Gupta 2013; Wara-aswapati et al. 2013; Kang et al. 2016). Bacterial LPS have been used by numerous in vitro studies to investigate the stimulatory or inhibitory effects of these bacterial toxins on different oral cells such as human gingival fibroblasts.

In the present study, both the *E. coli* and *P. gingivalis* LPS were used in the cell culture experiments to investigate the stimulatory effects of these two bacterial toxins on the hGFs. In a similar manner to the IL-1β experiments, the MMP-1 levels were measured first in the supernatants of the stimulated hGFs as an inflammatory response to the stimulation with these bacterial LPS. The study results revealed that, the *P. gingivalis* LPS stimulated the hGFs to produce significantly higher levels of MMP-1 in comparison to the control cells. In contrast to the *P. gingivalis* LPS*,* though the *E. coli* LPS stimulated the hGFs to produce numerically higher levels of MMP-1 than the control cells, the difference was not statistically significant. Williams et al. (2016), found that the *E. coli* LPS stimulatory effect on the hGFs in respect to the MMP-1 production was in a donor-dependent manner (i.e. hGFs obtained from some donors demonstrated higher levels of MMP-1 while cells derived from other donors did not). Nevertheless, the MMP-1 mRNA expression and protein levels were increased in the hGFs stimulated with the *E. coli* LPS; the difference was only significant when the *E. coli* LPS was synergized with leptin (Williams et al. 2016). These findings by Williams et al. (2016) are consistent with the present study findings with regard to the MMP-1 production by the *E. coli* LPS stimulated and control hGFs. Moreover, it has been reported that *P. gingivalis* LPS has the greatest influence on the hGFs production of inflammatory cytokines (such as IL-6, IL-8 and PGE_2) as compared to LPS from *A. actinomycetemcomitans,* and *E. coli* (Yamaji et al. 1995; Noguchi et al. 1996), this may also explain the difference in the MMP-1 production in response to *P. gingivalis* and *E. coli* LPS found by the present study.

The present study was the first to investigate the uPA and uPAR production in addition to the uPA activity in hGFs in response to stimulation by *E. coli* LPS. Results demonstrated that, though the uPA and uPAR levels were numerically higher in the supernatants of the stimulated cells as compared to the control cells, the difference was not statistically significant. Furthermore, no significant difference was found in the uPA activity between the stimulated and control cells. hGFs obtained from the same donor but from different anatomical locations on the periodontium may have different phenotypes therefore they may demonstrate different responses to

stimulation (Irwin et al. 1994; Jonsson et al. 2011), this may explain the hGFs responses to *E. coli* LPS found by the present study. In addition to that, inter-individual variability and differences in the hGFs sensitivity and responses to LPS have been reported by a number of studies (Tipton et al. 1991; Michel et al. 2001; Wurfel et al. 2005; Sukkar et al. 2007; Uehara and Takada 2007).

In contrast to *E. coli* LPS, the *P. gingivalis* LPS stimulated the hGFs to produce significantly higher levels of both uPA and uPAR in comparison to the control cells. Though it was in low levels as compared to what was found in saliva of periodontitis patients, the *P. gingivalis* LPS stimulated the hGFs to express higher uPA activity than the control cells. As uPAR binds and localizes the activity of uPA (Crippa 2007; Smith and Marshall 2010), the higher levels of both uPA and uPAR may explain the higher uPA activity measured in the supernatants of the stimulated hGFs. Furthermore, the high levels of MMP-1, uPA, uPAR, and uPA activity in the supernatants of the stimulated hGFs, might be explained by the fact that *P. gingivalis* is one of the well-known bacteria involved in the pathogenesis of periodontal diseases (Nickles et al. 2016; Kakuta et al. 2017), and that the inflamed hGFs response to the *P. gingivalis* LPS differs from that of the healthy cells by the expression and production of higher levels of different inflammatory cytokines and proteins (Kang et al. 2016).

Stimulation of receptors known as the toll-like receptors (TLRs) expressed by the hGFs induce these cells to demonstrate various responses. The hGFs express these surface receptors in order to activate inflammatory responses that may help to kill and remove any bacteria or bacterial products in the gingival tissues. The hGFs may express up to 10 TLRs, the most common are the TLR2 and TLR4 receptors. These receptors recognise bacterial-associated molecular patterns in the gingiva and dental plaque such as LPS (Wang et al. 2000; Kiji et al. 2007; Mahanonda et al. 2007; Uehara and Takada 2007; Yoshioka et al. 2008; Scheres et al. 2011; Kang et al. 2016; Williams et al. 2016; Palm et al. 2017). The TLR2 receptors are stimulated by TLR2 agonists such as *P. gingivalis* LPS (Hirschfeld et al. 2001; Darveau 2010; Scheres et al. 2011; Andrukhov et al. 2014; Kang et al. 2016). Whereas the TLR4 are stimulated by TLR4 agonists such as LPS of *A. actinomycetemcomitans* and enterobacteria such as *E. coli* (Lee et al. 2006; Andrukhov et al. 2014; Williams et al. 2016). Studies reported that periodontal pathogens (such as *P. gingivalis*) mostly

stimulate the destructive inflammatory responses of periodontitis by activating TLR2 receptors (Burns et al. 2006; Darveau 2010; Wara-aswapati et al. 2013; de Aquino et al. 2014). Other studies demonstrated that during inflammation the collagen degradation activity is increased after exposure of the hGFs TLR4 receptors to LPS such as *A. actinomycetemcomitans LPS* (Bhide et al. 2005; Takahashi et al. 2008). However, in addition to periodontal pathogens, commensal oral bacteria may also stimulate TLRs such as TLR2 in an attempt to induce a protective response that may prevent the host from developing disease (Darveau 2010). Furthermore, agonists and pathogens may stimulate receptors other than those known to be their specific targets, for instance a study have shown that *P. gingivalis* LPS induced hGFs to produce IL-1 via stimulating TLR4 receptor, and this IL-1 production was inhibited by anti-TLR4 antibody (Wang et al. 2000). Another study reported that in addition to TLR2, TLR1 and TLR7 in hGFs obtained from periodontitis patients also demonstrated strong affinity for induction by *P. gingivalis* (Scheres et al. 2011). Accordingly, hGFs that express TLR2 receptors are stimulated by *P. gingivalis* LPS, whereas cells that express TLR4 receptors are stimulated by *E. coli* LPS, this may explain the variation in the responses of the hGFs stimulated by these 2 bacterial LPS found by the present study.

A number of studies investigated the effects of LPS derived from *P. gingivalis* and other bacterial species on the hGFs in respect to the PA system proteins production and activities. In their research, Ogura et al. (1995) studied the stimulatory effects of LPS derived from the periodontal bacteria *Campylobacter rectus* on the hGFs in a time and dose dependent manner. Ogura et al. (1995), also investigated the effects of the *C. rectus* LPS along with LPS derived from other periodontal bacteria including the *P. gingivalis*, *Prevotella loescheii,* and *Porphyromonas endodontalis* on the production of PA system proteins by hGFs. Using the fluorogenic assay with a plasmin substrate, Ogura et al. (1995) found that the 24-hour incubation of hGFs with 10 µg/ml of the aforementioned LPS stimulated the PA activity determined by uPA and the highest activity was observed in the cells stimulated with the *C. rectus* and *P. endodontalis* LPS. Ogura et al. (1995), investigated the PA activity in a time dependent manner, they found that the incubation of hGFs with 10 µg/ml of the *C. rectus* LPS for 10, 24, 48, and 72 hours stimulated the cells to express significantly higher PA activity via uPA in comparison to the unstimulated cells. Moreover, the

dose dependent course showed that the incubation of hGFs with 0.4, 2 and 10 µg/ml of the *C. rectus* LPS for 72 hours stimulated the cells to demonstrate higher PA activity in comparison to the control cells (Ogura et al. 1995). Though they didn't carry out further investigations with the *P. gingivalis* LPS and focused on the *C. rectus* LPS, the Ogura et al. (1995) results may support the present study in regard to the higher production and activity of uPA by hGFs in response to the *P. gingivalis* LPS as both *P. gingivalis* and *C. rectus* are among the essential pathogens in periodontal diseases (Nickles et al. 2016). Furthermore, the PA activity via the uPA action on plasmin substrate measured by Ogura et al. (1995), is consistent with the uPA activity measured in the *P. gingivalis* LPS stimulated hGFs by the present study, and the Ogura et al. (1995) study was a further evidence for the role of uPA in the pathogenesis of periodontal diseases and may backup the present study for the identification of uPA as a biomarker for periodontal diseases.

Abiko et al. (1998), investigated the PA activity along with the PGE_2, IL-1β and IL-6 production by young and aged hGFs stimulated with *C. rectus* LPS for 24 hours. Abiko et al. (1998), measured the inflammatory mediators and the PA activity in the media of the stimulated cells in 3, 9, 12, and 24 hours following stimulation. The results showed that all the mediators were higher in both the stimulated aged and young cells (higher in the aged cells) in comparison to the controls. The PA activity assay using fluorogenic substrate, enabled Abiko et al. (1998) to find that the PA activity was higher in the stimulated cells as compared to the control cells. The semi-quantitative real time-PCR (RT-PCR) analysis showed that the gene expression for each of the inflammatory proteins was higher in the stimulated aged and young hGFs (more significant in the aged cells) than the control cells. Though they used *C. rectus* LPS, the Abiko et al. (1998) study is consistent with the present study in regard to the uPA activity measured in the hGFs stimulated with the *P. gingivalis* LPS. Furthermore, Abiko et al. (1998) found that the tPA gene expression was higher than that of the uPA in the stimulated cells, a finding that may explain the non-significant difference in the uPA levels produced by the *E. coli* LPS stimulated hGFs and the control cells found by the present study.

Mochizuki et al. (1999), investigated the effects of the *C. rectus* LPS on young and aged gingival fibroblasts obtained from rats and human donors (rGFs and hGFs) in relation to the plasminogen activators production and activity. The cells were

incubated with 0.01, 0.1, 1 and 10 µg/ml of *C. rectus* LPS for 8 hours, and the PA activity in the stimulated cells culture media was measured by the plasmin production from plasminogen substrate. Mochizuki et al. (1999), found that the PA activity was significantly elevated in both the young and aged hGFs and rGFs (more in the aged cells) in a dose dependent manner as compared to the control cells. Though they measured the PA activity in response to *C. rectus* LPS, the Mochizuki et al. (1999) study is also consistent with the present study in regard to the higher uPA activity measured in the *P. gingivalis* LPS stimulated hGFs as compared to the controls. Moreover, Mochizuki et al. (1999) detected high levels of tPA mRNA but not uPA in both the aged hGFs and aged rGFs stimulated with *C. rectus* LPS, a finding that might be in harmony with the present study results which revealed non-significant difference in the uPA levels produced by both the *E. coli* LPS stimulated and unstimulated hGFs.

The uPA and uPAR levels along with the PA activity were investigated in hGFs in response to stimulation with the *P. gingivalis* LPS (Ogura et al. 1999). Ogura et al. (1999), measured the PA activity using the Pfeilschifter et al. (1990) activity assay which measures the release of chromogenic plasmin substrate by the hGFs lysates and the releasable PA activity from the PI-PLC treated hGFs. The uPA and uPAR levels were measured using the slot blot analysis, the relative mRNA expression for the both proteins was measured by the RT-PCR. Ogura et al. (1999), incubated the cells with 1 µg/ml of LPS for 24 hours, the PA activity was measured at 4, 8, and 24 hours following stimulation. The PA activity was higher at all the time points in the stimulated cells as compared to the control cells, and the maximum increase in the activity was at the 8-hour time point. Ogura et al. (1999), also measured the PA activity in the hGFs following stimulation with 0.1, 1, and 10 µg/ml of LPS for 8 hours which increased the PA activity in the stimulated cells in a dose dependant manner and the maximum stimulation was at the 1 µg/ml of LPS. The 8-hour incubation of the hGFs with 1 µg/ml of *P. gingivalis* LPS increased the PI-PLC releasable PA activity by 3 folds in the stimulated cells as compared to the unstimulated cells. Considering the incubation time and the stimulating doses used by Ogura et al. (1999) in their experiments, the PA activity results of Ogura et al. (1999) are in agreement with the increased uPA activity measured in the hGFs supernatants following the 24-hour stimulation with *P. gingivalis* LPS found by the present study. Furthermore, Ogura et

al. (1999) found that the incubation of the hGFs with 1 µg/ml of *P. gingivalis* LPS for 8 hours increased both the protein levels and the relative mRNA expression for both uPA and uPAR in the stimulated cells in comparison to the control cells, these results were in congruence with present study results which also revealed that the *P. gingivalis* LPS stimulated the hGFs to produce higher levels of both uPA and uPAR in comparison to the unstimulated cells.

The production of tPA and its inhibitor PAI-2 were investigated in hGFs stimulated with LPS derived from *A. actinomycetemcomitans, P. gingivalis,* and *Fusobacterium nucleatum* periodontal bacteria and LPS from other non-periodontal bacteria including *E. coli* and *Salmonella enteritidis* (Xiao et al. 2001a). Follwoing a 24-hour incubation of the hGFs with 0.6 µg/ml of LPS from the aforementioned bacteria, Xiao et al. (2001a) measured the tPA and PAI-2 production using ELISA and RT-PCR assays which both revealed that there were significantly higher levels of tPA in the stimulated cells as compared to the control cells. The PAI-2 levels were increased in response to LPS from all bacteria except the *P. gingivalis* and the greatest increase was in response to the *E. coli* LPS. Moreover, Xiao et al. (2001a) found that the ratio of tPA to its inhibitor PAI-2 was greater following stimulation with LPS from all the periodontal bacteria and the highest ratio was in response to the *P. gingivalis* LPS. Xiao et al. (2001a), explained the *P. gingivalis* LPS stimulation of tPA as compared to its inhibior PAI-2, by the high plasminogen activation and inhibition of the plasminogen inhibitors (i.e. plasminogens activators production is stimulated and plasminogen inhibitors production is inhibited) possessed by *P. gingivalis* in comparison to other bacterial species such as *A. actinomycetemcomitans, T. forsythus* and *F. nucleatum* (Grenier 1996). Therefore, Xiao et al. (2001a) concluded that the periodontal bacteria had the greatest stimulatory effect on the plasminogen activators (represented by tPA), and that these bacteria affect the ratio or balance between the activators and their inhibitors in the favour of the activators resulting in degradation of the supporting periodontal tissues during periodontitis. Although, they investigated tPA and its inhibitor PAI-2 but not uPA or uPAR, the Xiao et al. (2001a) results are consistent with the increased levels of the PA system proteins (uPA and uPAR) produced by the hGFs in response to the *P. gingivalis* LPS stimulation found by the present study.

Otsuka et al. (2001), investigated the plasminogen activity in the hGFs of subjects with and without Down syndrome (DGFs and NDGFs) in response to the LPS derived from *A. actinomycetemcomitans.* The PA activity was measured by the Pfeilschifter et al. (1990) method. The cells were incubated with 0.01, 0.1, 1, and 10 µg/ml of LPS for 5 hours. Otsuka et al. (2001), found that the LPS stimulated the PA activity in both the DFGs and NDGFs in a dose dependent manner, and this activity was significantly higher in the stimulated cells as compared to the unstimulated cells, and was more significant in the stimulated DGFs. Otsuka et al. (2001), also assayed the PA activity after they incubated the cells for 3, 6, 9, 12 and 24 hours with 1 µg/ml of LPS. The results revealed that the PA activity was stimulated in a time dependent manner, and it was significantly higher in both the stimulated DGFs and NDGFs at all-time points than in the unstimulated cells and again the most significant effect was observed in the DGFs. Despite the fact they used other source of bacterial LPS and carried out the study on subjects with and without Down syndrome, the Otsuka et al. (2001) study may support the present study in regard to the high uPA activity measured in the hGFs stimulated with *P. gingivalis* LPS as compared to the unstimulated cells.

The production of the MMPs (2, 3, & 9) and tissue inhibitors of MMPs (TIMP-1 & TIMP-2) along with the activities of MMP-2 and uPA were investigated in hGFs in response to stimulation with LPS obtained from *A. actinomycetemcomitans*, *P. gingivalis,* and *Fusobacterium nucleatum subsp.nucleatum* (Bodet et al. 2007). Bodet et al. (2007), found that there was an overproduction of MMP-2 more than MMP-3, whereas MMP-9 was not expressed, and there was an overproduction of TIMP-1 but not TIMP-2 in response to the stimulation with 1 µg/ml of *A. actinomycetemcomitans* LPS for 6, 24, and 48 hours. The 48-hour incubation of the hGFS with 1 µg/ml of *P. gingivalis,* and *F. nucleatum* LPS also stimulated the cells to produce higher levels of MMP-2 and TIMP-1 in comparison to the controls. Bodet et al. (2007), used the zymography assay to assay the activity of MMP-2 and uPA. The 24/48-hour stimulation of the cells with 1 µg/ml of the 3 bacterial LPS stimulated the MMP-2 activity. In a similar manner to the MMP-2 activity, the incubation of the hGFs with 1 µg/ml of LPS obtained from the 3 bacteria for 24-48 hours also stimulated the cells to express significantly higher uPA activity in comparison to the control cells, and the most potent stimulation was in response to the *A. actinomycetemcomitans* and *P. gingivalis* LPS. Though they did not measure MMP-1, the Bodet et al. (2007) study is

in agreement with the present study in regard to the high levels of MMP-1 produced by the hGFs in response to *P. gingivalis* LPS. Furthermore, the Bodet et al. (2007) study was in harmony with the present study which also found high uPA activity in the hGFs stimulated with *P. gingivalis* LPS as compared to control cells. On the other hand, Bodet et al. (2007) attributed the higher uPA activity in response to the bacterial LPS, to the overproduction of uPA from the stimulated hGFs, which is consistent with the higher uPA levels and activity produced by the hGFs stimulated with *P. gingivalis* LPS found by the present study.

Na et al. (2014), investigated the mRNA expression and production levels of PAI-1 by hGFs in response to stimulation with *P. gingivalis* LPS. First, Na et al. (2014) checked the response of the cells to LPS derived from *P. gingivalis*, *E. coli,* and *F. nucleatum* by measuring the mRNA expression of both PAI-1 and TNF-α. Na et al. (2014), found that the PAI-1 mRNA expression was significantly higher in the cells stimulated with the *P. gingivalis* LPS in comparison to that of other bacteria, whereas the TNF-α mRNA expression was higher in response to the *E. coli* LPS. Na et al. (2014), found that the PAI-1 mRNA expression and protein levels were increased in both dose and time dependent manner in response to stimulation with *P. gingivalis* LPS (0.1,1, 2, 5 & 10µg of LPS for 2 hours, and 2µg of LPS for 30 minutes -24 hours). Though they investigated the PA system protein (PAI-1) but not uPA or uPAR, Na et al. (2014) study may support the present study which also found that the *P. gingivalis* LPS stimulated the hGFs to produce the PA proteins (uPA and uPAR) in higher levels as compared to the unstimulated cells. Therefore, the findings of both the present study and the Na et al. (2014) study along with the findings of the above-mentioned studies support the role of the periodontal bacteria *P. gingivalis* LPS in stimulating the hGFS to produce different PA system proteins.

6.3.3 The PA system proteins in other experimental studies

Though they did not stimulate the cells, Schmid and Chambers (1988) investigated the local PA system proteins production and activity in human gingival biopsies obtained from 2 healthy sites, 4 inflamed sites, and 10 apparently healthy sites 6 weeks after periodontal treatment. To differentiate between the specific PA system proteins, anti-tPA and anti-uPA antibodies were added to the prepared tissue samples. The results revealed that both the PA proteins production and activity were related to the tPA protein, and that the tPA activity was limited in both the healthy and

treated gingiva while in the inflamed tissues the entire epithelium demonstrated tPA activity. Schmid and Chambers (1988), concluded that tPA is the predominant PA protein in both the healthy and diseased human gingiva. Despite the fact that the PA activity detected was related to tPA, the Schmid and Chambers (1988) study may support the present study and the aforementioned studies in section (6.3.2) for the detection of PA activity in the hGFs in response to periodontal pathogen stimulus. On the other hand, the present study and the studies mentioned in sections (6.3.1, and 6.3.2) proved that tPA is not the predominant protein produced by the healthy and diseased hGFs, as high levels of uPA, uPAR, and PAI-2 were also detected in the stimulated and control hGFs by these studies. Furthermore, the uPA activity detected by the present study and the studies in sections (6.3.1, and 6.3.2) proved that the PA activity in the hGFs is not only related to tPA but uPA also plays a significant role in the PA activity especially in the presence of a periodontal pathogen stimulus.

In a similar manner to Schmid and Chambers (1988), Schmid et al. (1991) examined the local PA activity in gingival tissue biopsies obtained from 4 healthy subjects, 11 treated periodontitis patients (6 weeks after treatment), and 4 untreated periodontitis patients. The PA activity observed in the samples was a tPA fibrinolytic activity, which was limited to local areas in the healthy and treated samples, and distributed to the entire tissue in the diseased samples. Therefore, Schmid et al. (1991) found that the PA activity has a different pattern in the healthy and diseased tissues, and that the tPA activity was higher in the diseased tissues. Schmid et al. (1991), suggested that their findings may support the hypothesis that the PA system plays a role in the degradation of the periodontal tissues which is in agreement with the present study hypothesis. Though they also suggested that tPA and its activity are both prominent in the diseased gingival tissues; in contrary to Schmid and Chambers (1988), Schmid et al. (1991) found that adding anti-tPA antibodies to the samples did not totally inhibit the PA activity suggesting the possibility that uPA could be found in the tissues and exerts its activity during periodontitis. This finding may explain the high uPA levels and activity detected in the hGFs stimulated by periodontal bacteria LPS found by the present study and the studies in section (6.3.2).

In their study, Kinnby et al. (1999) investigated the PA system proteins (uPA, uPAR, tPA , PAI-1 and PAI-2) in gingival tissue samples obtained from 9 patients undergoing periodontal surgery (gingivectomy) preceded by a 6-week period of

treatment with scaling and oral hygiene instructions. The localization of the tPA and PAI-2 was carried out by the means of in situ hybridization and immunohistochemistry, whereas the uPA and PAI-1 were investigated by in situ hybridization only. Kinnby et al. (1999), revealed that the tPA protein and its inhibitor PAI-2 were found predominantly in the sulcular and junctional gingival epithelium. The uPA and its receptor uPAR were also found in single cells within the sulcular and junctional epithelium, as well as adjacent to the blood vessels close to the junctional epithelium. The PAI-1 was invariably found in the connective tissues associated with the blood vessels. The Kinnby et al. (1999) findings indicated the local production of PA proteins in gingival tissue which may explain the production of these proteins in the LPS stimulated hGFs found by the present study and the studies in section (6.3.2). Based on their findings, Kinnby et al. (1999) suggested that tPA and its inhibitor PAI-2 to be the most prominent PA proteins in the periodontal tissues exposed to bacterial invasion. However, it was found by the present study and the studies in section (6.3.2), that uPA and its receptor uPAR were produced in significantly high levels by the hGFs in the presence of periodontal bacterial stimulus such as LPS.

Fleetwood et al. (2015), investigated the production and activity of uPA in mice and human macrophage matrix culture models, and in mice periodontitis models in response to stimulation with the RgpA-Kgp complex of *P. gingivalis*. The RgpA-Kgp is a protease complex produced by *P. gingivalis* and is among its virulence factors. Fleetwood et al. (2015), found that, the RgpA-Kgp complex stimulated the uPA production in both the mice and human macrophages, increased the macrophage matrix degradation in a uPA dependent manner, and increased the uPA proteolytic activity resulting in enhanced alveolar bone loss in the mice periodontitis models. The Fleetwood et al. (2015) study indicated that *P. gingivalis* and its' RgpA-Kgp complex can induce alveolar bone loss via the activation of uPA proteolytic activity, this was another evidence for the uPA role in the pathogenesis of periodontitis, and successively supports the present study for the detection of uPA in high levels in saliva of periodontitis patients and for the uPA production by hGFs stimulated with the *P. gingivalis* LPS.

6.3.4 Summary of findings

IL-1β at doses of 5 and 0.05 ng/ml stimulated the hGFs to produce significantly higher levels of MMP-1 than the control cells. Both the 5 and 0.05 ng/ml of IL-1β stimulated the hGFs to produce numerically higher levels of uPA; however, there was no statistically significant difference as compared to the control cells. IL-1β did not stimulate the hGFs to express high uPA activity. IL-1β at doses of 5 and 0.05 ng/ml stimulated the hGFs to produce significantly higher levels of uPAR than the control cells. Accordingly, the results suggested that IL-1β may be more related to the stimulation of uPAR production by hGFs than the uPA.

Nevertheless, the 24-hour incubation with 100 ng/ml of *E. coli* LPS stimulated the hGFs to produce numerically higher levels of MMP-1, uPA and uPAR in comparison to the control cells, the difference was not statistically significant. Moreover, the *E. coli* LPS did not stimulate the hGFs to express high uPA activity. Therefore, the results suggested that the *E. coli* LPS might not be an effectual stimulant for the uPA and uPAR production by the hGFs.

The 24-hour incubation of the hGFs with 100 ng/ml of *P. gingivalis* LPS, was able to stimulate the cells to produce MMP-1, uPA and uPAR in significantly higher levels than the unstimulated cells. *P. gingivalis* LPS also stimulated the hGFs to express uPA activity. Hence, the study results suggested that the *P. gingivalis* LPS might be a potent stimulant for the uPA and uPAR production by the hGFs.

Thus the in vitro investigations of the present study provided evidence that both uPA and uPAR are produced locally by the hGFs in response to an inflammatory and bacterial stimuli, and that these two biomarkers might have roles in the pathogenesis of periodontitis. Therefore, uPA and uPAR are qualified as plausible biomarkers for periodontal diseases.

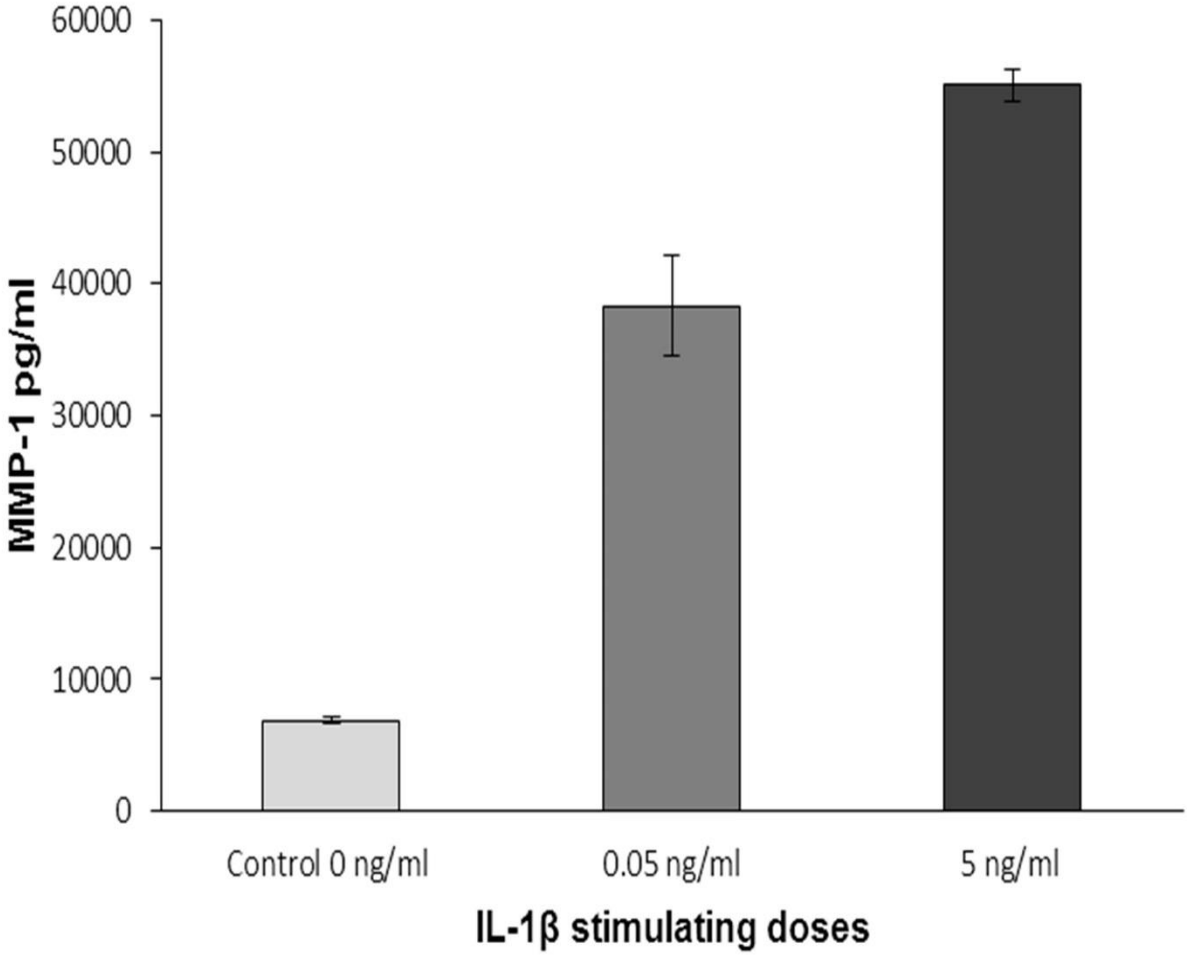

Figure 6.1: MMP-1 production by hGFs after stimulation with IL-1β- preliminary experiment.

hGFs obtained from 1 donor were cultured in three T25 flasks at density of (2-4x10^5 cell/flask) till the cells reached confluent growth (80-90%), then stimulated for 24 hours with 5 ml/flask of IL-1β in 2 doses (5 ng/ml and 0.05 ng/ml) as compared to control (0 ng/ml), n=1 flask for each dose. MMP-1 levels were measured by ELISA in the supernatants of the stimulated cells. Data represent the mean ±SEM of the ELISA duplicate measures for each dose.

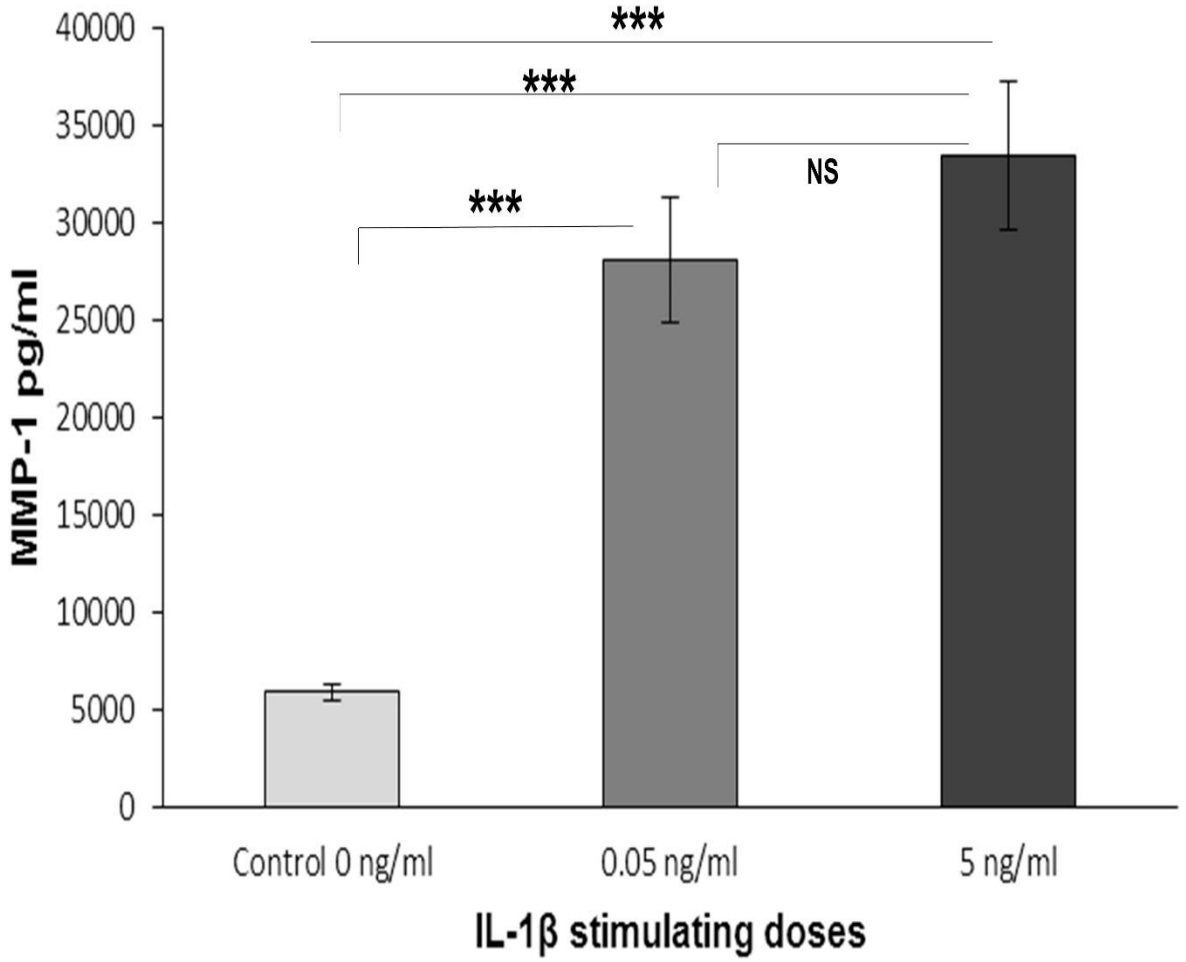

Figure 6.2: MMP-1 production by hGFs after stimulation with IL-1β.

hGFs were cultured in the 6 multi-well plates at density of ($2x10^5$ cell/well) till the cells reached confluent growth (80-90%), then stimulated with 2 ml/well of IL-1β in 2 doses (5 ng/ml and 0.05 ng/ml) in comparison to control (0 ng/ml) for 24 hours. MMP-1 levels were measured by ELISA in the supernatants of the stimulated cells. Data shown as mean values ±SEM (n=10 per dose) for cells from 3 donors stimulated in independent experiments. ***=$p<0.001$ respectively, and NS= non-significant (One-way ANOVA test with Bonferroni correction, and the Independent samples t-test).

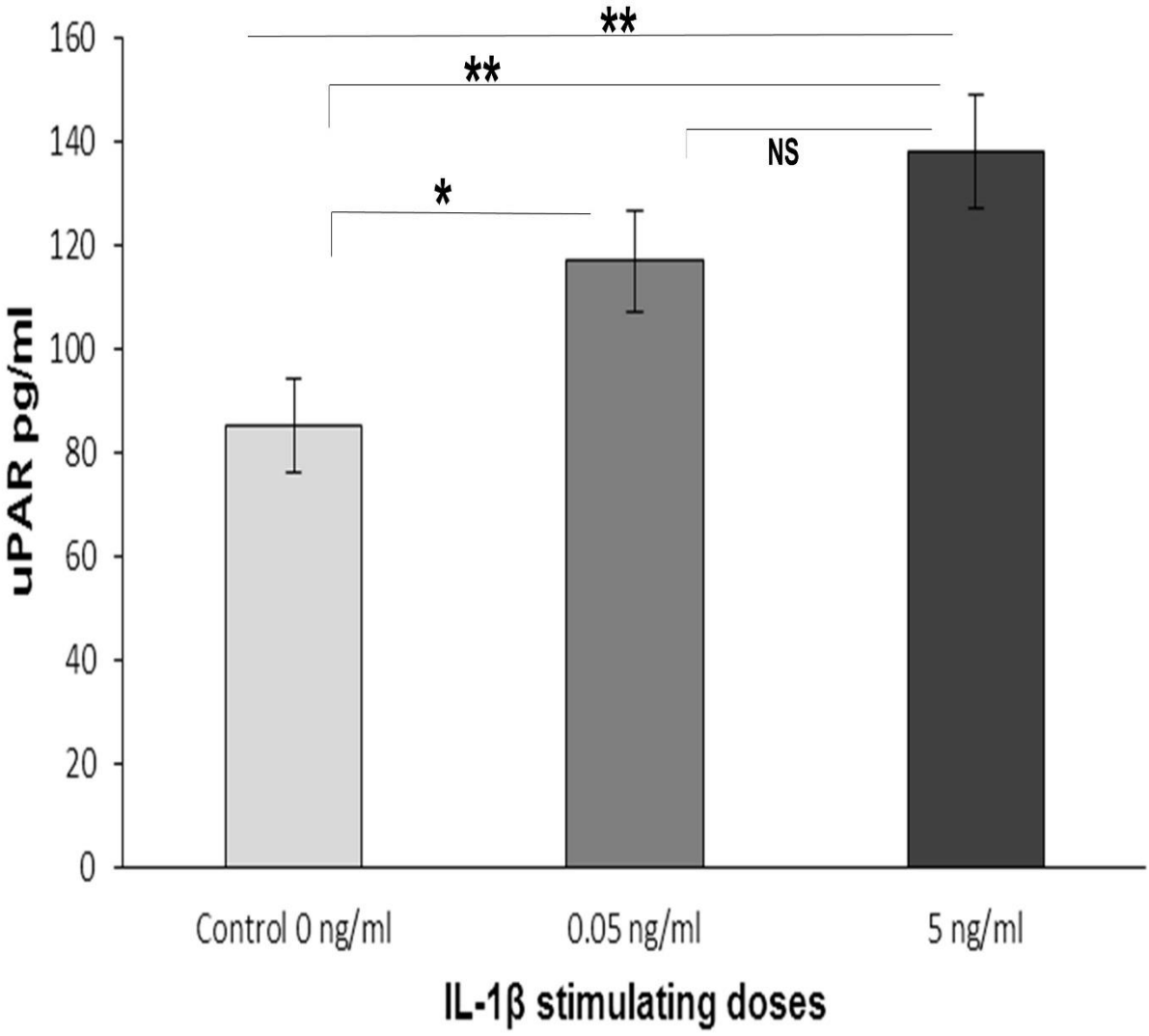

Figure 6.3: uPAR production by hGFs after stimulation with IL-1β.

hGFs were cultured in the 6-multi-well plates at density of ($2x10^5$ cell/well) till the cells reached confluent growth (80-90%), then stimulated for 24 hours with 2 ml/well of IL-1β in 2 doses (5 ng/ml and 0.05 ng/ml) as compared to control (0 ng/ml). uPAR levels were measured by ELISA in the supernatants of the stimulated cells. Data presented as mean values ±SEM (n=10 per dose) for hGFs from 3 donors stimulated in independent experiments. **=p<0.01, *=p<0.05 respectively, and NS= non-significant (One-way ANOVA test with Bonferroni correction, and the Independent samples t-test).

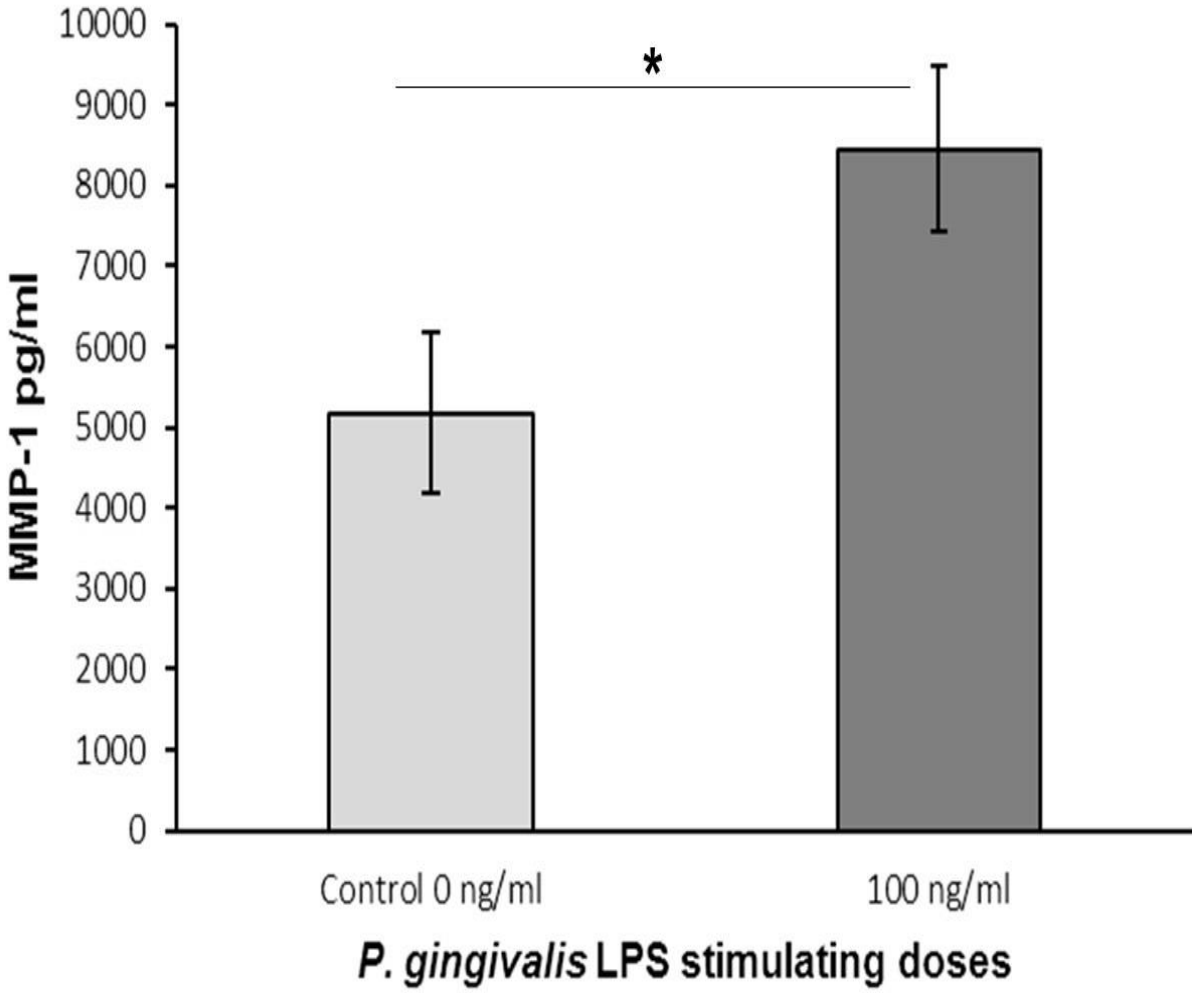

Figure 6.4: MMP-1 production by hGFs after stimulation with *P. gingivalis* LPS.

hGFs were cultured in the 6-multi-well plates at density of ($2x10^5$ cell/well), allowed to grow till (80-90%) confluency, then stimulated for 24 hours with 2 ml/well of 100 ng/ml of *P. gingivalis* LPS in comparison to control (0 ng/ml). MMP-1 levels were measured by ELISA in the supernatants of the stimulated cells. Data shown as mean values ±SEM (n=6 per dose) for cells from 2 donors stimulated in independent experiments. *****=$p<0.05$ (Independent samples t-test).

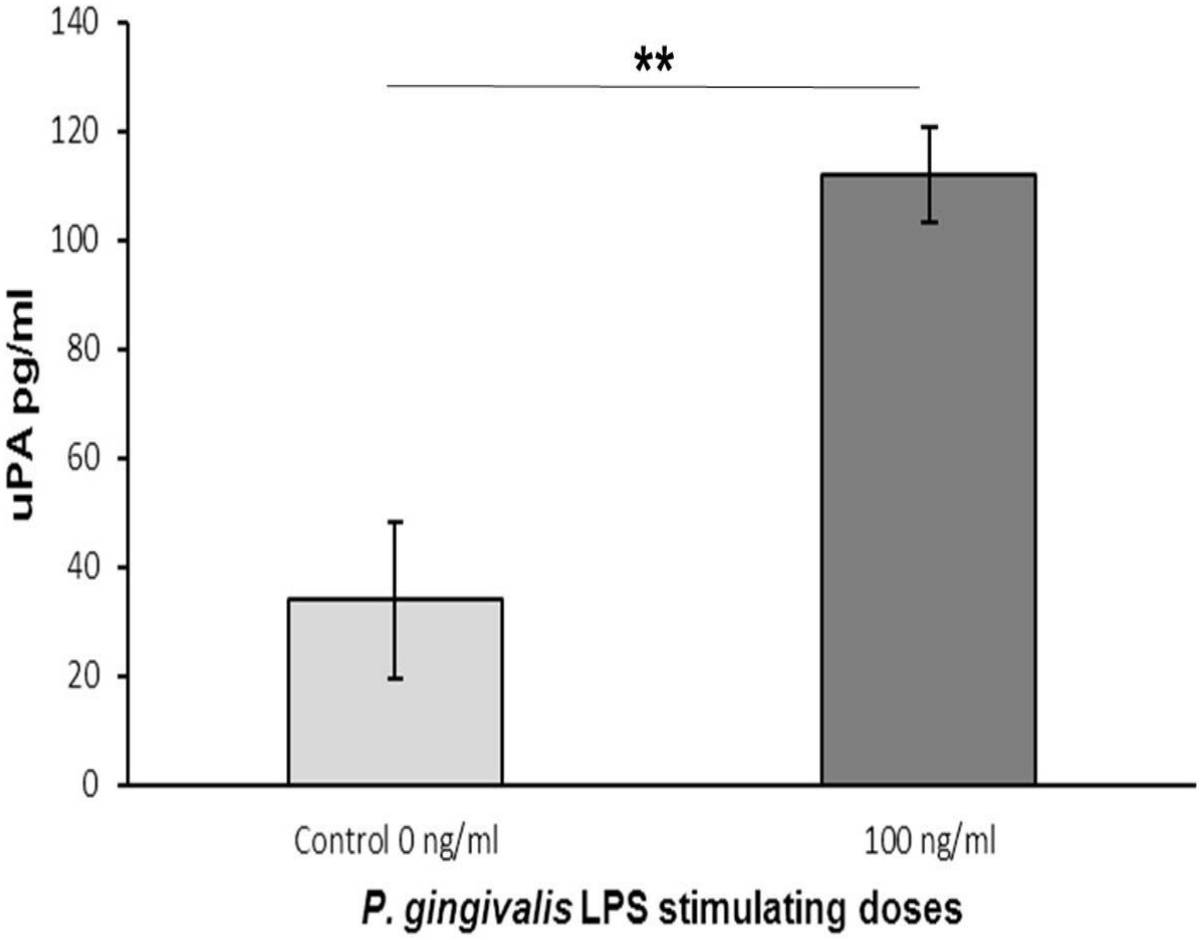

Figure 6.5: uPA production by hGFs after stimulation with *P. gingivalis* LPS.

hGFs were cultured in the 6-multi-well plates at density of ($2x10^5$ cell/well), till the cells reached (80-90%) confluent growth, then stimulated for 24 hours with 2 ml/well of 100 ng/ml of *P. gingivalis* LPS as compared to control (0 ng/ml). uPA levels were measured by ELISA in the supernatants of the stimulated hGFs. Data presented as mean values ±SEM (n=6 per dose) for hGFs from 2 donors stimulated in independent experiments. ******=$p<0.01$ (Independent samples t-test).

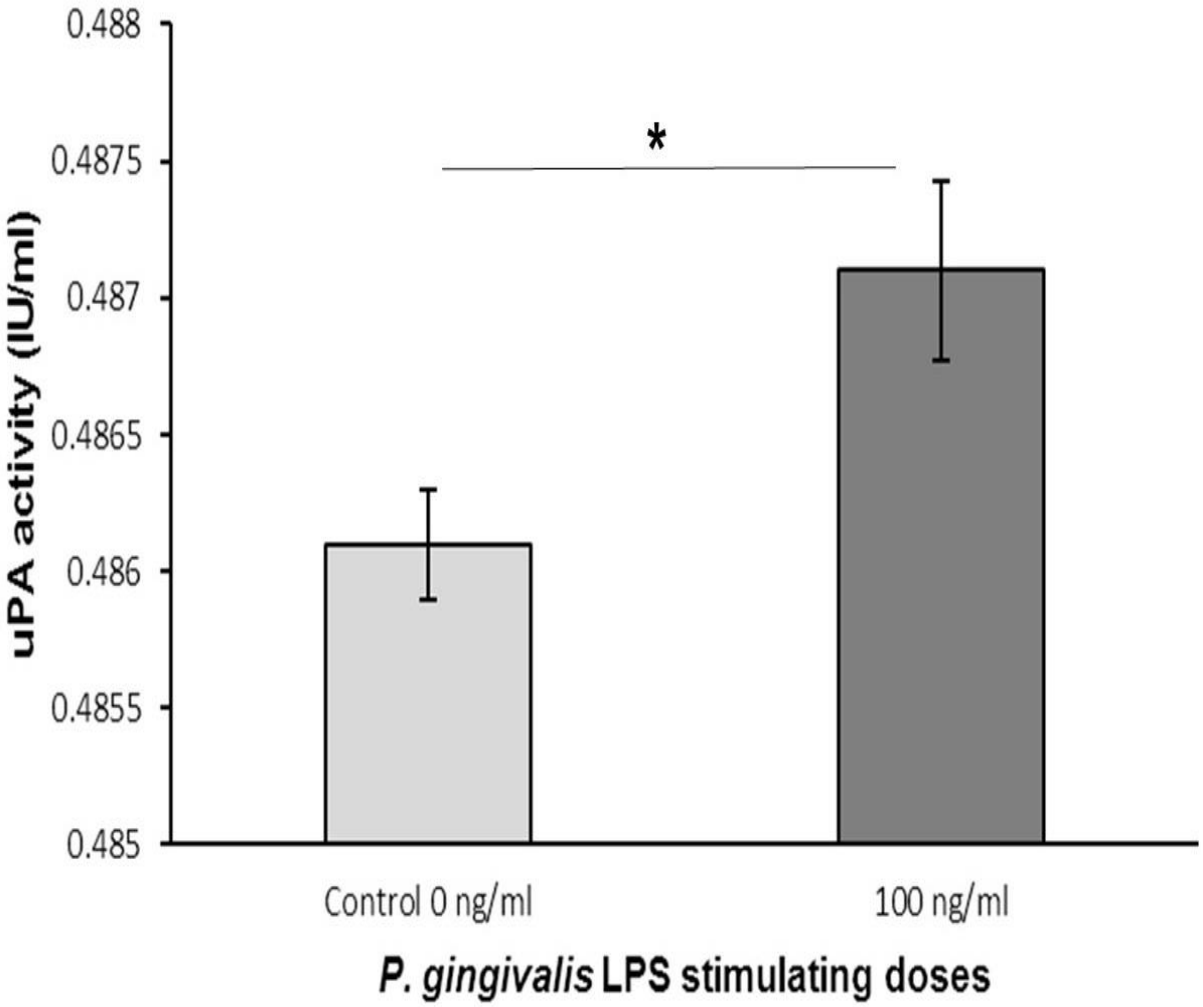

Figure 6.6: uPA activity in supernatants of hGFs stimulated with *P. gingivalis* LPS.

hGFs were seeded into the 6-multi-well plates at density of ($2x10^5$ cell/well), cultured till the cells reached (80-90%) confluent growth, then stimulated for 24 hours with 2 ml/well of 100 ng/ml of *P. gingivalis* LPS in comparison to control (0 ng/ml). The uPA fluorometric activity assay was used to measure the uPA activity in the supernatants of the stimulated hGFs. Data shown as mean values ±SEM (n=6 per dose) for cells from 2 donors stimulated in independent experiments. *=$p<0.05$ (Independent samples t-test).

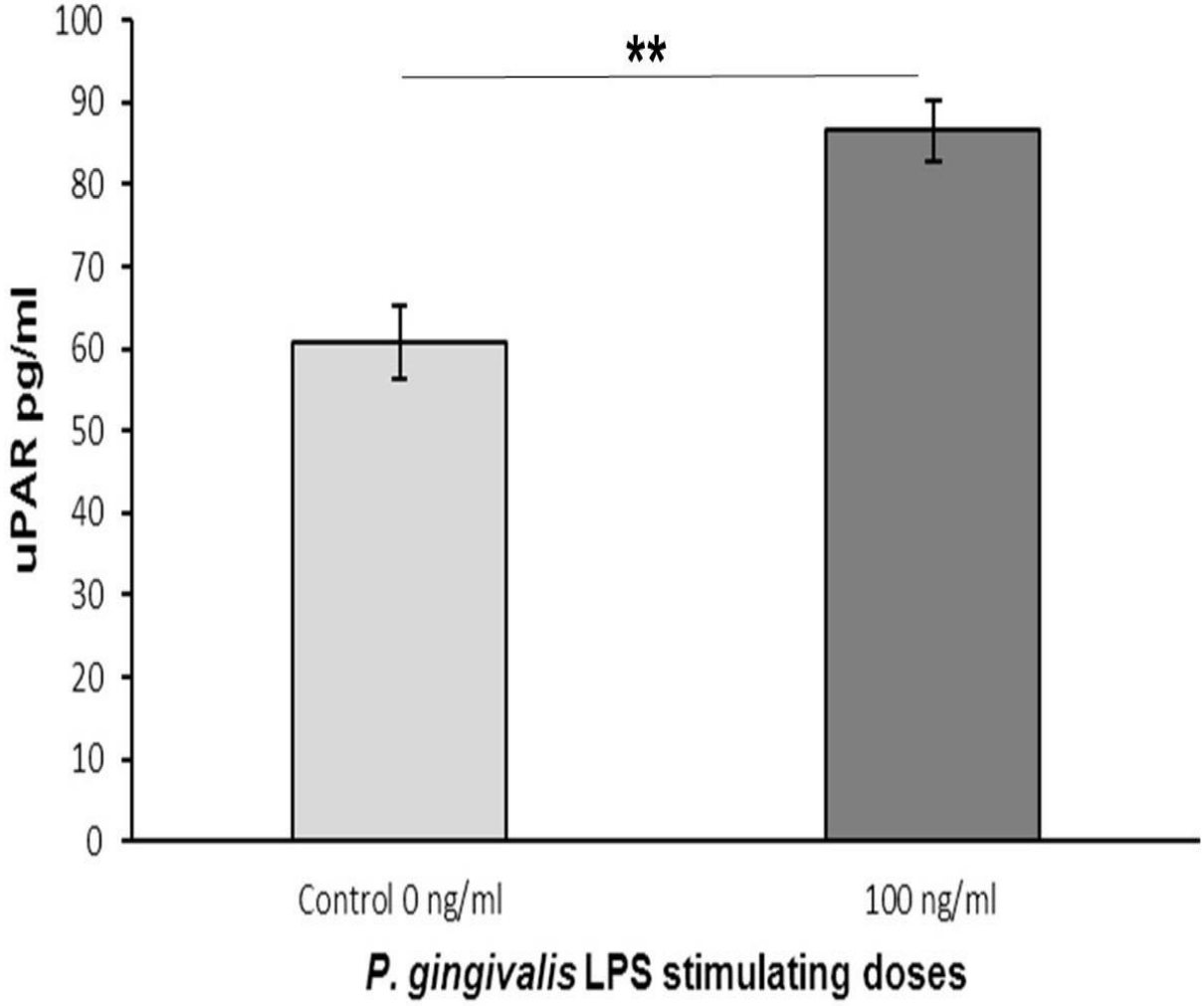

Figure 6.7: uPAR production by hGFs after stimulation with *P. gingivalis* LPS.

hGF cells were cultured in the 6-multi-well plates at density of ($2x10^5$ cell/well), allowed to grow till (80-90%) confluent, then stimulated for 24 hours with 2 ml/well of 100 ng/ml of *P. gingivalis* LPS as compared to control (0 ng/ml). uPAR levels were measured by ELISA in the supernatants of the stimulated cells. Data presented as mean values ±SEM (n=6 per dose) for hGFs from 2 donors stimulated in independent experiments. ******=p<0.01 (Independent samples t-test).

Chapter 7 General discussion, suggestions and summary

7.1 General discussion

Periodontitis and gingivitis are the most common oral inflammatory diseases affecting adults in the UK and worldwide. The progression of periodontal diseases is slow and painless, therefore if not diagnosed and prevented at early stages, can result in loss of teeth which requires complicated and expensive restorative treatments that exert a huge burden on the dental health care (Petersen and Ogawa 2012; White et al. 2012). Further to the conventional clinical diagnosis based on the examination of teeth and their supporting periodontal tissues, investigations of oral fluids including saliva and GCF have been used in the diagnosis and monitoring of periodontal diseases (Yin et al. 2000; Buduneli et al. 2005; Sorsa et al. 2010; Rathnayake et al. 2013; Schafer et al. 2014; Taylor 2014). Despite the fact that there are number of well-known salivary biomarkers such as IL-1β and MMP-8 (Kaushik et al. 2011; Ebersole et al. 2013; Rathnayake et al. 2013; Miricescu et al. 2014), in order to understand the complicated pathogenesis of periodontitis and its progression, in addition to the inter-individual variations, smoking, and systemic diseases which may confound the identification of the well-known biomarkers, there is always a need for new salivary biomarkers that can predict the onset of the disease, reflect its severity, follow the disease course and response to treatment. On this basis, the present study aimed to identify and characterize novel salivary biomarkers for periodontal diseases.

Data analysis of the PPA assays, which were used for the first time on saliva samples by the present research, revealed that there was a difference in the proteome profile of the whole unstimulated saliva obtained from patients with untreated chronic periodontitis as compared to healthy subjects. This difference in the salivary proteome profile might be attributed to the effects of periodontitis on the salivary proteins, a finding that was in agreement with a number of saliva and GCF proteomic studies in periodontal diseases (Kojima et al. 2000; Goncalves Lda et al. 2010; Goncalves Lda et al. 2011; Range et al. 2012; Salazar et al. 2013). Among the proteins identified by the PPA assays, were the two PA system proteins (uPA and uPAR), and the carrier protein VDBP. The 3 proteins were selected as candidate salivary biomarkers for periodontitis.

The present study investigated for the first time the relationships of the identified biomarkers with periodontitis. Results revealed that the 3 biomarkers were elevated in saliva of the periodontitis patients as compared to the healthy controls, and these elevated levels were positively correlated with the periodontal disease indices used for the clinical assessment of periodontitis. Therefore, the 3 salivary proteins were suggested as good biomarkers for the diagnosis of periodontitis and indicating the disease severity. Investigating the effects of non-surgical periodontal treatment on the biomarkers showed that only the uPA levels were significantly reduced following treatment. Thus, the results suggested that salivary uPA was the only useful biomarker to follow the clinical course of periodontitis in response to treatment as compared to uPAR and VDBP. Moreover, the present study was the first to investigate the enzymatic activity of salivary uPA in relation to periodontitis, results revealed that the uPA activity was significantly elevated in saliva of the periodontitis patients in comparison to the healthy controls and this activity was positively correlated with both the salivary uPA levels and the clinical measures of periodontitis. These findings suggested that uPA is not only elevated in saliva but it has a high activity during periodontitis.

For further confirmation of the results, the present study investigated the hypothesis that during periodontitis and in the presence of an inflammatory or bacterial stimulus, the hGFs will produce uPA and uPAR. The in vitro experimental investigations revealed that, IL-1β stimulated the hGFs to produce significantly elevated levels of uPAR as compared to the unstimulated cells. The *P. gingivalis* LPS stimulated the cells to produce significantly higher levels of both uPA and uPAR in comparison to the unstimulated cells.

As previously mentioned, there are well-known or robust salivary biomarkers for periodontitis such as IL-1β and MMP-8. Several cross-sectional studies demonstrated a thorough positive association between the high levels of salivary IL-1β and periodontitis, indicating that salivary IL-1β is a good biomarker for distinguishing periodontitis patients from healthy subjects (Miller et al. 2006; Ng et al. 2007; Scannapieco et al. 2007; Tobon-Arroyave et al. 2008; Fine et al. 2009; Gursoy et al. 2009; Mirrielees et al. 2010; Gursoy et al. 2011; Kaushik et al. 2011; Yoon et al. 2012; Ebersole et al. 2013; Rathnayake et al. 2013; Salminen et al. 2014). However, some studies were not able to find the same positive association between salivary IL-

1β and periodontitis (Christodoulides et al. 2007; Ramseier et al. 2009; Teles et al. 2009). A number of studies reported that systemic diseases or their treatment may confound the identification of robust salivary biomarkers, for instance Mirrielees et al. (2010) investigated the effects of rheumatoid arthritis on the levels of selected salivary biomarkers for periodontal disease, Mirrielees et al. (2010) found that periodontitis patients with rheumatoid arthritis treated with the anti-TNF-α antibody-based disease modifying therapy had significantly lower levels of salivary IL-1β and TNF-α as compared to systemically healthy periodontitis patients. Numerous cross-sectional studies demonstrated significantly positive association of the high salivary MMP-8 levels and/or activity with periodontitis (Iijima et al. 1983; Gangbar et al. 1990; Uitto et al. 1990; Hayakawa et al. 1994; Makela et al. 1994; Ingman et al. 1996; Matsuki et al. 1996; Miller et al. 2006; Herr et al. 2007a; Rai et al. 2008; Ramseier et al. 2009; Costa et al. 2010; Gursoy et al. 2010; Mirrielees et al. 2010; Gursoy et al. 2011; Ebersole et al. 2013; Rathnayake et al. 2013; Miricescu et al. 2014). On the other hand, in spite of its substantial value as a biomarker for the diagnosis of periodontitis, the use of salivary MMP-8 may face some limitations. Smoking, which is one of the major risk factors for periodontitis, is considered as one of the important limitations for the use salivary MMP-8 as a biomarker (Genco and Borgnakke 2013; Sorsa et al. 2016). Lower levels of MMP-8 have been measured in saliva of smokers as compared to former smokers or non-smokers (Ding et al. 1994; Liede et al. 1999; Heikkinen et al. 2010). Therefore, the low levels of MMP-8 in saliva of smokers may confound its use as a diagnostic biomarker for periodontitis in such patients (Sorsa et al. 2016).

In a similar manner to IL-1β and MMP-8, the cross sectional investigations of the present study revealed that the 3 candidate biomarkers were significantly higher in saliva of the periodontitis patients as compared to the healthy controls, positively associated with the clinical measures of periodontitis, able to diagnose periodontitis, indicate the disease severity, and distinguish periodontitis patients from healthy subjects. In comparison among the 3 candidate biomarkers, the receiver operating characteristic curve analysis (ROC) (see Chapter 2, section 2.4) was used to investigate which salivary protein among the 3 candidates was the best as a biomarker to differentiate between periodontitis and health status. The ROC curve, analysed the biomarkers by the means of their sensitivity (true positive rate) and 1-

specificity (false positive rate) as measures of accuracy for the biomarker ability to diagnose the disease. Results were expressed as the area under the curve (AUC) values ranging between 0-1, values of 0.5 means that the biomarker is half true positive-half false positive, values below 0.5 means that the biomarker is more toward the false positive rate indicating that it is not truly able to differentiate between health and disease status, whereas values above 0.5 means that the biomarker is more toward the true positive rate indicating its ability to differentiate between disease and health status (Fawcett 2006; Hajian-Tilaki 2013). The statistical analysis revealed that the AUC values for salivary uPA, uPAR and VDBP were: 0.95, 0.75, and 0.81 respectively. Salivary uPA possessed the highest AUC value, indicating that it was the best among the three biomarkers to discriminate periodontitis patients from healthy subjects (Figure 7.1). Thus, the present study cross-sectional results and the ROC curve analysis, along with the high salivary uPA activity measured in saliva of the periodontitis patients, all suggested salivary uPA as a potentially better biomarker for periodontitis when compared to both salivary uPAR and VDBP.

One of the cross-sectional studies carried out on IL-1β and MMP-8 was the Ebersole et al. (2013) study, which investigated the salivary levels of IL-1β, IL-6, MMP-8, IFN-α, and albumin in 50 periodontitis patients in comparison to 30 healthy adults. Results revealed that levels of IL-1β, IL-6, MMP-8, and albumin were significantly elevated in saliva of the patients as compared to the healthy controls, while the levels of IFN-α were consistently lower in saliva of the patients than in the healthy controls (Ebersole et al. 2013). Moreover, Ebersole et al. (2013) performed the ROC curve analysis on the biomarkers and found that salivary IL-1β, IL-6 and MMP-8 yielded high AUC values (0.963-0.984) for discriminating periodontitis from health. This finding might be in agreement with the present study ROC curve analysis which demonstrated that the 3 candidate biomarkers were also able to discriminate periodontitis from health, and salivary uPA had the highest AUC value. Another cross-sectional study was the Ramseier et al. (2009) study. Ramseier et al. (2009), used a combination of biomarkers including MMP-8, MMP-9, OPG and calprotectin along with quantifying the periodontal bacteria *P. gingivalis* to differentiate among 4 groups comprising: 18 healthy subjects, 32 gingivitis patients, 28 mild periodontitis patients, and 21 moderate/severe periodontitis patients. Results revealed that the biomarkers and bacteria were higher in the patients as compared to the healthy

subjects (higher in the periodontitis patients), and the biomarkers were able to differentiate between the patients groups with their different periodontal disease categories (Ramseier et al. 2009). These findings of Ramseier et al. (2009), were in harmony with present study which also found that the 3 candidate biomarkers were able to distinguish between periodontitis patients and healthy controls. Furthermore, the present study was in agreement with Ramseier et al. (2009) study in regard to the potential ability of salivary uPA levels and activity (as well as GCF uPA levels) in distinguishing between patients with different periodontal disease categories (gingivitis, chronic periodontitis, mild/moderate periodontitis, and advanced periodontitis). On the other hand, both the Ramseier et al. (2009) and Ebersole et al. (2013) studies adopted the concept of “biomarker signatures” which implies using multiple biomarkers rather than depending on one biomarker only for more efficient diagnosis of periodontitis and more substantial association with periodontitis. This concept was in agreement with the present study which aimed to identify novel salivary biomarkers for periodontitis in an attempt to add candidate biomarkers to the field of salivary diagnostics especially when the identification of some of the well-known salivary biomarkers might be compromised in the presence of confounding factors such as smoking, systemic diseases and inter-individual variations, or even the methods or techniques by which the biomarkers to be identified and quantified (Mirrielees et al. 2010; Taylor 2014; Jaedicke et al. 2016; Sorsa et al. 2016).

As compared to cross-sectional studies, data derived from longitudinal investigations of salivary biomarkers might be limited or variable, this may be explained by the fact that some longitudinal studies were confined to investigating the changes in the GCF levels of some biomarkers (such as MMP-8) and GCF samples are known to be not easily collected and time consuming, or there were difficulties to record or archive some studies, and other studies only followed the natural course of the disease without investigating the effects of treatment (Taylor 2014). Hence, rather robust longitudinal clinical studies to investigate the changes in salivary biomarkers in response to treatment are still required (Buduneli and Kinane 2011). A 4-year cohort longitudinal study followed up the progression of periodontitis by measuring the pocket depths of 219 patients who were not offered any dental treatment and measured the levels of 9 salivary biomarkers (Kibayashi et al. 2007). Kibayashi et al. (2007), found that the disease progression was related to smoking but not to the

salivary levels of any of the 9 biomarkers (IL-1β, MMP-8, MMP-9, lactoferrin, IgA, albumin, AST, LDH, and ALP). A longitudinal case control study measured a number of biomarkers (osteonectin, HGF, IL-1β, IFN-γ, TNF-α, IL-4, IL-6, IL-8 and ICTP) in 40 patients with alveolar bone loss in a 5-year follow up period as compared to healthy subjects, the study results demonstrated a positive association between the progression of alveolar bone loss and each of IL-1β and HGF levels in saliva (Scannapieco et al. 2007). Another case control longitudinal study followed the changes in the salivary levels of MIP-1α and IL-1β in 41 subjects in relation to developing localized aggressive periodontitis in a 2-3 year monitoring period (Fine et al. 2009). Fine et al. (2009) found that, though both the MIP-1α and IL-1β levels were elevated in 7 subjects who developed LAgP, salivary IL-1β levels were elevated for 5-fold only as compared to 50-fold increase in MIP-1α salivary levels; however, the results indicated an association between salivary IL-1β and the progression of LAgP (Fine et al. 2009).

Other longitudinal studies investigated the effects of periodontal treatment on the levels of biomarkers in saliva of periodontitis patients, albeit the treatment regimens used were diverse and the data generated were not enough for comparing the effects of a precise periodontal treatment on a particular biomarker (Taylor 2014). One of these studies investigated the MMP-8 levels along with MMP-9 and TIMP-1 levels before and after treatment in 33 periodontitis patients (Gorska and Nedzi-Gora 2006). Gorska and Nedzi-Gora (2006), found that the MMP-8 levels but not MMP-9 were reduced significantly following non-surgical periodontal treatment without adjunctive doxycycline therapy (not if used together), whereas the TIMP-1 levels were increased after non-surgical treatment combined with doxycycline but not if non-surgical treatment was offered alone. Kinney et al. (2011), carried out a longitudinal study on a cohort of 100 patients, they measured a panel of salivary biomarkers during a 6-month monitoring phase without any periodontal treatment, the results revealed that there were no changes in the levels of any of the salivary biomarkers during the monitoring phase as compared to their baseline levels. Treatment was offered for the patients, followed by a disease-recovery phase for 6 months during which the response to treatment was assessed and the biomarkers were measured after treatment. The levels of salivary MMP-8, MMP-9, OPG and IL-1β were significantly reduced after treatment (Kinney et al. 2011). Another longitudinal study carried out

on 68 periodontitis patients in which salivary biomarkers were measured over a period of 28 weeks in 33 patients who received only OHI and 35 patients who received both OHI and non-surgical periodontal treatment (Sexton et al. 2011). The improvement in the periodontal health in the both treated groups was associated with significant reduction in the salivary MMP-8 levels. However, the detailed analysis of the biomarkers revealed that the salivary levels of both IL-1β and MMP-8 were reduced significantly only the group who received both OHI and non-surgical periodontal treatment in comparison to the patients treated with OHI only (Sexton et al. 2011). Furthermore, Sexton et al. (2011) revealed that the salivary biomarkers (MMP-8, OPG, MIP-1α, and IL-1β) were able to discriminate patients who responded to treatment from those who did not. Using the ROC analysis, Sexton et al. (2011) demonstrated that MMP-8 was the best biomarker to assess the response to treatment as compared to the other biomarkers. Though they performed the ROC curve analysis on the biomarkers after treatment, this finding by Sexton et al. (2011) may be in harmony with present study, which also found that salivary uPA was the best among the three candidate biomarkers that distinguished periodontitis patients from healthy controls.

As with the longitudinal studies of salivary IL-1β and MMP-8, longitudinal investigations were also carried out by the present research to study the effects of non-surgical periodontal treatment on the levels of uPA, uPAR and VDBP in saliva of periodontitis patients. The results revealed that among the 3 biomarkers only the uPA levels were significantly reduced in the whole unstimulated and whole stimulated saliva, as well as in the GCF of periodontitis patients. These findings of salivary uPA were in accordance with the previously mentioned longitudinal studies of salivary IL-1β and MMP-8. It was infeasible to perform the ROC curve analysis on the 3 candidate biomarkers in the longitudinal investigations because only the salivary uPA levels were reduced after treatment of the patients as compared to both salivary uPAR and VDBP. Thus, the present study longitudinal investigations results suggested salivary uPA as a potential biomarker that can assess the response of periodontitis patients to treatment and follow the clinical course of the disease.

7.2 Concluding remarks and suggestions

According to the results of the present study, it might be worthy to carry out further cross sectional and longitudinal studies on the candidate salivary biomarker uPA

along with uPAR as this receptor is biologically related to uPA. Such clinical studies might give further information about salivary uPA and its relationship with periodontitis. Moreover, clinical studies along with measuring the levels of uPA and uPAR in saliva of periodontitis patients may confirm the findings of the present study. Furthermore, as uPA and uPAR were both identified and measured in saliva of non-smokers with periodontitis by the present study, it might be worthy to investigate both uPA and uPAR in saliva of smokers with periodontitis to study the effects of smoking on these two biomarkers. Though the in vitro production of uPA and uPAR by stimulated hGFs has been investigated by the present study and a number of previous studies, it might be reasonable to perform further in vitro studies. As suggestions for future work, investigating the in vitro production of uPA and uPAR by hGFs in response to IL-1β and *P. gingivalis* LPS stimulation in both a dose and time-dependent manner, and investigating the presence of uPA and uPAR in periodontal tissue biopsies obtained from patients with untreated periodontal diseases, such cell culture and histopathological laboratory investigations would be interesting and may confirm the results of the clinical studies. Future clinical and laboratory studies should validate salivary uPA as a biomarker for periodontitis and provide substantial data which might open the door for utilizing salivary uPA and uPAR as diagnostic biomarkers in daily clinical practice using chairside devices which could be used domestically by patients as well. Examples of chairside devices are the point-of-care devices (POC) (see Chapter 1, section 1.4.2) which have been developed and used for the identification of a number of biomarkers such as MMP-8 and IL-1β. Such devices enabled the clinicians to confirm the diagnosis of periodontitis in their practice, helped researchers while carrying out surveys for salivary biomarkers in rural areas or areas were highly equipped laboratories might not be available, and helped patients to monitor their disease progression and response to treatment as well (Wong 2006; Christodoulides et al. 2007; Herr et al. 2007b; Herr et al. 2007a; Sorsa et al. 2011; Fuentes et al. 2014). However, as previously mentioned, MMP-8 and IL-β may not be detected in saliva of some patients, therefore, it might be useful to add further biomarkers to the field of POC diagnostics such as salivary uPA following the validation of this biomarker.

7.3 Limitations of the study

There were no serious limitations in the design of the study; however, some points were noted. The challenges of sensitivity and cross-reactivity of the PPA assays may affect the reliability of the data generated. Therefore, as previously mentioned (see Chapter 2, section 2.3.1, and Chapter 3, sections 3.2.1 & 3.2.2) to overcome these challenges and for data reliability, the assays were repeated 6 times for each array. However, the budget of any project should be considered as these arrays are quite expensive and each array kit “currently” is supplied with 4 membranes only, which are enough for 2 study and 2 control samples.

As the GCF samples analysed in the present study were obtained from another project in the periodontal research group, the volumes were limited. This may explain the limited number of GCF samples in comparison to the saliva samples. However, the results of the GCF uPA investigations did yield useful data. Moreover, uPA and other PA system proteins have been investigated in GCF of periodontal diseases patients by previous studies. Therefore, the GCF samples number was not a serious issue for the present study, and the GCF uPA results were further support for the novel salivary uPA results.

7.4 Summary of findings

In conclusion, the study results highlighted the following:

The proteome profiler arrays can be used to investigate the salivary protein profile and to identify candidate salivary biomarkers, an approach that may contribute positively to the diagnosis of periodontal diseases.

Salivary uPAR is a candidate biomarker for the diagnosis of periodontitis, and indication of the disease severity. However, further longitudinal investigations are suggested to investigate the salivary uPAR ability to follow the clinical course of periodontitis in response to treatment.

Salivary uPA is a candidate biomarker for the diagnosis of periodontitis, has the potential to indicate the disease severity and progression, discriminate periodontitis from health, and follow its clinical course in response to treatment.

Salivary VDBP is a candidate biomarker for the diagnosis of periodontitis and indication of the disease severity. As this protein presents in high levels following inflammatory diseases, it might not be a useful biomarker to follow the clinical course of periodontitis in response to treatment; however, this could be investigated in further longitudinal studies.

Both uPA and uPAR can be locally produced by the human gingival fibroblasts in response to an inflammatory and periodontal bacterial stimuli, indicating that these two proteins might play roles in the pathogenesis of periodontitis (Figure 7.2). Thus, uPA and uPAR are qualified as plausible biomarkers for periodontal diseases.

Finally, the findings of the present study may contribute positively to the field of salivary diagnostics of periodontal diseases.

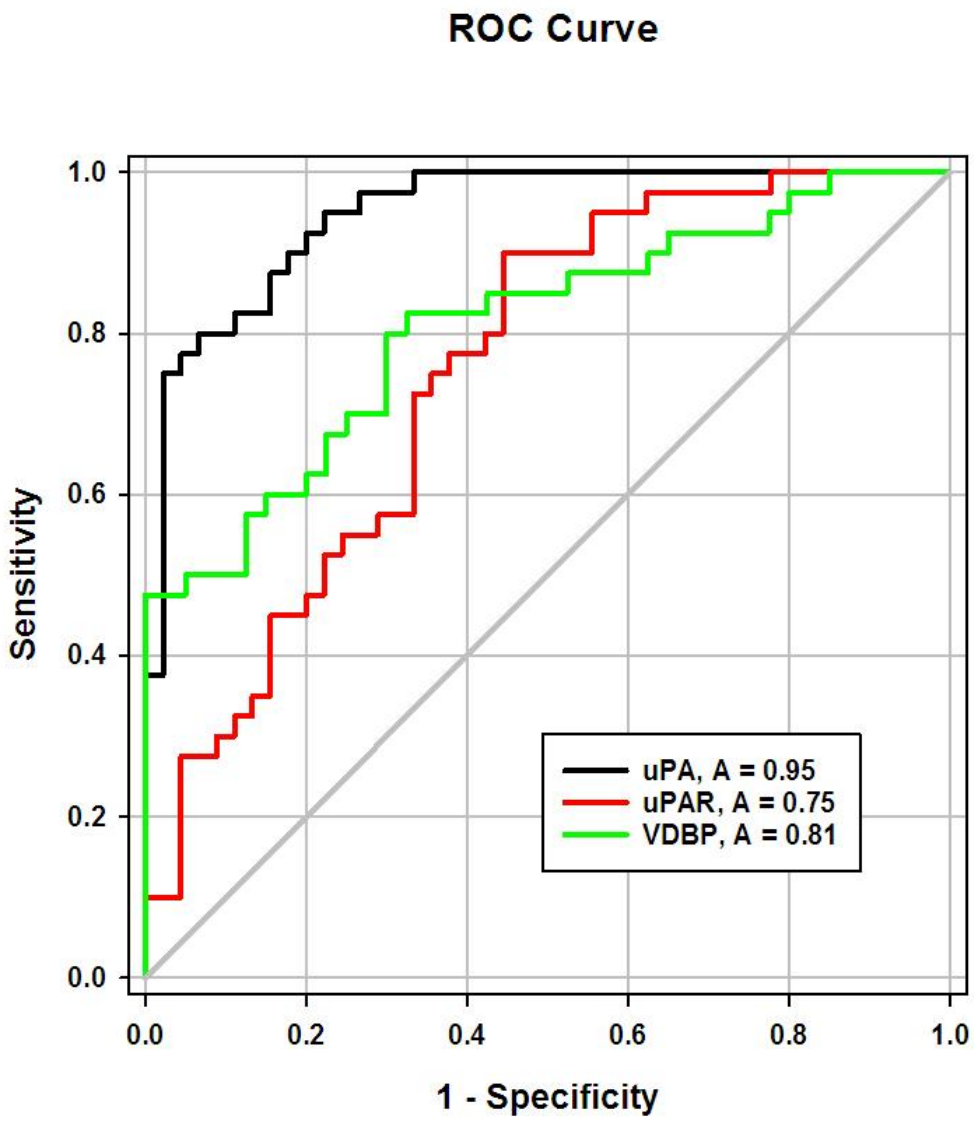

Figure 7.1: The ROC curve analysis of the candidate salivary biomarkers.

The ROC curve analysis was carried out on the salivary uPA, uPAR and VDBP levels in 45 pre-treatment periodontitis patients and 40 healthy control subjects. Salivary uPA was the best among the three candidate biomarkers to discriminate periodontitis from healthy status.

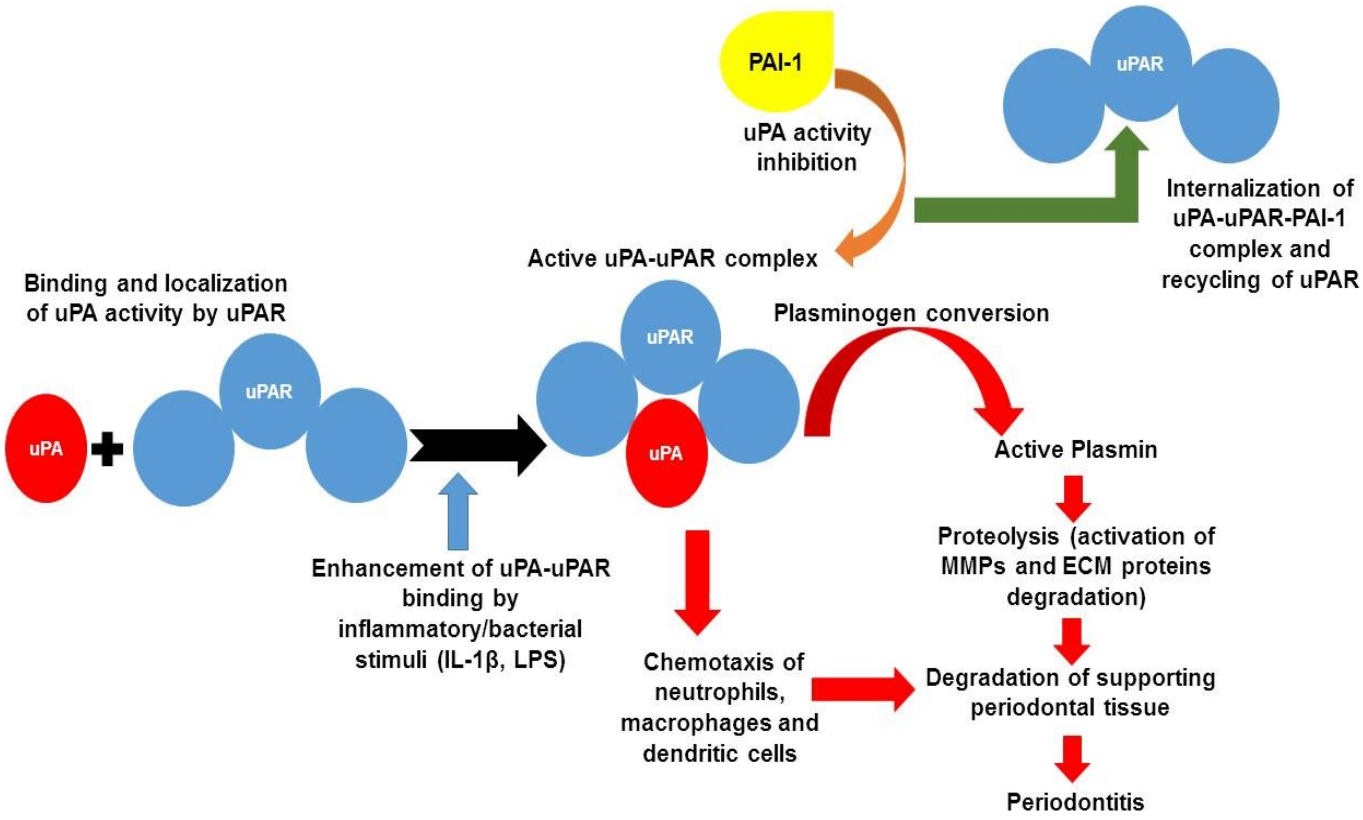

Figure 7.2: Potential roles of uPA and uPAR in the initiation of tissue destruction in periodontitis.

Schematic diagram demonstrating the potential roles of uPA and uPAR in the pathogenesis of periodontal diseases. During inflammation, uPA binds to uPAR, a binding that will activate and localize uPA leading to the conversion of plasminogen into active plasmin, thereby initiating the process of proteolysis. In the presence of inflammatory stimuli (such as IL-1β from infiltrating neutrophils and local periodontal tissues) or bacterial stimuli (such as LPS from *P. gingivalis*), the uPA/uPAR binding might be enhanced leading to more production of plasmin stimulating the proteolysis of supporting periodontal tissues associated with periodontitis. In addition to proteolysis, active uPA/uPAR complex exert chemotactic activity on inflammatory cells such as dendritic cells, macrophages and neutrophils which in turn release cytokines, as well as, proteolytic and lysosomal enzymes that also degrade the supporting periodontal tissues “collateral damage”. Hence the elevated levels of uPA/uPAR in saliva, GCF and local periodontal tissues, may indicate the extent of periodontal tissue destruction during periodontitis. After proteolysis, the uPA inhibitor PAI-1 inhibits the uPA activity by binding to the active uPA-uPAR complex, in the presence of low density lipoprotein receptor family (LDL proteins), uPAR mediates the internalization of the uPA-uPAR-PAI-1 complex, ending with uPA degradation by PAI-1 and releasing of uPAR to be available for further binding.

Appendices

Salivary proteases	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
ADAM9	1661 ±720	2416 ±508	+ 1.45	NS
ADAMTS1	1302 ±766	1597 ±298	+ 1.23	NS
ADAMTS13	2453 ±1709	1811 ±456	- 1.35	NS
Cathepsin A	24698 ±6670	22183 ±5984	- 1.11	NS
Cathepsin B	25108 ±8206	21943 ±7159	- 1.14	NS
Cathepsin C	9160 ±5511	5789 ±2627	- 1.58	NS
Cathepsin D	26306 ±9832	20405 ±5998	- 1.29	NS
Cathepsin E	1625 ±838	2126 ±602	+ 1.31	NS
Cathepsin L	1844 ±1298	1947 ±880	+ 1.06	NS
Cathepsin S	22957 ±7030	21755 ±4889	- 1.06	NS
Cathepsin V	20114 ±8573	11813 ±3239	- 1.7	NS
Cathepsin X/Z/P	22371 ±10560	14969 ±5793	- 1.49	NS
DPPIV/CD26	30794 ±9329	27497 ±7991	- 1.12	NS

Salivary proteases	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Kallikrein 3/PSA	3439 ±2655	1828 ±240	- 1.88	NS
Kallikrein 5	33989 ±10178	21312 ±8702	- 1.59	NS
Kallikrein 6	18294 ±5639	18771 ±6535	+ 1.03	NS
Kallikrein 7	8674 ±2382	6349 ±2092	- 1.37	NS
Kallikrein 10	33000 ±10051	26081 ±6185	- 1.27	NS
Kallikrein 11	29341 ±10132	29875 ±7959	+ 1.02	NS
Kallikrein 13	15622 ±5376	16185 ±6983	+ 1.04	NS
MMP-13	1964 ±1911	1494 ±545	- 1.31	NS
Presenilin-1	353 ±460	833 ±320	+ 2.36	NS
Proprotein Convertase 9	190 ±229	878 ±352	+ 4.61	NS

Appendix A. Table 1: Relative expression of salivary proteases in periodontitis samples as compared to healthy samples, single sample PPA assays.

The relative expression of 23 salivary proteases identified in 3 single sample protease PPA assays. Data are presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant).

Salivary proteases	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
ADAM9	665 ±112	5290 ±1025	+ 7.96	<0.05
ADAMTS1	697 ±79	2490 ±800	+ 3.57	NS
ADAMTS13	1266 ±136	6316 ±2461	+ 4.99	NS
Cathepsin A	12367 ±1055	17803 ±1612	+ 1.44	<0.01
Cathepsin B	14168 ±1301	16963 ±833	+ 1.2	<0.05
Cathepsin C	7220 ±555	10806 ±1683	+ 1.5	NS
Cathepsin D	14125 ±945	16159 ±2035	+ 1.14	NS
Cathepsin E	1095 ±198	8242 ±3267	+ 7.52	NS
Cathepsin L	682 ±81	2836 ±926	+ 4.15	NS
Cathepsin S	10739 ±702	16401 ±1733	+ 1.53	<0.05
Cathepsin V	10031 ±846	15130 ±1822	+ 1.51	<0.05
Cathepsin X/Z/P	14707 ±954	17998 ±1791	+ 1.22	NS
DPPIV/CD26	16206 ±1733	19476 ±996	+ 1.2	NS

Salivary proteases	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Kallikrein 3/PSA	871 ±120	2792 ±906	+ 3.2	<0.05
Kallikrein 5	8795 ±1221	14241 ±3648	+ 1.62	NS
Kallikrein 6	8441 ±204	13083 ±778	+ 1.55	<0.01
Kallikrein 7	3935 ±782	9271 ±1892	+ 2.36	<0.01
Kallikrein 10	14103 ±1879	19591 ±2645	+ 1.39	NS
Kallikrein 11	15435 ±612	18274 ±1415	+ 1.18	<0.05
Kallikrein 13	11330 ±328	15104 ±1182	+ 1.33	<0.05
MMP-13	662 ±159	2675 ±923	+ 4.04	NS
Presenilin-1	133 ±44	2550 ±1121	+ 19.12	<0.05
Proprotein Convertase 9	48 ±26	1724 ±685	+ 35.87	NS

Appendix A. Table 2: Relative expression of salivary proteases in periodontitis samples as compared to healthy samples, pooled sample PPA assays.

The relative expression of 23 salivary proteases identified in 3 pooled sample protease PPA assays. Data are presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant).

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Aggrecan	545 ±473	2533 ±1731	+ 4.64	NS
Angiogenin	6625 ±4482	7685 ±5484	+ 1.16	NS
Angiopoietin-1	ND	1377 ±1493	NA	NA
Angiopoietin-2	ND	ND	NA	NA
B-cell activating factor (BAFF)	ND	6913 ±6831	NA	NA
Brain-derived neurotrophic factor (BDNF)	ND	242 ±429	NA	NA
Complement component C5/C5a	ND	5678 ±4233	NA	NA
Cluster of differentiation 14 (CD14)	3556 ±2251	5380 ±4019	+ 1.51	NS
CD30 (TNFRSF8)	ND	1183 ±1053	NA	NA
CD40 ligand (CD40L)	ND	1696 ±1516	NA	NA
Chitinase 3-like 1	21860 ±11342	24126 ±9258	+ 1.1	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Complement Factor D	746 ±424	6058 ±4520	+ 8.12	<0.05
C-reactive protein	ND	1190 ±1354	NA	NA
Teratocarcinoma-derived Growth Factor (Cripto-1)	ND	ND	NA	NA
Dickkopf-1 (Dkk-1)	ND	402 ±548	NA	NA
Dipeptidyl-peptidase IV	495 ±25	8124 ±6965	+ 16.38	NS
ENA-78 (CXCL5)	10149 ±7193	4186 ±4217	- 2.42	NS
Endoglin	ND	783 ±773	NA	NA
Fas Ligand	ND	128 ±266	NA	NA
Basic fibroblast growth factor (FGF basic)	ND	ND	NA	NA
Fibroblast growth factor 7 (FGF-7)	ND	ND	NA	NA
Fibroblast growth factor 19 (FGF-19)	ND	417 ±392	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
FMS-like tyrosine kinase 3 ligand (Flt-3 Ligand)	ND	ND	NA	NA
Granulocyte-colony stimulating factor (G-CSF)	ND	125 ±288	NA	NA
Growth/differentiation factor 15 (GDF-15)	ND	2575 ±2609	NA	NA
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	ND	553 ±638	NA	NA
GRO-α (CXCL1)	1911 ±2155	9244 ±9297	+ 4.84	NS
Growth hormone	ND	125 ±246	NA	NA
Hepatocyte growth factor (HGF)	ND	184 ±329	NA	NA
Intercellular Adhesion Molecule 1 (ICAM-1)	ND	425 ±591	NA	NA
Interferon gamma (IFN-γ)	ND	ND	NA	NA
Insulin like growth factor binding protein 2 (IGFBP-2)	ND	3727 ±3353	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Insulin like growth factor binding protein 3 (IGFBP-3)	ND	ND	NA	NA
IL-1α	ND	5860 ±4251	NA	NA
IL-1β	ND	2471 ±2610	NA	NA
IL-1ra	11374 ±6613	12662 ±7520	+ 1.11	NS
IL-2	ND	349 ±361	NA	NA
IL-3	ND	426 ±413	NA	NA
IL-4	ND	998 ±1046	NA	NA
IL-5	ND	756 ±880	NA	NA
IL-6	ND	897 ±818	NA	NA
IL-10	ND	4188 ±4273	NA	NA
IL-11	ND	292 ±355	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
IL-12p70 (IL-12)	ND	29 ±171	NA	NA
IL-13	ND	72 ±212	NA	NA
IL-15	ND	76 ±281	NA	NA
IL-16	ND	1882 ±1781	NA	NA
IL-17A (IL-17)	212 ±59	1699 ±1188	+ 8	NS
IL-18 BPa	ND	774 ±660	NA	NA
IL-19	ND	4149 ±4121	NA	NA
IL-22	ND	485 ±471	NA	NA
IL-23	6 ±76	1286 ±796	+ 200.45	NS
IL-24	ND	367 ±411	NA	NA
IL-27	ND	64 ±207	NA	NA
IL-31	ND	ND	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
IL-32 α/β/γ	ND	432 ±542	NA	NA
IL-33	ND	41 ±217	NA	NA
IL-34	ND	ND	NA	NA
IP-10 (CXCL10)	ND	763 ±856	NA	NA
ITAC (CXCL11)	ND	350 ±334	NA	NA
Kallikrein 3	562 ±69	1938 ±1445	+ 3.44	NS
Leptin	ND	340 ±429	NA	NA
Leukemia inhibitory factor (LIF)	ND	356 ±315	NA	NA
Lipocalin-2	17346 ±7613	16915 ±6309	- 1.03	NS
Monocyte chemoattractant protein 1 (MCP-1)	4022 ±4163	1726 ±1557	- 2.33	NS
Monocyte chemoattractant protein 3 (MCP-3)	ND	ND	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Macrophage colony-stimulating factor (M-CSF)	ND	172 ±264	NA	NA
Migration inhibitory factor (MIF)	4924 ±3758	8948 ±5968	+ 1.82	NS
MIG (CXCL9)	ND	426 ±555	NA	NA
Macrophage inflammatory proteins (MIP-1α/MIP-1β)	ND	53 ±211	NA	NA
Macrophage inflammatory protein (MIP-3α)	ND	1424 ±1538	NA	NA
Macrophage inflammatory protein (MIP-3β)	ND	653 ±573	NA	NA
MMP-9 (Gelatinase B)	14035 ±8909	17003 ±10033	+ 1.21	NS
Osteopontin	ND	528 ±466	NA	NA
Platelet-derived growth factor (PDGF-AA)	ND	7162 ±6981	NA	NA
Platelet-derived growth factor (PDGF-AB/BB)	ND	89 ±209	NA	NA
Pentraxin-3	ND	5427 ±5184	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Platelet factor 4 (PF4) (CXCL4)	1161 ±2619	73 ±232	- 15.86	NS
Receptor for advanced glycation endproducts (RAGE)	ND	ND	NA	NA
RANTES (CCL5)	ND	189 ±299	NA	NA
Retinol binding protein 4 (RBP4)	1389 ±1571	10268 ±538	+ 7.39	<0.05
Relaxin-2	ND	662 ±614	NA	NA
Stromal cell-derived factor 1 (SDF-1α) (CXCL12)	ND	1284 ±1018	NA	NA
Serpin E1	119 ±190	6452 ±5839	+ 53.88	NS
Sex hormone-binding globulin (SHBG)	ND	1691 ±1681	NA	NA
ST2 (IL-1 R4, IL1RL1)	ND	811 ±722	NA	NA
TARC (CCL17)	ND	308 ±421	NA	NA
Trefoil factor 3 (TFF3)	16177 ±7436	18636 ±9577	+ 1.15	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Transferrin receptor (TfR)	ND	346 ±394	NA	NA
Transforming growth factor alpha (TGF-α)	ND	ND	NA	NA
Thrombospondin-1	ND	1028 ±1245	NA	NA
Tumour necrosis factor-alpha (TNFα)	ND	1206 ±160	NA	NA

Appendix A. Table 3: Relative expression of salivary cytokines in periodontitis samples as compared to healthy samples, single sample PPA assays.

The relative expression of 92 salivary cytokines identified in 3 single sample cytokine PPA assays. Data are presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant). ND not detected or negatively expressed. NA not applicable.

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Aggrecan	14145 ±13634	11925 ±10291	- 1.19	NS
Angiogenin	17423 ±13663	23042 ±16514	+ 1.32	NS
Angiopoietin-1	13757 ±13663	12749 ±11092	- 1.08	NS
Angiopoietin-2	11193 ±11237	12585 ±12205	+ 1.12	NS
B-cell activating factor (BAFF)	11982 ±11863	20827 ±19790	+ 1.74	NS
Brain-derived neurotrophic factor (BDNF)	10757 ±10841	13863 ±13572	+ 1.29	NS
Complement component C5/C5a	12377 ±12229	27546 ±22117	+ 2.23	NS
Cluster of differentiation 14 (CD14)	14737 ±13563	19898 ±16030	+ 1.35	NS
CD30 (TNFRSF8)	14751 ±14725	14213 ±13673	- 1.04	NS
CD40 ligand (CD40L)	12310 ±12013	13709 ±12604	+ 1.11	NS
Chitinase 3-like 1	16555 ±9198	72061 ±58216	+ 4.35	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Complement Factor D	25096 ±23622	17563 ±11876	- 1.43	NS
C-reactive protein	9570 ±9587	15962 ±13429	+ 1.67	NS
Teratocarcinoma-derived Growth Factor (Cripto-1)	9125 ±9214	11005 ±10733	+ 1.21	NS
Dickkopf-1 (Dkk-1)	10317 ±10367	12072 ±11615	+ 1.17	NS
Dipeptidyl-peptidase IV	12947 ±10982	34267 ±28260	+ 2.65	NS
ENA-78 (CXCL5)	10453 ±8283	30579 ±27756	+ 2.93	NS
Endoglin	43741 ±43580	9279 ±8420	- 4.71	NS
Fas Ligand	10703 ±10721	6361 ±5854	- 1.68	NS
Basic fibroblast growth factor (FGF basic)	7655 ±7721	8157 ±7686	+ 1.07	NS
Fibroblast growth factor 7 (FGF-7)	7623 ±7754	7922 ±7689	+ 1.04	NS
Fibroblast growth factor 19 (FGF-19)	19660 ±19623	9877 ±9143	- 1.99	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
FMS-like tyrosine kinase 3 ligand (Flt-3 Ligand)	9167 ±9297	8463 ±8251	- 1.08	NS
Granulocyte-colony stimulating factor (G-CSF)	17248 ±17379	10889 ±10582	- 1.58	NS
Growth/differentiation factor 15 (GDF-15)	17599 ±17691	11040 ±10105	- 1.59	NS
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	10764 ±10794	9692 ±9059	- 1.11	NS
GRO-α (CXCL1)	8106 ±8033	13162 ±11879	+ 1.62	NS
Growth hormone	22306 ±22249	5717 ±5253	- 3.9	NS
Hepatocyte growth factor (HGF)	7372 ±7380	5265 ±4898	- 1.4	NS
Intercellular Adhesion Molecule 1 (ICAM-1)	5929 ±5927	5770 ±5230	- 1.03	NS
Interferon gamma (IFN-γ)	5768 ±5874	6078 ±5813	+ 1.05	NS
Insulin like growth factor binding protein 2 (IGFBP-2)	6832 ±6677	12148 ±9952	+ 1.78	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Insulin like growth factor binding protein 3 (IGFBP-3)	7352 ±7472	7453 ±7306	+ 1.01	NS
IL-1ra	8305 ±6311	18741 ±13916	+ 2.26	<0.05
IL-2	5434 ±5584	7799 ±7413	+ 1.44	NS
IL-3	10503 ±10595	9479 ±9273	- 1.11	NS
IL-4	7398 ±7325	5283 ±4747	- 1.4	NS
IL-5	4991 ±4968	4243 ±3831	- 1.18	NS
IL-6	5019 ±4915	4573 ±3836	- 1.1	NS
IL-10	4391 ±4505	4647 ±4463	+ 1.06	NS
IL-11	3914 ±4048	6121 ±5691	+ 1.56	NS
IL-12p70 (IL-12)	3781 ±3943	6417 ±6221	+ 1.7	NS
IL-13	4730 ±4817	7526 ±7314	+ 1.6	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
IL-15	5259 ±5394	4368 ±4182	- 1.2	NS
IL-16	10609 ±10611	5600 ±5074	- 1.9	NS
IL-17A (IL-17)	5525 ±5374	9002 ±7334	+ 1.63	NS
IL-18 BPa	8498 ±8593	7860 ±7539	- 1.08	NS
IL-19	4777 ±4508	4721 ±3333	- 1.01	NS
IL-22	3432 ±3324	3960 ±3337	+ 1.15	NS
IL-23	2327 ±2124	5202 ±4417	+ 2.23	NS
IL-24	3625 ±3599	5189 ±4577	+ 1.43	NS
IL-27	2754 ±2869	1508 ±1359	- 1.83	NS
IL-31	2684 ±2786	4249 ±4141	+ 1.58	NS
IL-32 α/β/γ	3049 ±3120	6380 ±5935	+ 2.09	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
IL-33	3722 ±3862	3311 ±3149	- 1.12	NS
IL-34	3871 ±4008	4887 ±4729	+ 1.26	NS
IP-10 (CXCL10)	1944 ±2037	3985 ±3692	+ 2.05	NS
ITAC (CXCL11)	3934 ±4019	5131 ±4885	+ 1.3	NS
Kallikrein 3	6178 ±6045	10165 ±8731	+ 1.65	NS
Leptin	2012 ±1904	437 ±295	- 4.6	NS
Leukemia inhibitory factor (LIF)	199 ±182	748 ±359	+ 3.75	NS
Lipocalin-2	5780 ±2876	50423 ±41098	+ 8.72	NS
Monocyte chemoattractant protein 1 (MCP-1)	372 ±303	5494 ±3223	+ 14.74	NS
Monocyte chemoattractant protein 3 (MCP-3)	566 ±703	609 ±527	+ 1.08	NS
Macrophage colony-stimulating factor (M-CSF)	474 ±613	2311 ±2099	+ 4.87	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Migration inhibitory factor (MIF)	156 ±1190	22224 ±18528	+ 142.46	NS
MIG (CXCL9)	2016 ±2115	2572 ±2270	+ 1.28	NS
Macrophage inflammatory proteins (MIP-1α/MIP-1β)	3464 ±3583	2459 ±2137	- 1.41	NS
Macrophage inflammatory protein (MIP-3α)	3455 ±3539	3159 ±2961	- 1.09	NS
Macrophage inflammatory protein (MIP-3β)	2179 ±2268	4136 ±3714	+ 1.9	NS
MMP-9 (Gelatinase B)	4887 ±2606	49397 ±43481	+ 10.11	NS
Myeloperoxidase	ND	14650 ±12373	NA	NA
Osteopontin	ND	ND	NA	NA
Platelet-derived growth factor (PDGF-AA)	16636 ±16385	10125 ±7671	- 1.64	NS
Platelet-derived growth factor (PDGF-AB/BB)	460 ±555	ND	NA	NA
Pentraxin-3	ND	4710 ±2997	NA	NA

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Platelet factor 4 (PF4) (CXCL4)	ND	1204 ±1028	NA	NA
Receptor for advanced glycation endproducts (RAGE)	2041 ±2187	64 ±151	- 31.81	NS
RANTES (CCL5)	3414 ±3512	503 ±304	- 6.78	NS
Retinol binding protein 4 (RBP4)	5986 ±2985	28659 ±15122	+ 4.79	NS
Relaxin-2	2166 ±2227	1937 ±1229	- 1.12	NS
Stromal cell-derived factor 1 (SDF-1α) (CXCL12)	22635 ±22603	3892 ±3035	- 5.82	NS
Serpin E1	ND	1192 ±889	NA	NA
Sex hormone-binding globulin (SHBG)	ND	ND	NA	NA
ST2 (IL-1 R4, IL1RL1)	ND	672 ±398	NA	NA
TARC (CCL17)	605 ±3065	ND	NA	NA
Trefoil factor 3 (TFF3)	2904 ±3349	36414 ±28307	+ 12.54	NS

Salivary cytokines	Healthy samples PI mean values ±SEM (n=3)	Periodontitis samples PI mean values ±SEM (n=3)	Fold change (periodontitis vs health)	p-value of the log-fold change
Transferrin receptor (TfR)	ND	ND	NA	NA
Transforming growth factor alpha (TGF-α)	1698 ±1915	ND	NA	NA
Thrombospondin-1	1907 ±1954	ND	NA	NA
Tumour necrosis factor-alpha (TNFα)	6589 ±6560	3430 ±654	- 1.92	NS

Appendix A. Table 4: Relative expression of salivary cytokines in periodontitis samples as compared to healthy samples, pooled sample PPA assays.

The relative expression of 91 salivary cytokines identified in 3 pooled sample cytokine PPA assays. Data are presented as mean ±SEM of the PI values. Proteins compared by the means of fold change and p-value of the logarithm-fold change (Statistics: One sample t-test, NS= non-significant). ND not detected or negatively expressed. NA not applicable.

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Appendix C: Presentations and scientific meetings

1. Identification and Characterisation of Novel Salivary Biomarkers for Oral Inflammatory Disease. ICM - ICM Postgraduate Students Research Seminar. 18/02/2015.
2. Three minutes presentation of the study in the COHR research afternoon 13/05/2015. Runner up in the postgraduate oral presentation competition.
3. Presentation of the study in the COHR event “It’s OK to ask. Patient and Public Event. Dental Clinical Research Facility, School of Dental Sciences” 20/05/2015.
4. Presentation of the study in the COHR presentation programme 08/07/2015.
5. Identification of novel salivary biomarkers for chronic periodontitis. Abstract in the BSODR Annual Meeting - Cardiff 14-16 September 2015. Ahmed Khudhur, John Taylor, Philip Preshaw.

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