Furthering knowledge of the pathogenesis of Periodontal Disease in the evaluation of biomarkers for monitoring disease susceptibility and activity.

A thesis submitted in fulfilment of the requirements of the degree of

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2015

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Summary

The development of periodontitis is a multifactorial process initiated by a bacterial-induced inflammation which is further modified by genetic and environmental factors contributing towards an exaggerated host response and associated tissue destruction. Pro-inflammatory mediators such as cytokines play an important role in the resulting inflammation leading to degradation of periodontal connective tissue, several of which may be detected in GCF and may be of diagnostic and prognostic value. However, our limited understanding of the molecular mechanisms involved in the pathogenesis of periodontal disease restrains the effectiveness of current diagnostic and management techniques in assessing true periodontal health, identify susceptible patients, monitor response to therapy or implement the use of biomarkers which may assist in the management of patients with periodontal disease.

Therefore, this thesis aims to further our understanding of the pathology of periodontal disease through a series of *in-vitro* and *in-vivo* studies. In recognising that as a consequence of periodontal disease is the degradation of the extracellular matrix, clinical studies investigated the release of proteoglycan components from the periodontal tissue in patients with chronic periodontitis. Increasing levels of chondroitin sulphate (CS), a proteoglycan metabolite was observed with progressive clinical attachment loss, highlighted periods of activity and inactivity, with only a few sites demonstrating disease activity over a 21 month period. Furthermore, *in-vitro* studies investigated the cellular synthesis of proteoglycan in a pathological condition by examining the biological effects of *P. gingivalis* LPS on PDL cells. In the presence of *P. gingivalis* LPS, an alteration in cell behaviour was observed with an increase in cell proliferation and a decrease in matrix formation, further suggesting that the degradation products detected in GCF such as decorin and biglycan, were as a consequence of tissue destruction and not as a result of repair or remodelling. Collectively, these results highlight the potential of CS present in GCF as a marker of disease activity.

Due to the multifactorial nature of the disease, it is highly unlikely that any one marker may provide information that may be of diagnostic as well as prognostic value. Rather, the use of
a panel of markers may further corroborate the implementation of biomarkers in periodontal disease management. Therefore, in considering the prominent role of cytokines in tissue destruction, this thesis further examined the prognostic value of cytokine profiling in identifying the high risk patient. On cross-sectional evaluation of cytokine profiles in GCF from patients with different types of periodontal disease using bead array technology, a variable response was observed in the severe type, which was suggestive of an imbalance in Th1/Th2/Th17/Treg responses, further affirming the predominant role of an altered host response in disease progression. Further, resident PDL cells were also considered as potential contributors to the variation in response. Consequently, in-vitro studies demonstrated that although TLR receptors were present on PDL cells, no cytokines were released on exposure to *P. gingivalis* LPS further suggesting that the cytokines detected in GCF were produced as a consequence of an altered host response which brings about progression of disease.

In conclusion, the development of rapid, non-invasive, site based risk assessment and comprehensive screening for biomarkers may be possible in the near future as a result of the rapid development of new diagnostic technologies such as microarray and microfluidics along with the use of oral fluids such as GCF. Therefore, in the future enhanced patient assessment may be possible which will enable provision of customized therapies that target treatment at individual level.
CONTENTS

Chapter 1: Review of literature

1.1 Introduction ........................................................................................................... 1
1.2 Global periodontal tissue epidemiology ............................................................. 5
1.3 The periodontal tissues in health ....................................................................... 6
  1.3.1 Extracellular matrix of the periodontium ...................................................... 11
    1.3.1.1 Proteoglycans ...................................................................................... 12
    1.3.1.2 Glycoproteins .................................................................................... 24
    1.3.1.3 Proteins associated with bone and cementum ..................................... 25
1.4 Turnover in the periodontium ............................................................................ 26
1.5 Aetiology of periodontal disease ......................................................................... 29
1.6 Pathogenesis of periodontal disease .................................................................. 31
  1.6.1 Role of P. gingivalis in the pathogenesis of periodontal disease ............... 32
    1.6.1.1 Receptors for LPS ............................................................................... 35
      1.6.1.1.1 CD14 receptors ............................................................................. 36
      1.6.1.1.2 TLR receptors .............................................................................. 40
1.7 Histopathological and immunopathological changes leading to periodontal tissue destruction ......................................................................................... 46
  1.7.1 Cytokines of the advanced lesion ................................................................. 46
  1.7.2 Role of T-helper subsets ............................................................................... 49
    1.7.2.1 Pro-inflammatory cytokines of T-cell lineage in host response .......... 51
      1.7.2.1.1 Th1 subset ..................................................................................... 51
      1.7.2.1.2 Th2 subset ..................................................................................... 52
      1.7.2.1.3 Th17 subset ................................................................................... 52
      1.7.2.1.4 Treg ............................................................................................. 53
1.8 Degradation of periodontal tissue ..................................................................... 55
  1.8.1 Connective tissue destruction ....................................................................... 55
  1.8.2 Bone resorption ......................................................................................... 56
1.9 Resolution of inflammation ................................................................................. 58
  1.9.1 Anti-inflammatory cytokines ....................................................................... 59
1.10 Gingival crevicular fluid .................................................................................... 64
1.11 Biomarkers for elucidation of pathology .......................................................... 66
  1.11.1 Bacterial products in GCF .......................................................................... 66
  1.11.2 Inflammatory and immune markers ............................................................ 67
  1.11.3 Hydrolytic and cytosolic enzymes ............................................................... 67
  1.11.4 Markers of connective tissue degradation .................................................. 68
1.12 Aims ..................................................................................................................... 69
Chapter 2: An increase in proteoglycan metabolites in GCF represents periods of disease activity: a longitudinal study in patients with chronic periodontitis.

2.1 Introduction.................................................................................................................. 70

2.2 Materials and Methods.............................................................................................. 75
  2.2.1 Longitudinal monitoring of proteoglycan metabolites in GCF......................... 76
    2.2.1.1 Validation of methodology
      2.2.1.1.1 Inclusion/Exclusion criteria.............................................. 77
      2.2.1.1.2 Sample collection.............................................................. 77
      2.2.1.1.3 Measurements of clinical parameters............................. 78
        2.2.1.1.3.1 Florida Probe................................................................. 78
        2.2.1.1.3.2 Investigator calibration............................................... 79
        2.2.1.1.3.3 Longitudinal assessment of periodontal health........... 79
    2.2.1.1.4 Evaluation of release of sGAG in GCF during collection period........... 79
    2.2.1.1.5 Cellulose Acetate Electrophoresis........................................... 80
    2.2.1.1.6 Assessment of methods for GAG quantification....................... 81
    2.2.1.1.7 Statistical Analysis................................................................... 82
  2.3 Results......................................................................................................................... 84
    2.3.1 Investigator calibration...................................................................................... 84
    2.3.2 Assessment of methods for quantification of GAGs................................. 84
    2.3.3 Longitudinal analysis of proteoglycan metabolites in GCF with disease severity.......................... 85
    2.3.4 Cross-sectional analysis of proteoglycan metabolites in GCF with disease severity.................................................. 99

2.4 Discussion................................................................................................................... 101

Chapter 3: P. gingivalis LPS inhibits the reparative and regenerative processes during periodontal disease by influencing PDL cell behaviour and altering the biological events associated with matrix synthesis.

3.1 Introduction................................................................................................................. 108

3.2 Materials and Methods............................................................................................ 113
  3.2.1 Extraction of LPS from P. gingivalis................................................................. 113
  3.2.2 Characterisation of LPS..................................................................................... 114
    3.2.2.1 SDS- Polyacrylamide Gel Electrophoresis...................................... 115
    3.2.2.2 Staining with Coomassie Brilliant Blue for detection of protein contamination.......................... 116
    3.2.2.3 Staining with Silver for characterisation of LPS............................. 116
    3.2.2.4 Staining with Ethidium Bromide for assessing contamination with nucleic acids.......................... 117
    3.2.2.5 LAL assay....................................................................................... 117
3.2.3 Isolation of PDL cells……………………………………………………………118
3.2.4 Characterization of PDL cells………………………………………………119
3.2.5 Determination of seeding density using MTS assay………………………120
3.2.6 Determination of sub-toxic levels of LPS on cell viability…………………121
3.2.7 Effect of sub-toxic levels of LPS on cell proliferation……………………..122
3.2.8 Effect of sub-toxic levels of LPS on alkaline phosphatase activity………123
3.2.9 Effect of subtoxic levels of LPS on matrix formation………………………...124
  3.2.9.1 RT- PCR……………………………………………………........124
  3.2.9.1.1 RNA extraction……………………………………………124
  3.2.9.1.2 Quantification of RNA……………………………………..126
  3.2.9.1.3 Reverse transcription of total RNA…………………………126
  3.2.9.1.4 Polymerase chain reaction………………………………127
  3.2.9.1.5 Visualisation of PCR products on Agarose gels…129
3.2.9.2 Western blot analysis………………………………………… 129
  3.2.9.2.1 Protein extraction……………………………………….129
  3.2.9.2.2 Protein quantification using BCA assay…..130
  3.2.9.2.3 Protein separation using SDS-PAGE………...130
  3.2.9.2.4 Western Blotting…………………………….131
3.3 Results..........................................................................................134
  3.3.1 Characterisation of P. gingivalis LPS………………………………134
  3.3.2 Isolation and characterization of PDL cells…………………………137
  3.3.3 LAL assay.…………………………………………………………..141
  3.3.4 Evaluation of seeding density by MTS assay……………………….141
  3.3.5 Effect of P. gingivalis LPS on cell viability………………………….144
  3.3.6 Effect of sub-toxic levels of LPS on cell behaviour…………………146
  3.3.7 Effect of sub-toxic levels of LPS on alkaline phosphatase activity……………148
  a) Protein quantification……………………………………..150
  b) mRNA level………………………………………………..150
  c) Protein synthesis………………………………………….151
3.4 Discussion...................................................................................161

Chapter 4:  P. gingivalis LPS upregulates TLR2 and CD14 expression on PDL cells.

4.1 Introduction……………………………………………………………………169
4.2 Materials & Methods........................................................................177
  4.2.1 RT-PCR…………………………………………………………………177
  4.2.2 Immunolocalisation……………………………………………………179
  4.2.3 ELISA…………………………………………………………………..180
  4.2.4 Slot Blot………………………………………………………………..181
4.3 Results.........................................................................................182
  4.3.1 RT PCR………………………………………………………………….182
  4.3.2 Immunolocalisation……………………………………………………186
  4.3.3 ELISA………………………………………………………………...191
  4.3.4 Dot blot………………………………………………………………191
4.4 Discussion..................................................................................192
## Index to Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1</td>
<td>activated protein 1</td>
</tr>
<tr>
<td>aMEM</td>
<td>alpha minimal essential media</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>β-actin</td>
<td>beta actin</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3'-indolyl phosphate</td>
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<tr>
<td>Bis-Tris</td>
<td>bis (2-hydroxyethyl)-amino-tris (hydroxymethyl)-methane</td>
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<tr>
<td>BMMSc</td>
<td>bone marrow-derived mesenchymal stem cells</td>
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<td>BMP-4</td>
<td>bone morphogenic protein</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribo nucleic acid</td>
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<td>CD14</td>
<td>cluster of differentiation 14</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<td>chondroitin sulphate</td>
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<td>DNase</td>
<td>deoxyribonuclease</td>
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<td>ethylenediamine tetra acetic acid</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GCF</td>
<td>gingival crevicular fluid</td>
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<td>GPI</td>
<td>glycosyl-phosphatidylinositol</td>
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<td>IGF-1</td>
<td>insulin like growth factor</td>
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<td>interferon gamma</td>
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<td>Abbreviation</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IL-1RA</td>
<td>interleukin 1 receptor antagonist</td>
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<tr>
<td>IRAK</td>
<td>Interleukin 1 receptor associated kinase</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LBP</td>
<td>lipopolysaccharide binding protein</td>
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<td>Min</td>
<td>minutes</td>
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<td>µl</td>
<td>microlitres</td>
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<tr>
<td>MAPKs</td>
<td>mitogen--activated protein kinases</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metallo proteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene (88)</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro-blue tetrazolium</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NK cells</td>
<td>natural killer cells</td>
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<tr>
<td>nM</td>
<td>nanometre</td>
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<tr>
<td>OC</td>
<td>osteocalcin</td>
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<tr>
<td>ON</td>
<td>osteonectin</td>
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<tr>
<td>OP</td>
<td>osteopontin</td>
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<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>PGs</td>
<td>proteoglycans</td>
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<tr>
<td>PGE$_2$</td>
<td>prostaglandin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>pNA</td>
<td>para-nitro aniline</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor κβ</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κβ ligand</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLRP</td>
<td>small leucine rich proteins</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered solution</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor-6 (TRAF6)</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1: Review of literature

1.1 Introduction

Periodontal diseases are chronic inflammatory diseases of multifactorial aetiology (Page and Kornman 1997), common in all human populations, which may result in gradual destruction of the supporting tissues of the teeth and ultimately tooth loss. The prevalence of periodontal disease within the U.K as reported by the Adult Dental Health Survey (2008) is 45%, of which 9% suffer from the severe form of the disease (White et al. 2012). The survey reported a marked reduction in moderate chronic periodontitis when compared to the 1998 survey, which was attributed to an improvement in oral hygiene practices and promotion of oral health. However, it is interesting to note that prevalence of severe disease has increased from 6% to 9% which may be attributed to current diagnostic and management techniques which are limited in their ability to identify high risk individuals, assess true periodontal status and monitor response to therapy. Therefore, in recent years, extensive research has focussed on scrutinizing the qualitative changes in the composition of potential markers released in GCF as adjunctive tools in the management of periodontal disease.

Accumulation of plaque has been accepted as the primary initiator of periodontal disease (Loe et al. 1965) but it is not solely responsible for the destruction that may follow. The destruction seen in periodontal disease is a consequence of the interaction between host and microorganisms present in plaque which result in the activation of the host inflammatory and immune responses, leading to loss of collagen and bone supporting the tooth (Kornman 2008). Although the role of bacteria is undisputed in the initiation of periodontitis, the quantity and types of bacteria have not been sufficient to explain the significant differences in disease severity between individuals (Page and Kornman 1997). The wide variations in susceptibility to periodontal disease seem to
be associated with a number of secondary genetic, environmental and behavioural risk factors (Salvi et al. 1997). All forms of the disease, however, have a common series of underlying events leading to tissue breakdown and loss of attachment. Although multiple factors have been cited as influencing the progression of periodontal disease, there is overwhelming evidence that, it is the uncontrolled inflammatory and immune responses that largely drive tissue destruction (Page et al. 1997) and therefore the important role played by the host inflammatory and immune response has been the focus of much research in the last several years.

Diagnosis of periodontal disease is based on clinical and radiographic examination. Clinical examination involves assessment of loss of attachment and bleeding on probing. However, there are limitations with regards to reproducibility and accuracy. False positive results with bleeding on probing are common, most of the time (Lang et al. 1986). Radiographs can be used to assess if there has been bone loss, but it only provides information on historical bone loss, rather than activity at the time of examination. The diagnostic examination therefore, only informs that tissue loss has occurred since the last evaluation and fails to inform whether bone is being lost actively at the time of examination. Consequently, it is estimated that the absence of an objective, specific and quantitative test for active periodontal disease has led to the misdiagnosis of periodontal disease in over 70% of patients resulting in costly and inappropriate treatment (Lang et al. 1986). Identification of mechanisms likely to result in periodontal breakdown will help us to target and manage ‘high risk’ patients and determine the efficacy of treatment procedures.

In recent years, research has elucidated the contribution of host-microbe interaction to both disease initiation and disease progression. Epidemiological and clinical studies indicate variation in susceptibility to periodontal disease despite the long-term presence of the oral bio-film (Baelum et al. 1986; Loe et al. 1986; Baelum et al. 1988), in addition to an increased susceptibility and greater severity of periodontal disease in patients with an impaired immune response (Feller and Lemmer 2008; Mealey and
Rose 2008). These variations may be attributed to an altered host response which are regulated by inflammatory mediators such as cytokines, which play a prominent role in amplifying the immune response resulting in degradation of the periodontal tissue and subsequent collection of host and bacterial products in the GCF. It has been suggested that these components may reflect the status of various components of the periodontium, that might serve as potential diagnostic and prognostic markers for the progression of periodontitis (Embey et al. 2000).

Therefore, extensive research has been carried out in the last few years to identify biomarkers that have the potential to diagnose accurately disease activity, prognosis and response to therapy (Bakri et al. 2013; Leppilahti et al. 2013; Miricescu et al. 2013; Tsuchida et al. 2013; Zein Elabdeen et al. 2013; Carneiro et al. 2014; Kumari et al. 2014; Leppilahti et al. 2014). However, our limited understanding of the pathogenesis of periodontitis limits the implementation of markers as adjunctive diagnostic and prognostic tools in the management of patients with periodontal disease.

Of particular interest to this project are proteoglycan metabolites such as chondroitin sulphate (CS) which is released as a consequence of periodontal tissue degradation and cytokines which are released as a consequence of microbial challenge. Previous clinical and biomedical studies by Waddington and Embey (Waddington et al. 1989; Waddington and Embey 1991; Waddington et al. 1994; Waddington et al. 1996; Waddington et al. 1998; Embey et al. 2000; Waddington and Embey 2001; Waddington et al. 2003a) have highlighted the quantification of CS which is known to derive from the degradation of alveolar bone as a promising diagnostic marker for active bone resorption. In recent years, cytokine interactions and their role in immune regulation in periodontal pathogenesis have been investigated extensively (Javed et al. 2012; Di Benedetto et al. 2013; Sima and Glogauer 2013; Souza and Lerner 2013; Yucel-Lindberg and Bage 2013; Javed et al. 2014; Khalaf et al. 2014; Kumari et al. 2014). Cytokines pooled in GCF have the potential to provide information of the inflammatory changes occurring in the underlying tissues and therefore cytokine
profiling may be of prognostic value in identifying high risk patients and thereby contribute towards timely and efficient management of these patients.

Against this background, the aim of this project is to further our understanding of the biological mechanisms that play an important role in modulating chronic inflammation and alveolar bone loss in periodontal disease through a series of *in-vitro* and *in-vivo* studies. Clinical studies will evaluate proteoglycan metabolites and cytokine profile in GCF of patients with periodontal disease to assess its value as potential markers in the management of patients with periodontal disease. Further, *in-vitro* studies will examine activity of the PDL cells in a pathological situation and thereby provide biological justification for the use of biomarkers in GCF for monitoring periodontal disease.
1.2 Global periodontal disease epidemiology

Based on previous data available from the WHO Global Oral Health Data Bank, Peterson and Ogawa 2005, reported that gingivitis affects more than 90% of the population, whereas only 10–15% of the adult population worldwide, was affected by the severe form of the disease (Petersen and Ogawa 2005). Among the countries that participated in these surveys, few countries reported findings using comparable methodologies that permit assessment of general trends. For example, USA, Australia, Vietnam have used the National Health and Nutrition Examination Survey (NHANES) protocol, whereas countries such as the UK, Germany and Canada have used the WHO protocol. These protocols vary in the types of probes used for measurement, various probing locations, full mouth assessment vs partial mouth assessment, probing depths vs loss of attachment, all of which make comparisons between studies difficult.

Recent epidemiological surveys of periodontal disease have revealed a 5-15% prevalence of advanced periodontitis in the adult global population (Dye 2012). Within the asian continent, the prevalence of severe periodontitis was reported to be 15-20% (Corbet and Leung 2011). In the U.S, the National Health and Nutrition Survey (NHANES) 2009-2010, reports the prevalence of periodontitis to be over 47% in the general population, with severe periodontitis affecting 8.5% of the adult population (Eke et al. 2012). In the UK, the Adult Dental Survey 2008 reported that 45% of the general population had periodontitis, with 9% suffering from the severe form of the disease (White et al. 2012). Interestingly, these recent surveys have reported that the prevalence of mild to moderate periodontitis has decreased in Australia, UK and USA but has increased in Germany and Hungary (Dye 2012). Promotion of oral health and an improvement of oral hygiene practice among the public, may contribute to the improvement of periodontal health in the population susceptible to mild to moderate periodontitis in countries such as the UK, USA and Australia. However, despite this
increase in periodontally healthy individuals, there has been an increase in the prevalence of severe periodontitis in some countries, for example the UK, from 6% in 1998 to 9% in 2009 (White et al. 2012). Epidemiological data from Sweden showed an unchanged 6–8% prevalence of advanced periodontitis from 1973 to 2003, despite an increase from 8% to 44% in the prevalence of periodontally healthy individuals during the same time period (Hugoson et al. 2008). Despite the differences in the provision of dental services and oral hygiene measures in various countries, the occurrence of advanced periodontitis is surprisingly similar. These reports may possibly reflect the limitations of current diagnostic and treatment modalities in the management of the high-risk patient.

Therefore, current epidemiological studies and national surveys are shifting away from focussing only on pocket formation (WHO protocol), to include assessment of loss of attachment and possibly self-reported questions and blood sampling, with a view to promote the creation and use of standardised case definitions for population based studies, as it appears that there is not enough current information to refute or support earlier estimates of severe periodontitis, which ranged from 5-15% of the adult global population (Dye 2012).

1.3 The periodontal tissues in health

The periodontium represents the supporting tissues of the teeth and comprises of the gingivae, the periodontal ligament, the alveolar bone and the cementum, which together maintain the function of a tooth. Although each component has unique tissue architecture and characteristic biochemical composition, each of these components influences the cellular activities of adjacent structures (Bartold and Narayanan 2006). The main function of the periodontium is to attach the teeth to the jaws and support them effectively during function. Its structure is such that it is able to accommodate
the small horizontal and lateral movements caused by the occlusal load during function and proper functioning of the periodontium is only achieved through structural integrity and interaction between its components.

The epithelial component of the gingiva provides the first line of defence in the periodontal region and presents with regional morphological variations such as oral, sulcular and junctional epithelium that are a reflection of tissue adaptation to the tooth and alveolar bone. Although its main role is one of protection to the underlying connective tissues, the epithelium is now recognised as having an active role in the innate host defence (Dale and Fredericks 2005). By rapid renewal and constant shedding of the epithelial cells, in addition to an increase in gingival crevicular fluid flow, bacterial colonization is efficiently inhibited. Bacterial internalization in a tissue culture model and in-vivo in severe periodontitis followed by epithelial cell apoptosis has been demonstrated (Andrian et al. 2004; Vitkov et al. 2005) and a decrease in mitosis and increased apoptosis of gingival epithelial cells at sites exhibiting severe inflammation has been reported (Carro et al. 1997). Additionally, in-vitro experiments have demonstrated expression of Toll-like receptors and protease activated receptors by oral epithelial cells in response to stimulation with P. gingivalis, leading to secretion of pro-inflammatory cytokines (Lourbakos et al. 2001; Ren et al. 2005). Recent research has reported that antimicrobial peptides such as defensins and LL-37 contribute to host defence and homeostasis by recruiting immune cells in times of health and disease (Greer et al. 2013). These antimicrobial peptides present in the epithelium contribute to the protection of the host tissue from dental plaque that resides around the tooth and root surfaces (Dale and Fredericks 2005). For example, defensins and LL-37 have the ability to enhance phagocytosis by macrophages (Yang et al. 2004a; Kohlgraf et al. 2010), they can also serve as chemo attractants for monocytes, macrophages, T-lymphocytes and immature dendritic cells (Chaly et al. 2000; Yang et al. 2004a; Yeung et al. 2011). Defensins have the ability to enhance antigen-specific immune response (Kohlgraf et al. 2010; Yeung et al. 2011) and
suppress the production of pro-inflammatory cytokines of certain microbial agents (Kohlgraf et al. 2010). The defensins and LL-37 can activate and degranulate mast cells (Yang et al. 2004a; Kohlgraf et al. 2010) as well as regulate the complement system (Yang et al. 2004a; Kohlgraf et al. 2010). A recent clinical study evaluating human beta defensin (HBD) levels in GCF of patients with localised aggressive periodontitis reports that the levels of defensins decreased significantly post treatment, thereby suggesting that the appropriate expression of HBD peptides in health and disease may contribute to the maintenance of periodontal homeostasis, possibly through its antimicrobial effects and the promotion of adaptive immune responses (Ebrahem 2013). The cells of the junctional epithelium actively facilitate leukocyte recruitment to the site of inflammation by expressing chemotactic factors such as IL-8, intercellular adhesion molecules (ICAM) and E-selectin that aid leukocyte migration from the blood vessels (Moughal et al. 1992; Nylander et al. 1993; Gemmell et al. 1994; Tonetti et al. 1994; Tonetti 1997; Tonetti et al. 1998). Calprotectin, a protein expressed in neutrophils, monocytes, and gingival keratinocytes protects against binding and infection by *P. gingivalis* (Nisapakultorn et al. 2001). Collectively, the above studies demonstrate the important role of the cells of the epithelium in maintaining an active defence mechanism.

The connective tissues of the periodontium are composed of two soft tissues (gingival connective tissue and periodontal ligament) and two hard tissues (alveolar bone and cementum). Although each component has a unique tissue architecture and characteristic biochemical composition, each of these components influences the cellular activities of the adjacent structures and the interactions between these components not only determines tissue health, but also reflects events associated with tissue damage, repair and regeneration (Bartold and Narayananan 2006). The extracellular matrix within each periodontal component comprises both fibrous and nonfibrous elements including collagens, elastin, fibronectin, laminin, osteopontin, bone sialoprotein, a variety of growth factors and other noncollagenous proteins,
proteoglycans, lipids, minerals and water. The cellular component of the periodontium comprises of differentiated cells such as fibroblasts, osteoblasts, cementoblasts, epithelial cells (cell rests of Malassez), endothelial cells, as well as cells participating in the immune and inflammatory responses. It also contains a population of stem/progenitor cell population which originate from the ectomesenchymal cranial neural crest cells which possess the capacity to differentiate into periodontal ligament fibroblasts, cementoblasts and osteoblasts (Sodek and McKee 2000; Bartold et al. 2006; Hynes et al. 2012). It is speculated that these progenitor cell population play an active role in repair and regeneration. Collectively these cells play a major role in the formation as well as degradation of the extracellular matrix which is in a state of constant renewal. The main role of fibroblasts is the synthesis and secretion of collagen, glycosaminoglycans, proteoglycans and glycoproteins. Whilst the main role of gingival fibroblasts is to maintain the synthesis and integrity of the gingival connective tissue, the periodontal ligament fibroblasts have specialised functions which are concerned with the formation and maintenance of the periodontal ligament, including its repair or regeneration following damage (Berkowitz, 1992). The fibroblasts within the PDL are a heterogeneous population (McCulloch and Bordin 1991) both around the same tooth and between different teeth. Compared to other fibroblasts, PDL fibroblasts are unique in that they possess the capacity to differentiate into cementoblasts and osteoblasts (Roberts et al. 1982; McCulloch and Melcher 1983). This osteogenic differentiation capacity is reflected in that, PDL fibroblasts in-vivo possess alkaline phosphatase activity (Rooker et al. 2010). Furthermore, variations in alkaline phosphatase activity have been reported between subsets of PDL fibroblasts in that, PDL fibroblasts towards the tooth side express low alkaline phosphatase activity and PDL fibroblasts towards the bone side express high alkaline phosphatase activity (Rooker et al. 2010). In addition to alkaline phosphatase activity, the PDL fibroblasts are characterised by a high rate of collagen turnover (Sodek 1977) which occurs by simultaneous synthesis and degradation of collagen.
matrix. These cells have been shown to possess osteoblast like characteristics including the production of osteonectin (Somerman et al. 1990; Nohutcu et al. 1996) as well as osteocalcin (Nojima et al. 1990b). From the above studies it is clear that phenotypically distinct and functional sub populations of cells of both fibroblast as well as osteoblast lineage exist in the periodontal ligament.

Other cells present in the periodontium are the osteoblasts, cementoblasts, osteoclasts and cementoclasts which may be found lining the endosteal and periosteal bone surfaces and cementum surfaces, more obvious in the stages of active deposition or resorption. Osteoblasts synthesize and regulate the deposition of bone organic matrix including collagen type I, proteoglycan, osteonectin, osteocalcin, bone sialoprotein and osteopontin. These cells also express and release alkaline phosphatase, which has been shown to be closely associated with new bone formation. The process of mineralisation is controlled by osteoblasts, which may become trapped in their own secretion and subsequently become incorporated in the matrix as osteocytes. Osteoclasts, the large multinucleated cells are responsible for resorbing bone. Cementoblasts are morphologically and functionally identical to osteoblasts and their function is the synthesis and secretion of the components of the organic matrix of the cementum. Similar to bone formation, during the formation of cementum, cementoblasts become trapped and are then referred to as cementocytes. Unlike bone, there is no evidence of cementum remodelling, however there is continuous, slow apposition of surface cementum as cementoblast activity continues at a low level throughout life. Resorption of cementum is carried out by cementoclasts and occurs in response to excessive occlusal stress, orthodontic movement, pressure from tumors or cysts, or due to deficiency of vitamin A and D, where bone and tooth mineralization is altered leading to malformed and weak bones and teeth, premature tooth loss and tooth infections.
1.3.1 Extracellular matrix of the periodontium

Extracellular matrix assembly and subsequent mineralization in cementum and bone occur through successive, highly ordered steps with a lag phase existing between matrix deposition and mineralization. As part of this process, extracellular matrix proteins are secreted, sometimes modified or cleaved by enzymes and then organized into macromolecular assemblies, which together is then structured into a mature fibrillar matrix receptive to mineral deposition (McKee et al. 2005). These events are orchestrated by the osteoblasts and cementoblasts with each being associated with a thin layer of unmineralised matrix that subsequently mineralizes at the 'mineralization front' to form the completed tissue. Additional mineralization and mineral changes such as further carbonate substitution in the hydroxyapatite lattice (Cazalbou et al. 2004) occur slowly over time (Boskey and Coleman 2010), along with changes in the organic phase of the extracellular matrix (Kaartinen et al. 2002; Kaartinen et al. 2005) and proteolytic degradation, in a maturation process that ultimately provides an adequate final functional state to provide for the biomechanical demands placed on each tissue.

The extracellular component of the periodontium is made up of collagen I, III, IV, V, VI and XII. In the periodontal ligament, type I and III predominate. Much of the type I collagen which is fibrillar, is gathered together to form bundles which are about 5μm in diameter and these are known as principal fibres. Type III is more elastic in nature and may be important in maintaining the integrity of the ligament during the small vertical and horizontal movements which occur during chewing. The main collagen in the organic matrix of bone and cementum is type I and this is virtually insoluble due to cross-links which provide the structural and mechanical stability for normal function (Bartold et al. 2006). In addition to collagen fibres, the oxytalan fibres which play an important role in support, are present in the periodontal ligament.
1.3.1.1 Proteoglycans

Numerous non-collagenous components exist in the periodontal tissues. Within the matrix, proteoglycans (PG) provide important roles in matrix assembly, cell signalling and mineralisation.

Proteoglycans are characterized by a protein core to which one or more anionic GAG chains are attached. The proteoglycans are divided into extracellular matrix proteoglycans and cell surface proteoglycans (Fig 1.1).
Fig. 1.1: Classification of proteoglycans (PGs) based on their location and binding. The heterogeneous group of PGs include those of the extracellular matrix, such as small leucinerich PGs (SLRP; e.g., decorin) and modular PGs. Modular PGs are divided into hyalectans (hyaluronan- and lectin-binding PGs) and the non-hyaluronan binding PGs of the basement membrane. The third group of cell-surface PGs encompasses mainly the membrane spanning syndecans (e.g. syndecan-4) and glypicains. Serglycin is an intracellular PG found in hematopoietic and endothelial cells (Schaefer and Schaefer 2010).
The physical characteristics and biological functions of the PGs are determined by the physicochemical characteristics of the GAG chains and partly by the structure of the core proteins interactions between PGs and other extracellular molecules (Embery et al. 2000). GAGs are covalently bound to core proteins and are all sulphated with the exception of hyaluronan which exists unattached to a core protein and is non sulphated (Prehm 1984). Seven species of GAGs exist (Fig 1.2), chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), dermatan sulphate (DS), heparan sulfate, heparin, keratan sulfate and hyaluronan (Embery et al. 2000). In the periodontium, the non-sulphated hyaluronic acid, and the sulphated heparin sulphate, chondroitin-4-sulfate and chondroitin-6-sulfate are present, of which condroitin-4-sulphate predominates (Larjava et al. 1992). The turnover rate of these molecules is faster than collagen and they have many functions including ion and water binding and exchange, cell signalling, control of collagen fibrillogenesis and fibre orientation. The water binding function provides the ligament with a hydraulic cushion effect in resisting the forces of mastication as well as traction on the ligament fibres. Cell adhesion and growth are regulated by the proteoglycans which also have the capacity to bind and regulate growth factor activity (Bartold and Narayanan 2006). In recent years, it has been firmly established that the small leucine-rich proteoglycans (SLRPs), whose distinctive feature is the presence of 7 to 24 leucine-rich repeats in the core protein, are functionally involved in normal bone development and homeostasis (Nikitovic et al. 2012). Among the SLRPs, decorin and biglycan, which are conjugated to dermatan sulphate or chondroitin sulphate GAGs, with one or two GAG chains respectively, have been identified in several connective tissues. Studies have confirmed that these molecules have specific designated roles during all phases of bone formation including periods relating to cell proliferation, organic matrix deposition, remodelling and mineral deposition (Wilda et al. 2000; Soto-Suazo et al. 2002). These SLRPs influence cell behaviour by various means such as, act as cytokine reservoirs in the extracellular matrix (Tillgren et al. 2009), matrix barriers restricting molecular diffusion (Magzoub et
al. 2008) and matrikines directly interacting with cell-surface receptors (Schaefer and Lozzo 2008).

Following synthesis, the SLRPs are secreted into the pericellular matrix where they either diffuse and bind to the components of the extracellular matrix such as collagens or remain in free form. The localisation of the SLRPs in the ECM appears to be strictly predetermined with specific SLRPs being predominantly distributed variably in the ECM ‘proper’ whereas others are localised both to the ECM and pericellular matrix (Henry et al. 2001; Schaefer and Lozzo 2008). The SLRPs located in the ECM ‘proper’ bind to various types of collagens thereby regulating the kinetics, assembly and special organisation of fibrils in tendon, bone and cornea (Lozzo 1997; Reed and Lozzo 2002; Chakravarti et al. 2006). Besides being mainly sequestered in the ECM, these SLRPs can also exist as soluble molecules, as when released from the ECM by proteolytic digestion of injured tissues. In both the bound as well as the soluble form, the SLRPs interact with various growth factors including TGF-β (Hildebrand et al. 1994), BMP-4 (Chen et al. 2004), PDGF (Nili et al. 2003), TNF-α (Tufvesson and Westergren-Thorsson 2002) and IGF-1 (Schonherr et al. 2005). The biological interactions modulate growth factor bioavailability through the formation of specific concentration gradients. Pericellular localisation of SLRPs allows these molecules to interact with various molecules, ligands and cell surface receptors, thereby modulating a wide range of cell matrix interactions (Schaefer and Lozzo 2008).
1.3.1.1.1 Role of SLRPs in modulating cellular activity

Waddington et al (2003b), reported that dermatan sulphated (DS) biglycan is expressed during phases of proliferation and differentiation, whereas chondroitin sulphated (CS) biglycan is expressed at onset of mineralisation (Waddington et al. 2003b). Their results were suggestive of a potential role for decorin and biglycan as signalling molecules in that, the early expression of DS biglycan may be associated with directing proliferation or development of the osteoblast phenotype and the later expression of decorin may indicate a role in down regulation of cell proliferation.

Biglycan is suggested to favour proliferation during osteoblast development (Waddington et al. 2003b) and proliferation of vascular smooth muscle cells by CDK2- and p27- dependent pathways (Shimizu-Hirota et al. 2004). In addition to its proliferative capacity, biglycan has anti-proliferative effects such as inhibition of tumor cells in pancreatic cancer (Weber et al. 2001) as well as inhibition of BMMSC (Inkson et al. 2009). Biglycan also influences cellular differentiation, and is shown to control osteoblast differentiation by modulating BMP4 (Chen et al. 2004). Additionally, it has been shown that biglycan is a critical component in organising the niche of tendon stem/progenitor cells, and its absence affects tendon progenitor differentiation by modulating BMP signalling (Bi et al. 2007).

Several studies have reported that biglycan controls key signalling pathways regulating osteogenic program, including the activity of TGF-β (Bi et al. 2005), BMP-4 (Ye et al. 2012), Wnt and NFκB (Berendsen et al. 2011), which influence both the number of available osteogenic precursors as well as their subsequent development, differentiation and function in bone formation (Nikitovic et al. 2012). These studies suggest that biglycan may regulate proliferation in a cell-specific manner via specific receptor and signalling pathways, or by an indirect and unidentified mechanism. Moreover biglycan is implicated as a co-regulator of growth factors such as FGF2 (Hou
et al. 2007) and TGF-β (Chen et al. 2002) which are important factors for progenitor proliferation and mineralisation. Additionally, biglycan plays a role as a signalling molecule important to the innate system whereby, on tissue stress or injury, sequestered and immunologically inactive biglycan is released from the ECM by proteolytic mechanisms (Nikitovic et al. 2012). Decorin, signals through IGF-IR to exert anti-apoptotic effects under physiological conditions, thereby favouring normal cell growth (Schonherr et al. 2005) and also has anti-tumor properties (Reed et al. 2002). It has been suggested that in the absence of decorin and biglycan, the proper sequestration of TGF-β within the extracellular matrix is prevented. Initially decorin binds to TGF-β (Kresse and Schonherr 2001) followed by biglycan to form SLRP/TGFβ complexes which may be excreted or in the presence of collagen I are sequestered in the ECM thus downregulating TGF-β signalling (Abdel-Wahab et al. 2002). However, the interaction between the SLRPs and TGF-β could also enhance the bioactivity of TGF-β as seen in the case of decorin during the process of bone formation during remodelling (Takeuchi et al. 1994) or muscle formation (Riquelme et al. 2001). Therefore, the direct binding of excess TGF-β to its receptors could cause a switch of fate from growth to apoptosis and thus lead to decreased number of osteoprogenitor cells and subsequent reduced bone formation (Bi et al. 2005). Therefore SLRPs influence cell behaviours including differentiation, apoptosis, proliferation and migration through multiple means such as act as cytokine reservoirs in the ECM, as matrix barriers restricting molecular diffusion and matrikines directly interacting with cell surface receptors as mentioned above in section 1.3.1.1.
1.3.1.1.2 Role of SLRPs in matrix assembly

Although collagen fibrils can self-assemble, the cell also participates in organization of the fibrils through interactions involving integrins, fibronectin and other minor collagens such as thrombospondins, tenascins etc (Kadler et al. 2008). SLRPs have the ability to interact with collagen molecules and facilitate fibril formation thereby playing an important role in the provision of a collagenous framework which allows for further mineral deposition. The cell-matrix interactions facilitated by the SLRPs, may be by directly interfering with plasma membrane receptors and pericellular matrix molecules. For example, decorin inhibits cell attachment through fibronectin (Schmidt et al. 1991) thrombospondin (Davies Cde et al. 2001), tenascin (Minamitani et al. 2004), whereas lumican (Zeltz et al. 2010), osteoadherin (Lucchini et al. 2004) and chondroadherin (Camper et al. 1997), all have high affinity for integrins. Biglycan on the other hand, regulates muscle cell behaviour by binding plasma membrane α-dystroglycan through its GAGs (Bowe et al. 2000) playing a role in muscular dystrophies. Cell-membrane associated SLRP such as Nyctalopin act by integrating cell receptors and pericellular matrix proteins to modulate cell behaviour (Cao et al. 2011).

Studies have shown that an altered expression of SLRPs, disrupts matrix integrity resulting in structural deficiency in that, SLRP-deficient mice exhibit phenotypes that are consistent with dysfunctional matrix assembly in connective tissue such as skin, bone, cartilage and teeth (Haruyama et al. 2009), as well as non-connective tissues such as liver (Baghy et al. 2011) and pregnant uterus (Sanches et al. 2010; Wu et al. 2012b). For instance, targeted disruption of the biglycan gene leads to osteoporosis-like phenotype in mice (Xu et al. 1998), biglycan/fibromodulin deficient mice have abnormal collagen fibrils in tendons that lead to gait impairment, ectopic ossification and osteoarthritis (Ameye et al. 2002), and Ehlers-Danlos like changes such as skin laxity and fragility as well as joint laxity are found in decorin and biglycan deficient mice as well as lumican and fibromodulin deficient mice (Corsi et al. 2002). Indeed, altered
expression of SLRPs has been observed in a broad range of human diseases such as Marfan's syndrome (Raghunath et al. 1993), localised scleroderma (Beavan et al. 1993), infantile progeroid patients (Beavan et al. 1993), osteogenesis imperfect (Fedarko et al. 1995), systemic sclerosis (Westergren-Thorsson et al. 1996) and carbohydrate-deficient glycoprotein syndrome (Gu and Wada 1995).

1.3.1.1.3 Role of SLRPs in mineral deposition

Studies by Waddington et al (2003a and b), have demonstrated that DS decorin is associated with early matrix deposition and maturation, while CS decorin presents during matrix mineralisation (Waddington et al. 2003b). Furthermore, these studies suggest that GAG degradative products may contribute towards bioactivity, whereby DS may act to inhibit mineralisation, and conversely CS could control and/or promote mineralisation (Waddington et al. 2003a), having the capacity to bind five-fold more calcium than DS (Embery et al. 1998). Preceding studies investigating molecular mechanisms have suggested CS adopts a rigid structure in solution, whilst DS is more flexible in nature, adopting several energetic conformations, thus providing a mechanism by which the SLRPs play an important role in interacting with crystal mineral while allowing for crystal growth (Embery et al. 1998). These differences may imply that these PGs perform differing functions during matrix formation.

1.3.1.1.4 Role of SLRPs in bone formation

The key role of biglycan in bone development is corroborated by the observation that biglycan deficiency leads to structural abnormalities in collagen fibrils in bone, dermis and tendon (Corsi et al. 2002) and delayed osteogenesis (Chen et al. 2003). Targeted deletion of genes encoding for decorin and biglycan within mice have demonstrated that, following single deletion of the gene within the mouse genome, the most striking effect was observed for biglycan knockouts which developed as osteoporotic
phenotype, failing to achieve peak bone mass due to decreased bone formation with significantly short femurs (Ameye et al. 2002). *In-vitro* experiments, demonstrated that the number and responsiveness of bone marrow stromal cells to TGFβ and hence osteogenic precursor cells, decreased dramatically with age while apoptosis rates increased (Chen et al. 2002). These effects were also observed within teeth, where transition from predentine to dentine appeared to be impaired and the thickness of enamel was dramatically increased (Goldberg et al. 2002). For biglycan knockouts, the collagen fibrils in bone were very irregular in size, demonstrating variability in size along the length of the individual fibril with notches and protuberances frequently observed (Corsi et al. 2002; Goldberg et al. 2002). Conversely, for the decorin knockouts, bone mass was not affected, with bone appearing both at histological and macroscopic levels, to be similar to the wild type (Corsi et al. 2002). Interestingly, for the double knockouts, the decrease in bone mass was more severe and developed earlier than the single biglycan knockout, suggesting the effects of decorin and biglycan to be synergistic within bone (Corsi et al. 2002). These studies led to the conclusion that although single deletion of either decorin or biglycan would suggest that they provide distinct functions, the gross effects witnessed from the double knockout indicate that a deficiency in one may be compensated by the presence of the other (Ameye and Young 2002).

Moreover, recent studies have demonstrated a link between SLRPs and aortic valve stenosis. Both decorin and biglycan are also expressed in healthy arteries (Bianco et al. 1990; Yeo et al. 1995) with the adventitia of aorta being a major site for biglycan deposition (Heegaard et al. 2007). However, biglycan plays a somewhat sinister role in the development of atherosclerotic plaques and aortic valve stenosis in that, biglycan promotes lipid deposition through binding to Toll-like receptor, induction of cytokine production and inflammation. It binds to Toll-like receptors 2 and 4 of macrophages, and thus functions as one of mediators of innate immunity (Schaefer and Iozzo 2008).
It also induces the expression of BMP-2 and alkaline phosphatase in human aortic valve interstitial cells primarily through Toll-like receptor 2, and thus contributes to the osteogenic process and calcifications during pathogenesis of aortic valve stenosis (Song et al. 2012).

In carcinoma cells, decorin signals mainly via the epidermal growth factor receptor (EGFR) (Moscatello et al. 1998), whereas in endothelial cells and renal fibroblasts decorin binds to the insulin-like growth factor-I receptor (IGF-IR), thereby regulating apoptosis and synthesis of other extracellular matrix constituents (Schonherr et al. 2005; Schaefer et al. 2007). In contrast, biglycan, is an endogenous pro-inflammatory ligand for the TLR2 and TLR4 in macrophages (Merline et al. 2009) and on its release from the extracellular matrix, it signals through the MAP kinases p38 and ERK and through the NF-κB pathways (Schaefer et al. 2005) resulting in enhanced infiltration of mononuclear cells into the lung in experimental sepsis and into the kidney in unilateral ureteral obstruction (Schaefer et al. 2002; Schaefer et al. 2005).

On the other hand, dermatan sulphate proteoglycan play an important role in extracellular matrix organisation during the wound healing process by binding to FGF2 (Turnbull et al. 1992) and promoting FGF mediated cell proliferation via FGFR1 (Penc et al. 1998). In addition to a functional role in wound healing, the dermatan sulphate molecule itself may have a role in reparative processes as opposed to deleterious effects during inflammation. However, it has also been speculated that dermatan sulphate may contribute towards pathological conditions (Malavaki et al. 2008).

From the evidence presented in the literature, it is very clear that SLRPs have several important roles in a variety of biological and pathological processes, which include regulating cell proliferation, migration and differentiation in early developmental stages, regulate matrix assembly in later stages and further, as an indispensable structural component of the ECM. In pathological conditions, such as during inflammation and wound healing, SLRPs facilitate tissue repair and regeneration (Ansorge et al. 2012;
Dunkman et al. 2013). Additionally, recent research has highlighted that SLRPs may be associated with tissue changes associated with aging (Dunkman et al. 2013). The ECM are regulated by SLRPs during assembly, however, these matrices also regulate the dynamic distribution and function of SLRPs during development and disease. Therefore it has been suggested that the SLRPs in ECM matrices may provide us with biomarkers for bone diseases and perhaps enable novel therapeutic interventions in management of a broad range of diseases (Theocharis et al. 2010; Nikitovic et al. 2012; Chen and Birk 2013).
1.3.1.2 Glycoproteins

The main glycoproteins present in the periodontium are fibronectin and tenascin. Fibronectin is a high molecular weight insoluble fibre-forming glycoprotein present both extra and intracellularly (Midwood et al. 2006) and binds to cells as well as other sites that bind to collagen, heparin and fibrin (Mariotti 1993). This is thought to promote the attachment of cells to the substratum and especially to collagen. Additionally, cells preferentially adhere to fibronectin which may be involved in cell migration and orientation (Berkovitz et al., 1992). In view of the high turnover rate of the periodontal ligament, it is possible that fibronectin may have considerable biological significance. Immunohistological localization techniques have revealed that fibronectin is uniformly distributed throughout the periodontal ligament, both during eruption and in fully erupted teeth (Steffensen et al. 1992; Romanos et al. 1993). However, fibronectin is expressed particularly strongly along attachment sites of the periodontal ligament collagen fibres to cementum but not to alveolar bone (Matsuura et al. 1995). It is also found in the endosteal spaces, periosteum and bone lining cells at their interface with alveolar bone (Steffensen et al. 1992). In the cementum, its expression is weaker than in the periodontal ligament and ultrastructural studies have localised fibronectin over collagen fibres and at certain sites at the cell collagen interface (Zhang et al. 1993). Fibronectin has also been localised in the basement membrane and lamina propria (Steffensen et al. 1992) with a fibrillar and diffuse distribution (Romanos et al. 1993). In addition to its main role as an adhesive protein, fibronectin is involved in blood coagulation, wound healing and chemotaxis (Mariotti 1993). During the terminal maturation of many connective tissue matrices, a general loss of fibronectin has been observed. However, its continued presence in the periodontal ligament may be indicative of either the ligament retaining immature characteristics or its high turnover. On the other hand, expression of tenascin is only maintained during wound healing and in a few adult tissues including bone marrow and the periodontal tissues. Unlike
fibronectin, it is not uniformly localised throughout the periodontal ligament but rather concentrated adjacent to the alveolar bone and the cementum (Steffensen et al. 1992; Becker et al. 1993). It is found between less densely packed collagen fibrils of the periodontal ligament (Zhang et al. 1993) and accumulated towards the alveolar bone and cementum (Steffensen et al. 1992) with only weak expression throughout the alveolar bone matrix. Weak expression of tenascin is also observed in cementum which may have been deposited prior to mineralisation (Zhang et al. 1993). Elastin, laminin (Mariotti 1993), vitronectin (Steffensen et al. 1992) are other glycoproteins that play a role in cell attachment and are present in small amounts within the periodontal tissues.

1.3.1.3 Proteins associated with bone and cementum

Osteonectin, osteocalcin, bone sialoprotein (BSP) and osteopontin are the main proteins associated with the hard tissues of the periodontium. Osteonectin, secreted by osteoblasts, is one of the major non-collagenous proteins of bone, with a strong affinity for calcium ions thought to play an important role in the initial stages of mineralisation (Termine et al. 1981). It has been located in the basal lamina (Bilezikian, 1996) as well as in the periodontal ligament particularly strongly around the Sharpey's fibres, at the attachment sites between the ligament and alveolar bone and cementum (Matsuura et al. 1995). Osteocalcin, a small protein that is mainly secreted by osteoblasts and becomes incorporated into the mineralised matrix soon after its secretion, is thought to plays a crucial role in mineralisation (Mariotti 1993). BSP expression marks a late stage of osteoblast differentiation and an early stage of matrix mineralisation (Lekic and McCulloch 1996; Gordon et al. 2007). A weak expression is also observed in the periodontal ligament at attachment sites with alveolar bone and cementum (Matsuura et al. 1995). Additionally, BSP is expressed by cells lining the
root surface at early stages of cementogenesis during tooth development (MacNeil et al. 1996). Although the precise function of BSP is unknown, it may serve as an attachment factor as it has an affinity for collagen fibres. Osteopontin, is found primarily in bone and has an affinity for calcium ions (MacNeil et al. 1995). It is expressed prior to mineralisation and appears to be involved in the attachment and movement of osteoblasts and osteoclasts and may possibly function as an inhibitor of mineralisation during periodontal ligament development (MacNeil et al. 1995).

1.4 Turnover in the periodontium

In health, the regulation of tissue turnover is dependent on the recruitment as well as stimulation of the appropriate cells under the influence of growth factors released by the functioning mature cells. These factors exert their effects by binding to specific transmembrane receptors on target cells which generate a cascade of intracellular molecular signals (Ioannidou 2006), thereby regulating the activation and proliferation of the signalled cells as well as regulating a number of other factors including cell migration and synthesis which are essential events in healing. Epithelial turnover is also affected by hormones, cytokines, growth factors such as EGF, PDGF, TGFs, as well as the underlying connective tissue. The turnover rate in the oral epithelium is 8-40 days and the junctional epithelium is 4-11 days, with the connective tissue turnover in the periodontal ligament being five times higher than alveolar bone and 15 times higher than the dermis of normal skin (Eley and Manson, 2010). The turnover rate in the periodontal ligament has been reported to be the highest when compared to other connective tissues in the body (Sodek 1977) resulting in constant renewal of the
periodontal ligament fibres, the alveolar bone proper as well as the Sharpey’s fibres, thereby facilitating adaptation to physical forces exerted on this tissue. This increased turnover in the periodontal ligament may also suggest that these cells are more susceptible to destruction in the presence of inflammatory mediators and therefore contribute towards the rapid destruction observed in periodontal disease.

Fibroblasts are responsible for both the synthesis and degradation of all components of the ECM with MMPs produced by fibroblasts playing a major role. Two pathways of collagen degradation have been postulated. The intracellular pathway involves the engulfment of the collagen fibrils by the fibroblast followed by digestion by MMP, followed by ingestion by phagosome and further degradation by cysteine proteinases. The extracellular pathway involves the digestion of relatively large amounts of collagen in the extracellular space under the influence of a variety of enzymes such as MMPs secreted by fibroblasts. Additionally, some cytokines may affect collagen, fibronectin and proteoglycan synthesis and secretion and these include FGF, PDGF, and TGF. Other cytokines such as IL-1 and IFN-γ, PDGF and TGF can stimulate collagenase secretion. In bone, turnover takes place continuously throughout life with deposition mediated by osteoblasts and resorption largely mediated by osteoclasts with additional support form osteoblasts. Stimulated osteoblast secrete pro-collagenase which, when activated can remove the non-mineralised collagenous surface of bone. Cytokines such as RANKL and M-CSF are secreted by osteoblasts which are essential for osteoclast differentiation (Kobayashi and Udagawa 2007). Osteoclasts then spread over the bone surface and beneath their ruffled borders secrete acid which dissolves the mineral phase. The process is regulated by PTH, Vit D₃ and calcitonin, in addition to locally produced factors such as PGE₂, leukotrienes and cytokines such as IL-2, IL-3 and IL-6 as well as growth factors such as TNF, TGF and PDGF.

Although in health, this turnover process is tightly regulated by cytokines, an imbalance between pro-inflammatory and anti-inflammatory cytokines occurs, in response to the presence of periodontal pathogens. As a consequence, remodelling becomes
imbalanced or dysregulated leading to rapid destruction and progression of disease. However, the most important characteristic of the periodontal ligament is its capacity for repair and regeneration which is reflected in the complex and heterogenous cell population within this tissue (Lekic and McCulloch 1996). The mesenchymal progenitor cell population present within the periodontal ligament proliferate, migrate and ultimately differentiate leading to the synthesis of new collagen fibres under the influence of proteins such as decorin. Additionally, high proliferation of endothelial cells and angiogenesis in the periodontal ligament has been observed during periods of repair (Fiedler et al. 2008) and signalling factors such as VEGF and PGE$_2$ from endothelial cells have been proposed to promote periodontal ligament progenitor cell osteogenesis (Wu et al. 2012a). A study examining location of stem cells in humans, reported its presence in healthy as well as diseased periodontal ligament and that the number of these cells were enhanced in the presence of inflammation associated with periodontitis (Chen et al. 2006). The origin and location of the progenitor stem cell population has been extensively investigated, with some reports suggesting that these cells are present perivascularly adjacent to blood vessels, which on appropriate stimuli may give rise to periodontal ligament fibroblasts or migrate towards bone and cementum to form osteoblasts and cementoblasts (Lekic and McCulloch 1996). Other studies suggest that these cells may be present in the vascular channels of the alveolar bone which may migrate towards the periodontal ligament (Melcher et al. 1987). Additionally, it is also possible that separate precursor cells may be present for each distinct mature cell type.
1.5 Aetiology of periodontal diseases

Bacterial plaque is the primary aetiological factor in periodontal disease and the disease will not develop in the absence of plaque. Studies have demonstrated a positive relationship between severity of gingivitis and the amount of plaque present when oral hygiene was ceased and resumed (Loe et al. 1965). Further studies showed improved oral hygiene in conjunction with non-surgical periodontal therapy resulted in a reduction in gingival inflammation in patients with periodontitis (Badersten et al. 1984).

In patients who failed to maintain adequate oral hygiene to remove microbial plaque post treatment, the periodontal treatment was generally unsuccessful, (Nyman et al. 1977; Axelsson et al. 1991). In addition, animal studies (Lindhe et al. 1970; Lindhe et al. 1975; Listgarten et al. 1979), microbiological studies (Socransky et al. 1964; Persson et al. 1990b) and immunological studies (Evans et al. 1992b; Persson et al. 1994) have contributed to the overwhelming evidence to support the role of plaque as the primary aetiological factor in periodontal disease.

The amount of bacterial plaque or the types of bacteria found in plaque do not by themselves appear to explain the prevalence, wide variation and severity of disease seen in the adult population (Socransky 1977; Page and Kornman 1997). Epidemiological studies have identified groups of patients who are susceptible and resistant to disease. In a classical study of the natural history of periodontal disease in a population of Sri-Lankan tea workers, Loe and co-workers showed that in the absence of conventional oral hygiene measures, the majority of workers exhibited moderate progression of disease whereas 8% suffered rapid progression and 11% did not develop disease (Loe et al. 1986). This evidence supports the view that plaque alone is insufficient for disease progression.
According to the current concept of disease activity, periodontal pockets go through periods of exacerbation and quiescence resulting from bursts of activity followed by periods of remission. Periods of quiescence (inactivity) are characterised by a reduced inflammatory response and little or no loss of bone or connective tissue attachment. A build-up of unattached plaque (subgingival biofilm), with its predominantly gram-negative, motile and anaerobic bacteria starts a period of exacerbation (activity) in which bone and connective tissue attachment are lost and the pocket deepens. This period may last for days or weeks or months and is eventually followed by a period of remission or quiescence in which gram-positive bacteria proliferate and a more stable condition is established. Periodontal destruction does not occur in all parts of the mouth at the same time or on only some aspects of some teeth at any given time. This is referred to as site specificity of periodontal disease. Sites of periodontal destruction are seen next to sites with little or no destruction. The site specificity and predilection in periodontitis and gingivitis probably relates to the retention of plaque in specific areas, such as in local areas where oral hygiene is impaired or difficult, in areas of calculus accumulation and in areas of restoration overhangs or poor crown margins. Therefore, the severity of periodontitis increases with the development of new sites and the increased breakdown of existing sites. Progression of disease occurs when the equilibrium between the destructive and protective mechanisms is lost either because an increase of destructive factors or a decrease in the effectiveness of protective mechanisms.
1.6 Pathogenesis of periodontal disease

It is now well established that periodontal disease activity is determined by a complex interplay between the immune system and periodontal pathogens (Gaffen and Hajishengallis 2008). The host response being highly complex, contains both protective and destructive elements and may be proactively modified by immune subverting pathogens (Gemmell et al. 2007; Kinane et al. 2011). In susceptible patients, a dysregulation of inflammatory and immune pathways leads to chronic inflammation, tissue destruction and disease.

Like most biofilms, the dental plaque is built in a continued process characterized by succession of different bacterial species, each one with relevant roles in every step of biofilm construction (Kolenbrander et al. 2002)

It is formed initially by the interaction of bacteria with the tooth surface and later by the physical and physiological interaction between different species within the microbial mass. Primary colonization on the acquired pellicle is dominated by facultative Gram-positive bacteria (such as Streptococcus species followed by Actinomyces species). These Gram positive cocci and rods coaggregate and multiply and provide receptors for the subsequent adhesion of Gram negative organisms (such as Fusobacterium nucleatum, Prevotella intermedia) which have a poor ability to directly adhere to the pellicle. The heterogeneity increases as plaque ages and matures. As a result of ecological changes, more Gram-negative strictly anaerobic bacteria colonize secondarily and contribute to an increased pathogenicity of the biofilm (Lindhe et al., 1997). The supragingival plaque matures to give a new ecological environment that strongly influences the growth, accumulation and pathogenic potential of subgingival plaque (Schiby et al., 1995). Although subgingival plaque harbours over 500 species, current data suggests that within plaque a limited number of specific pathogenic bacteria like Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter
actinomycetemcomitans, Prevotella intermedia, Campylobacter rectus, Treponema species are bacterial species strongly implicated in periodontitis (Socransky and Haffajee 1991; Lovegrove 2004). These specific pathogens are mostly gram-negative anaerobes producing lipopolysaccharides which play an important role in periodontal disease (Chen et al. 1995). For periodontal microorganisms to be pathogenic it must be able to first colonize the periodontal tissue, then evade host defence mechanisms that are aimed at eliminating these organisms from the periodontal environment and finally cause destruction of the host tissue. In periodontal disease, tissue destruction results from the interaction of bacteria or bacterial substances with host cells which directly or indirectly lead to the degradation of periodontal tissues.

1.6.1 Role of *P. gingivalis* LPS in the pathogenesis of periodontitis.

*P. gingivalis* is a gram-negative rod that is strongly associated with chronic and aggressive periodontitis. Several studies suggesting a significant positive correlation between *P. gingivalis* numbers and pocket depth (Kawada et al. 2004) as well as a reduction of *P. gingivalis* numbers was associated with resolution of disease at the affected site following treatment (Haffajee et al. 1997; Fujise et al. 2002) confirmed the association of *P. gingivalis* with periodontal disease. Moreover, experimental implantation of *P. gingivalis* in animal models induces an inflammatory response and periodontal bone loss (Evans et al. 1992a; Hajishengallis et al. 2011). This species possesses a number of potential virulence factors, such as cysteine proteinases (gingipains), lipopolysaccharide (LPS), capsule and fimbriae (Offenbacher 1996; Lamont and Jenkinson 1998). The carbohydrate capsule on its outer surface prevents opsonisation by complement and inhibits phagocytosis and killing by neutrophils. *P. gingivalis* produces an array of toxins and proteolytic enzymes with the potential to degrade collagen and other constituents of the extracellular matrix. This bacterium
releases copious amounts of outer membrane vesicles containing LPS (Grenier et al. 1995), which can penetrate periodontal tissue (Schwartz et al. 1972; Moore et al. 1986; McCoy et al. 1987) and thus participate in the destructive innate host response associated with disease. \textit{P. gingivalis} LPS (Fig 1.3) has been considered to be an important pathogenic component in the initiation and development of periodontal disease (Hamada et al. 1994; Tobias et al. 1997) because bacterial LPS is known to be a potent stimulator of various biological responses such as bone resorption, polyclonal B-cell activation, inhibition of bone formation and fibroblast proliferation. LPS from \textit{P. gingivalis} induces monocytes-macrophages to secrete TNF-α (Shapira et al. 1994; Shapira et al. 1998) and can also induce tissue necrosis (Amar 1996; Champagne et al. 1996). \textit{In-vitro} studies have confirmed that whole bacteria and their respective isolated LPS yield similar responses (Darveau et al. 1991) and \textit{in vivo} studies have validated the important role of LPS in triggering inflammation in response to bacterial infection (Khan et al. 1998; Somerville et al. 1999; Haziot et al. 2001). All Gram-negative LPS’s are known to activate the complement cascade by the alternative pathway which in turn generates prostaglandins resulting in bone resorption, with recent studies demonstrating that pathogens may not simply undermine complement or TLRs (or both) as separate entities, but may also exploit their crosstalk pathways (Roy and Mocarski 2007; Lambris et al. 2008; Wang et al. 2010).
Fig 1.3: General structure of bacterial LPS: Lipid A, core region and specific O-chain. Rough type LPS(R) do not contain specific O-chain, semi rough (SR) contain a repetitive unit of specific O-chain and smooth type (S) contain two or more repetitive units of specific O-chains (Pupo E and Hardy E, 2009).
1.6.1.1 Receptors for LPS

PDL cells are not only structural elements of the periodontium, but actively influence immune responses by interaction with innate immune cells (Konermann et al. 2012b). Evidence suggests that fibroblasts, which are known to produce paracrine immune modulators, are crucially involved in inflammation control and in the regulation of immune responses (Smith et al. 1997; Svensson and Kaye 2006) by the synthesis of immunomodulatory cytokines that influence the local response to infections (Rizzo et al. 2010). The release of inflammatory mediators occurs as a consequence of activation of cells through a complex mechanism involving CD14 and TLRs, which are a class of LPS receptors, resulting in the signal transduction in LPS responsive cells (Fig 1.3).

Pathogen-host interaction takes place on release of the bacterial components resulting in activation of host cells such as neutrophils, monocytes, macrophages, fibroblasts which generate an inflammatory response. Recognition of bacterial components by the host is a specific mechanism and involves two main components namely pattern recognition receptors (PRRs) such as TLRs, CD14 and pathogen associated molecular patterns (PAMPs) such as LPS, genetic material and fimbriae. Once PRRs recognise PAMPs and bind to PAMPs, other extracellular and intracellular proteins are attracted to this complex to form the receptor cluster.
1.6.1.1.1 CD14 receptors

The CD14 molecule which is primarily expressed on macrophages was the first protein to be identified as an LPS receptor for initial bacterial recognition and reported to bind with LPS and mediate LPS induced cell activation (Wright et al. 1990; Tobias et al. 1995). Many lipid containing molecules including LPS, microbial lipoproteins and the walls of streptococcal molecules can bind to CD14 (Pugin et al. 1994). Two forms of CD14 molecules have been identified. One is the glycosylphosphatidylinositol (GPI)-anchored membrane CD14 (mCD14) and the other is the soluble form of CD14 (sCD14), which lacks the GPI structure (Bazil et al. 1989). CD14 expression has been correlated with increased sensitivity of a number of cells to LPS and other microbial molecules in their ability to activate downstream signalling events and cytokine production (Wright et al. 1990; Ulevitch and Tobias 1995; Wright 1995). On its own, CD14 is unable to send signals into the cells as it is a GPI anchor protein and lacks a membrane bound domain and intracellular domain (Wright et al. 1990; Ulevitch and Tobias 1995; Wright 1995). Toll like receptors, mainly TLR-2 and TLR-4 (Hirschfeld et al. 1999; Hajishengallis et al. 2002) act as a co-receptor to CD14. The observation that CD-14 deficient mice are hypo-responsive to LPS, strongly suggests that CD14 does play a very crucial role in this process, particularly in cellular activation and cytokine production (Haziot et al. 1998). These receptors have been identified on by several studies on gingival fibroblasts, periodontal fibroblasts and osteoblasts (Amano et al. 1997; Kadono et al. 1999; Hatakeyama et al. 2003; Scheres et al. 2011).
Fig 1.4: Toll-like receptors are involved in the recognition of various infective agents. TLR2+TLR1/TLR6 bind lipoproteins, TLR3 binds dsRNA, TLR4 binds LPS, TLR5 binds flagellin, TLR7 binds viral components and TLR9 binds bacterial DNA.
Fig 1.5: Toll-like receptor (TLR) signaling: stimulation of TLRs by periodontal pathogen associated molecular patterns triggers the association of myeloid differentiation primary-response protein 88 (MyD88), which in turn recruits IL-1 receptor associated kinase-4 (IRAK) which is activated by phosphorylation and then associated with TRAF6 (TNF receptor associated factor 6) leading to activation MyD88 dependent or MyD88 independent signalling pathway resulting in activation of NFκB resulting in gene activation and secretion of pro-inflammatory cytokines (Kirkwood et al. 2007).
However, studies by different groups (Sugawara et al. 1998; Wang et al. 1998) report inconsistent results on CD14 expression of fibroblasts. These inconsistencies could possibly be because of difference in the origin of these fibroblast cells. For example, periodontal ligament fibroblasts have an origin from the cells of the dental follicle whereas gingival fibroblasts have their origin from the enamel organ and therefore the variation in response to bacterial components. Their findings suggest that fibroblast cells are not a homogenous cell population and that there is wide variation among fibroblast cells with respect to form, proliferation rate, expression of membrane markers, function and other characteristics. In addition, gingival fibroblasts heterogeneously express different levels of CD14 (Sugawara et al. 1998), IL-10 receptor (Wang et al. 1999a) and Toll-like receptor (Wang et al. 2001) and can be separated into several populations. Gingival fibroblasts exposed to LPS from *P. gingivalis* respond by increasing the mRNA and protein levels of the cytokines IL-1α, IL-1β, IL-6, IL-8 and TNF-α as well as receptors CD14, TLR-2 and 4 (Wang et al. 2003). LPS on release from the bacterial cells form LPS aggregates due to the amphiphilic nature of the molecule. LPS binding protein (LBP) is a lipid transfer molecule found in normal and acute phase serum that catalyses the movement of the LPS monomers from the LPS aggregates to CD14 (Hailman et al. 1994). Once the LPS-LBP-CD14 interaction takes place, downstream signalling events are activated resulting in cytokine production (Wright et al. 1990; Ulevitch and Tobias 1995; Wright 1995).
1.6.1.1.2 Toll like receptors (TLRs)

Toll like receptors are a family of receptors (TLR1-TLR13) involved in the recognition of a wide range of microbial molecules, such as lipopolysaccharide (LPS) from Gram negative bacteria and peptidoglycan from Gram positive bacteria. These are expressed by myelomonocytic cells as well as endothelial, epithelial and various other cells including gingival fibroblasts (Wang and Ohura 2002). Within periodontal tissues, TLR-2 and TLR-4 expression appears to be increased in severe disease states (Mori et al. 2003).

Each receptor recognizes a small range of conserved molecules from a group of pathogens for eg: TLR-2 recognises peptidoglycan, bacterial lipoproteins and atypical LPS (Fig 1.3). The LPS/LBP/CD14/TLR complex activates the ‘downstream’ transcription factor (NF-κB) pathway resulting in the production of inflammatory cytokines and enhances the cells antimicrobial killing mechanisms and antigen presenting capacity. TLRs all contain a common extracellular leucine rich domain and a conserved intracellular domain (Akira 2003). The intracellular tail of the receptor was shown to be homologous with the intracellular domain of the Interleukin-1 receptor type 1. The interaction of TLR with the pathogen associated molecule (Fig 1.4) results in the recruitment of specific adapter molecules such as MyD88 and Mal, which then bind to IL-1R associated kinase IRAK. The signal is then transmitted through a chain of signalling molecules common to all TLR’s involving TNF receptor associated factor-6 (TRAF6) and mitogen–activated protein kinases (MAPKs). Thereafter, activation of nuclear kappaB (NF-κB) and activated protein1 (AP-1) leads to transcription of genes involved in the activation of the innate host defense resulting in release of proinflammatory cytokines (Fig 1.5).
1.7 Histopathological and immunopathological changes leading to periodontal tissue destruction

The progression of gingivitis to periodontitis has classically been described as progressing through a series of stages as described by Page and Schroeder, into initial, early and established stages leading to the advanced stage which is periodontitis (Page and Schroeder 1976). The ‘initial lesion’ is the response of the resident leukocytes and endothelial cells to the bacterial biofilm. The metabolic products produced by the bacteria in the biofilm trigger the production of cytokines and neuropeptides resulting in vasodilatation of local blood vessels. Further, neutrophils leave the blood vessels and migrate toward the blood vessel and migrate towards the site of inflammation in response to chemokines.

The ‘early lesion’ results from the inability of the activated host response to clear the infection and is characterised by an increasing levels of lymphocytes, macrophages, engagement of capillary vasculature and the development of a perivascular inflammatory infiltrate (Seymour et al. 1983). The lesion was described to be a perivascular lymphocyte/macrophage lesion with predominantly T-lymphocytes with a CD4:CD8 ratio of 2:1 (Seymour et al. 1988). Complement proteins are activated and histologically, the epithelium proliferates to form rete pegs. Clinical signs of gingival inflammation such as bleeding is present in addition to an increase in GCF flow.

The ‘established lesion’ is characterised by a predominance of plasma cells and B-lymphocytes, where an intense chronic inflammatory reaction is observed (Page and Schroeder 1976; Seymour et al. 1981). Plasma cells invade the connective tissue not only immediately below the junctional epithelium but also deep into the connective tissue, around blood vessels and between bundles of collagen fibers. The ‘advanced lesion’ is the progression of the established lesion into the alveolar bone characterised by an infiltrate consisting of monocytes and lymphocytes including T-cells, B-cells and emigrating neutrophils (Page and Schroeder 1976). Cytokines produced by these
cells and antigens from the bacteria drive differentiation of B cells to specific antibody producing plasma cells. Microbial factors such as LPS have an effect on the resident cells such as fibroblasts, epithelial cells, endothelial cells and also on the recruited cells such as neutrophils, monocytes, macrophages resulting in the release of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, which in excess amplify the inflammatory host response (Bartold and Narayanan 2006). As a result, osteoblasts and stromal cells are activated leading to an increase in RANKL expression, activation of osteoclasts and bone destruction (Fig 1.6).

Histological studies show that periodontal lesion consists of predominantly lymphocytes and inflammatory cells with T-lymphocytes predominating in the stable lesion, B lymphocytes and plasma cells predominating in the progressive lesion (Seymour et al. 1981; Cole et al. 1987). Immunological studies also suggest that cell mediated response may be suppressed in active disease concluding that the active lesion in chronic periodontitis is predominantly B-lymphocyte mediated (Seymour and Gemmell 2001). It has been suggested previously, that the progression of periodontal lesion is related to a shift from a Th1 to a Th2 response (Gemmell and Seymour 2004). The dominance of B-lymphocytes and plasma cells in the progressive lesion suggests that a change from Th1 to Th2 response may lead to the possibility of tissue destruction as a result of unregulated release of cytokines such as IL-1, IL-6 and TNF.
Fig 1.6: Pathogenesis of periodontal disease: In the presence of virulence factors, resident and recruited cells release cytokines which amplify the host response (A). Unregulated cytokine expression results in activation of osteoblasts and stromal cells takes place resulting in increased expression of RANKL thereby activating osteoclasts leading to destruction of hard tissues of the periodontium (B)(Kirkwood et al. 2007).
However, with the identification of the Th17/Treg subsets and its signature cytokines, periodontal pathogenesis is currently considered under the extended Th1/Th2/Th17/Treg paradigm in which disease progression is considered to take place due to an imbalance between pro-inflammatory and anti-inflammatory cytokines, with the Th cells differentiating towards Th1, Th2, Th17/Treg subsets based on the cytokine microenvironment.

If the innate response to a pathogen is poor, it will fail to control the infection which may then result in polyclonal activation of B-cells and the subsequent production of IL-4 which would then stimulate the development of a Th2 response (Gemmell and Seymour 2004). The disease will not progress if the antibodies generated by this response are protective and successfully clear the infection. If however, they are non-protective, the lesion will persist and continued B-lymphocyte activation may then lead to unregulated production of IL-1 with subsequent tissue destruction (Gemmell and Seymour 2004). Microbial products such as LPS activate monocytes or macrophages to produce vasoactive substances such as prostaglandin E₂, interferon, TNF and interleukin-1 (Page and Kornman 1997). Macrophages activated by lipopolysaccharide produce IL-1α, IL-1β, TNF-α, MMP’s and PGE₂. IL-1β and TNF-α activate resident fibroblasts to produce PGE₂ and MMPs. Both activated cell types decrease production of TIMP’s resulting in greatly increased levels of MMPs. This destroys components of the extracellular matrix, creating space for the enlarging inflammatory cell infiltrate. The microbial cell infiltrate may extend apically and laterally. The epithelial cells activated by LPS can produce MMPs which can destroy attached collagen fibers at the apical terminus of the junctional epithelium, allowing apical extension of the epithelium, formation of additional pocket epithelium and pocket deepening. As this occurs, MMP’s mediate clinical attachment loss and PGE₂ mediates resorption of the alveolar bone and the gingival pocket progresses to become a periodontal pocket. Once inflammation reaches the bone by extension from the
gingiva, it spreads into the marrow spaces and replaces the marrow with a leukocytic and fluid exudate, new blood vessels and proliferating fibroblasts (Takata and Donath 1988). Multinuclear osteoclasts and mononuclear phagocytes increase in number and the bone surfaces appear lined with Howship’s lacunae. In the marrow spaces, resorption proceeds from within, causing a thinning of the surrounding bony trabeculae and enlargement of the marrow spaces followed by destruction of the bone and a reduction in bone height. Fatty bone marrow is partially or totally replaced by a fibrous type of marrow in the vicinity of the resorption. As the pocket deepens, the flora becomes more anaerobic and the host response becomes more destructive and chronic. Eventually, the periodontitis lesion progresses to such an extent that the tooth is lost (Kirkwood et al. 2007).

The production of proteinases and mediators as well as their inhibitors by host tissue cells are influenced by bacteria and regulatory molecules produced by host cells that are resident within or recruited to the periodontal tissues. These mediators include cytokines, proteinases, and prostaglandins. Page et al. (2000), suggested that uncontrolled production of IL-1 was a major mediator of tissue destruction in periodontal disease and other inflammatory disease. Although in health macrophages are the major source, very few macrophages are present in the progressive lesion (Gemmell and Seymour 2004). The most likely source of IL-1 in periodontal disease seems to be the B-lymphocytes which are present in large numbers in the progressive lesion (Gemmell and Seymour 2004). Furthermore, it has been demonstrated that *P. gingivalis* can induce the release of IL-1 from B-lymphocytes (Gemmell et al. 1998) thus providing a link between the microorganism, cytokine production and disease progression.
1.7.1 Cytokines of the advanced lesion

The term "cytokine" includes interferons, the interleukins, the chemokine family, mesenchymal growth factors, the tumor necrosis factor family and adipokines. These are low molecular weight soluble proteins involved in the initiation and effector stages of immunity and inflammation, in which they regulate the amplitude and duration of the response. The complex cytokine network that mediates the immune response includes pro-inflammatory cytokines, anti-inflammatory cytokines and specific cytokine receptors (Opal and DePalo 2000). As in other chronic inflammatory conditions such as rheumatoid arthritis, cytokines are considered to play an important role in the initiation, progression and the host modulation of periodontal disease (Bascones et al. 2005; Salvi and Lang 2005). Their primary role is protective with the aim of restoring homeostasis. However, tissue damage may occur as an unwanted side-effect from the over production of these mediators leading to the so called “by-stander” damage. They are produced transiently, are extremely potent, generally acting in picomolar concentrations and interact with specific cell surface receptors which are usually expressed in relatively low numbers. T-cells and macrophages are the major source of cytokines, although they are produced by a wide range of cells that play important roles in many physiological responses. Some cytokines are produced by a restricted type of cell, such as IL-2 produced by T-cells whereas others including IL-1 and IL-6 are produced by many different cell types. Many cytokines are pleiotropic, having multiple activities on different target cells or overlapping cell regulatory actions but despite this overlap, cytokine functions may not be identical (Dinarello and Savage 1989). The response of a cell to a given cytokine depends on the local concentration, the cell type and other cell regulators to which it is constantly exposed. Cytokines interact in a network by inducing each other, by transmodulating cell surface receptors, by synergistic, additive or antagonistic interactions on cell function (Balkwill and Burke 1989). The network of interactions within the immune system is very complex and it is
believed that this complexity is essential for overcoming the various defence strategies of microorganisms and hence could help in the preservation of homeostasis as well as being involved in autoimmunity and the pathogenesis of chronic inflammatory diseases. Examples of cytokine interactions in chronic diseases include: rheumatoid arthritis (Duff 1993), connective tissue disease such as scleroderma, systemic lupus erythematosus (Blakemore et al. 1994); inflammatory bowel disease (Carter et al. 2001), periodontal disease and arteriosclerosis (Duff 1994).

IL-1 is a principal mediator of inflammatory responses acting on many cell types and it has been suggested that uncontrolled production of IL-1 plays a major role in tissue destruction in periodontal and other inflammatory diseases (Page et al. 1997). In active periodontal disease an increase in the amount of IL-1 (both α and β) in gingival tissues has been reported and levels can be reduced by treatment of disease (Masada et al. 1990). Moreover, in patients with periodontal disease, levels of IL-1 in the gingivae were reported to be greater than in the gingivae of healthy patients (Honig et al. 1989) and IL-1 levels in GCF were higher in sites with periodontal disease than from healthy sites (Mathur et al. 1996). Furthermore, studies have reported that IL-1 has the potential to induce many cell types to secrete prostaglandins, increase adhesion of leucocytes to endothelial lining and endothelial cell proliferation, stimulate fibroblast proliferation and collagenase secretion, stimulate macrophage and PMN activation, activate B and T lymphocyte for Immune response, induce many cell types to secrete cytokines and MMP’s and induce bone resorption (Dinarello 1996; Apte and Voronov 2002; Apte et al. 2006a; Apte et al. 2006b; Apte and Voronov 2008; Voronov et al. 2013). As a result of the above and the ability of IL-1 to induce its own secretion by some cells by a positive feedback mechanism, the inflammatory response may be amplified and thus allows a greater potential to increase ‘bystander damage’.
TNFα is a pleotropic cytokine produced primarily by macrophages in response to agents such as LPS (Clemens 1991) and has a wide variety of biological effects similar to IL-1 (Le and Vilcek 1987). TNF-α is a key mediator of chronic inflammation and has the potential to initiate tissue destruction and bone loss in periodontal disease. TNFα mediates tissue destruction by stimulating collagenase (Dayer 1992) and degradation of type 1 collagen by fibroblasts leading to connective tissue destruction. TNF-α synergises with IL-1 in stimulating bone resorption (Stashenko et al. 1991) and execute major changes in the connective tissue matrix (Qwarnstrom et al. 1989). TNF-α molecules stimulate bone resorption by inducing the proliferation and differentiation of osteoclast progenitors and activating formed osteoclasts indirectly (Mundy 1991). On its own, TNF-α is one hundred times less potent than IL-1 in bone resorption assays (Stashenko et al. 1987). However, along with IL-1, it stimulates a number of events associated with periodontal disease (Graves and Cochran 2003; Takashiba et al. 2003) such as stimulation of MMPs and bone resorption. The use of antagonists to IL-1 and TNF-α in experimental periodontitis have demonstrated a cause and effect relationship between their activity and disease progression, thereby suggesting that much of the tissue damage that occurs during the active stage of the disease is as a consequence of altered host response by excessive production of IL-1 and TNF-α in response to periodontal pathogens.

IL-6 is a pleiotropic cytokine with important roles in the regulation of the immune response, inflammation and haematopoiesis (Gabay 2006; Nishimoto and Kishimoto 2006). It is produced by both haemopoietic and non-haemopoietic cells and induces immunoglobulin secretion in preactivated B cells and thereby induces the final maturation of B-cells into high rate immunoglobulin secreting cells (O’Garra 1989). Like IL-1, it appears to have a major role in the mediation of inflammatory and immune responses initiated by infection or injury and has been shown to be a potent stimulator of osteoclast differentiation and bone resorption (Roodman 1992) and bone formation (Hughes 1993). It has been shown to reduce osteoblast activity by by inhibiting
osteoblast differentiation (Hughes and Howells 1993). Additionally, it induces the expression of TGF-β1 and angiotensin II, inducers of extracellular matrix component (Wang et al 2004, Omori et al 2004). In periodontitis, IL-6 plays an important role by activating osteoblasts and inducing bone resorption. Within inflammatory periodontal lesions, IL-6 is present in abundance and activates fibroblasts in the presence of soluble IL-6 receptor. It also stimulates gingival fibroblasts to produce collagenolytic enzymes resulting in tissue destruction (Takashiba et al. 2003). Additionally, it also stimulates T-cell differentiation and is important in maintaining the balance between the T-cell subsets. An association between IL-6 gene polymorphism at several positions (-174, -572, -1363) and periodontal disease has been reported in many studies (Trevislattato et al. 2003; D’Aiuto et al. 2004; Brett et al. 2005; Tervonen et al. 2007). However, the results are conflicting with regards to the specific genotype which is responsible for the risk of periodontal damage.

1.7.2 Role of T helper cell subsets

In investigating the pathogenesis of periodontitis, the immunoregulatory role of T cells has been highlighted in the last several years. T helper cells are lymphocytes within the tissues which regulate both the humoral and cell-mediated response via cytokines. The cytokines provide a precise mechanism for the control of the immune response so that a sufficient response is produced to deal with the offending pathogen. Naïve CD4+ T-cell are stimulated by antigen in the context of an antigen presenting cell to differentiate into specific effector cells (Gaffen and Hajishengallis 2008). In the presence of IL-12, a newly activated Th cell develops into a Th1 phenotype, characterised by the secretion of IFN-γ, which activates macrophages, cytotoxic T cells and NK cells, as well as driving anti-viral signals in target cells. IFN-γ also provides a positive feedback signal to reinforce Th1 development by up-regulating IL-12 receptor.
Signals from Th1 cytokines also inhibit Th2 and Th17 differentiation. Conversely, a newly activated Th cell when exposed to IL-4, differentiates to a Th2 phenotype which is characterised by secretion of IL-4, IL-5 and IL-13. IL-4 provides an analogous positive reinforcement signal to drive expansion of this lineage (Gaffen and Hajishengallis 2008). Th17 cells are driven to differentiate by TGF-β, IL-6, IL-1 and IL-21 (Weaver et al. 2007). IL-23 is a key expansion and pathogenicity factor for Th17. Th17 cells provide important immunity against extracellular pathogens through activation of innate inflammation as well as being the key Th cell contributors to autoimmunity in various settings (Gaffen et al. 2006; Kramer and Gaffen 2007). The Treg cell population are is driven to develop in opposition to Th17, since signals from TGFβ in the absence of STAT3 (via IL-6 and/IL-21) serve to drive this lineage. IL-2 plays an important role in expanding this lineage, while simultaneously inhibiting Th17 development (Laurence and O'Shea 2007; Laurence et al. 2007)
1.7.2.1 Pro-inflammatory cytokines of T-cell lineage in host response

1.7.2.1.1 Th1 subset

IFNγ - the main cytokine associated with Th1 type response has been reported to be present in increased levels in biopsies from periodontal lesions (Garlet et al. 2003b) and high levels have been associated in tissue obtained from patients with severe forms of periodontal disease (Honda et al. 2006; Dutzan et al. 2009b). Animal studies have supported that IFN-γ is involved in the inflammatory process and bone resorption in response to stimulation with \textit{A. actinomycetemcomitans} and \textit{P. gingivalis} (Baker et al. 1999; Garlet et al. 2008). However, contradictory results have been reported by in vitro studies which have reported IFN-γ to systematically inhibit osteoclastogenesis by the rapid degradation of the RANK adapter protein TRAF6 resulting in the inhibition of RANKL signalling and its subsequent osteoclastogenic events (Takayanagi et al. 2005; Ji et al. 2009). These results support the previous hypothesis postulated by Gemmell et al, that Th1 cells were associated with stable lesions and Th2 cells were associated with disease progression (Gemmell et al. 2007). However, other studies have reported IFNγ to stimulate osteoclast formation and bone loss in vivo via T cell activation or through chemo attraction of RANKL+ cells (Gao et al. 2007; Garlet et al. 2008; Repeke et al. 2010). In addition, a recent study has demonstrated that Th1 cells are an important source of RANKL in experimental periodontitis (Repeke et al. 2010).

IL-12, which is the major Th1 inducing cytokine has been reported to mediate proinflammatory response in mice on stimulation with \textit{P. gingivalis} (Sasaki et al. 2008). However, conflicting results have been reported in human studies where IL-12 levels have been reported to be lower in diseased than in health (Johnson and Serio 2005) and levels in GCF following therapy were low (Thunell et al. 2010). In view of these conflicting results, further studies are needed to determine the potential role of Th1 related cytokines in the immunopathogenesis of periodontitis.
1.7.2.1.2 Th2 subset

The main cytokine secreted by Th2 cells is IL-4 which is necessary for the commitment and further action of Th2 cells in disease in addition to B-cell stimulation (Murphy and Reiner 2002; Appay et al. 2008; Sallusto and Lanzavecchia 2009). In addition to IL-4, IL-6 is thought to contribute to B-cell differentiation and antibody production (Cronstein 2007). Studies have reported that the majority of B-cells in periodontitis lesions are RANKL+ (Kawai et al. 2006) and on stimulation with periodontal pathogens, B-cells produce RANKL (Han et al. 2009). These results indicate that the Th2 type responses present in progressive lesions have an increased number of B-cells which are potentially RANKL producing cells and therefore bring about tissue destruction (Gemmell et al. 2002b; Kawai et al. 2006). In addition, autoantibodies against periodontal tissue components have been described in patients with chronic and aggressive periodontitis (Koutouzis et al. 2009) and autoreactive B cells have been reported in inflamed periodontal tissue (Donati et al. 2009). These studies indicate a destructive role of Th2/B-cells in the progression of periodontitis.

1.7.2.1.3 Th17 subset

Th17 cell lineage are characterised by selectively secreting IL-17. These cells develop through cytokine signals distinct from and antagonised by the products of the Th1 and Th2 lineages. Th17 cells have the capacity to produce IL-6 and up regulate IL-1β and TNF-α production thereby generating an inflammation amplification loop with consequent increase of MMP and RANKL expression (Beklen et al. 2007). Studies have also reported the presence of Th17 cells in chronic periodontal lesions (Cardoso et al. 2009; Adibrad et al. 2012) and the cytokines associated with these cells have been detected in periodontal lesions (Takahashi et al. 2005; Vernal et al. 2005; Ohyama et al. 2009).
A recent study has reported that human periodontal ligament cells on stimulation with pro-inflammatory cytokines such as IFN-γ, IL-17 produced significant increase of immunomodulatory cytokines and induced recruitment of leucocytes (Konermann et al. 2012a). The above studies confirm that the Th1, Th2 and Th17 subsets have a potentially destructive role in the pathogenesis of periodontitis.

### 1.7.2.1.4 Tregs

Within the periodontal environment, Tregs have been considered to have a protective role in attenuating disease progression (Garlet et al. 2010). Tregs regulate the activation, proliferation and effector function of activated conventional T-cells, determining the outcome of several immunological settings, ranging from infectious diseases to immunopathology and autoimmunity (Appay et al. 2008; Sallusto and Lanzavecchia 2009). Treg cells secrete TGFβ and IL-10 and have a role in regulating other T-cell subsets and prevent autoimmunity by maintaining tolerance against self-antigens (Josefowicz and Rudensky 2009). Within the periodontal environment, immunohistological, molecular and flow cytometry analysis have characterised Tregs in the periodontal tissues by the expression of their phenotypic markers such as FOXp3 (Nakajima et al. 2005a; Cardoso et al. 2008). TGFβ, a Treg associated cytokine is involved in the regulation of cell growth, differentiation and matrix production. It also plays an immunosuppressive role by down regulation of pro-inflammatory mediators such as IL-1β and TNFα, MMP’s (Okada and Murakami 1998) and RANKL (Dutzan et al. 2009a; Dutzan et al. 2009b) thereby reinforcing its protective role in tissue destruction.
Fig 1. 7: Cytokine networks in periodontal disease (Preshaw and Taylor 2011). Resident and infiltrating cells in the periodontium respond to the presence of microbial factors by release of cytokines. Upregulated cytokine activity leads to vascular changes, PMN activation and migration, and ultimately, osteoclastogenesis and osteoclast activation. Furthermore, these cytokines activate the immune response resulting in differentiation of Th1/Th2/Th17/Treg cells which is dependent on the local cytokine milieu thereby contributing towards further destruction of the periodontal tissues.
1.8 Destruction of periodontal tissue

1.8.1 Degradation of extracellular matrix

Interaction between host and microbial factors such as LPS result in activation of the host inflammatory and immune response. As a consequence, pro-inflammatory cytokines such as IL-1, TNF, IL-6 are released, which in excess, amplify the inflammatory response resulting in destruction of the soft and hard tissues of the periodontium. Connective tissue destruction is mediated by several proteolytic enzymes such as serine proteases, MMPs, cysteine proteases or aspartic proteinases. The MMPs are proteolytic enzymes which are dependent on intrinsic Zn$^{2+}$ and extrinsic Ca$^{2+}$ and are divided into true collagenases (MMP1,8,13), gelatinases (MMP2,9) stromelysins (MMP3, 10,11,19), matrilysins (MMP7, 26), metalloelastase (MMP 12) and membrane anchored MMPs (MMP14,15, 16,24)(Renaud et al. 2003). These enzymes can collectively degrade all the components of the extracellular matrix including collagens, gelatine, elastin, fibronectin, laminin, entactin and proteoglycans. The MMPs are inhibited by their tissue inhibitors (TIMPs) and an imbalance of their secretion, activity, inhibition and functions may play a key role in oral and periodontal disease (Sorsa et al. 2004). Inflammatory cells, particularly PMNs are a major source of MMPs and thought to play a major role in the destructive lesion (Golub et al. 1995; Lee et al. 1995; Golub et al. 1998). Cysteine and serine proteases may also contribute to the extracellular matrix destruction and contribute towards activation of each other in the proteolytic cascades (Everts et al. 1992). Proteoglycan degradation is carried out mainly by stromelysins such as MMP-3, 10, 11 (Reynolds et al. 1994; Reynolds 1996). Additionally, reactive oxygen species (ROS) have also been shown to damage proteoglycans (Moseley et al. 1995). With regards to periodontal diseases, elevated levels of MMPs in periodontal lesions were demonstrated by immunocytochemical studies (Ryan et al. 1996). The ability of MMP inhibitors such as doxycycline to retard periodontal destruction further supports the possible destructive role of these enzymes.
in periodontal disease (Golub et al. 1995; Golub et al. 1998). Additionally, the degradation products of proteoglycans have been detected in GCF (Embery et al. 1982), MMPs, elastase, cathepsins B, D and G, tryptase, chymotrypsin and aminopeptidases have been found in gingival tissue and/or crevicular fluid (Meikle et al. 1986; Cox and Eley 1987, 1989a, b, c; Uitto et al. 2003). Furthermore, the activity of a number of these proteases in GCF have been positively correlated with the severity of chronic periodontitis and also significantly decrease following periodontal treatment (Cox and Eley 1992; Eley and Cox 1992a, b).

1.8.2 Bone resorption

Bacterial products such as LPS, as well as products released by the host during the inflammatory and immune response may affect the bone turnover by either promoting differentiation and activation of osteoclasts or by inhibiting bone formation by osteoblasts. The main host derived bone resorbing factors are the prostaglandins and cytokines which are generated as a result of immune and inflammatory response. In vitro studies have demonstrated that IL-1α and β (Gowen and Mundy 1986), TNFα and β (Bertolini et al. 1986), TGF (Tashjian et al. 1985) and PDGF (Tashjian et al. 1982) stimulate bone resorption. Additionally, IL-6 released by fibroblasts, endothelial cells and osteoblasts may stimulate the formation of osteoclasts from precursor cells (Lowik et al. 1989). In periodontitis, IL-1 and TNF are considered to be the most potent stimulators of bone resorption. Both IL-1α and β have been found in GCF from periodontal sites in nanomolar concentrations which are sufficient to cause bone loss in vitro (Masada et al. 1990) and significant levels of IL-1β have been detected in inflamed gingivae when compared to healthy sites (Honig et al. 1989). TNFα has also been detected in GCF but in low levels which are below those necessary for bone resorption in vitro (Rossomando et al. 1990). The two key mediators of osteoclastic
activity are the RANKL and its natural inhibitor, osteoprotegerin (OPG). RANKL is responsible for the induction of osteoclastogenesis and bone resorption, whereas OPG can directly block this action (Bostanci et al. 2007a). An imbalance between these two cytokines may cause a switch from the physiological state to enhanced bone formation or resorption, as these two cytokines play a crucial role in regulating the bone remodelling process. An immunocytochemical study investigating levels of RANKL/OPG in inflamed connective tissue from patients with periodontitis compared to healthy subjects reported a significant higher level of RANKL protein in diseased compared to controls. Conversely, OPG was found to be significantly reduced in diseased when compared to controls (Crotti et al. 2003). A clinical study investigating levels of RANKL/OPG in GCF from patients with mild, moderate and severe periodontitis and healthy controls reported an increase in RANKL concentration compared to OPG in patients with periodontitis, compared to healthy controls (Sarlati et al. 2012). Additionally, the ratio of the concentration of RANKL to OPG in GCF was found to be significantly higher in patients with periodontitis compared to healthy controls (Mogi et al. 2004). Another study reported that RANKL and OPG expression are differentially regulated in various forms of periodontitis and the relative RANKL/OPG ratio appears to be indicative of the severity of disease thereby suggesting that this ratio could be of diagnostic and therapeutic value in treatment (Bostanci et al. 2007c). However, recent work by this same group has reported that, since RANKL/OPG levels do not reduce post treatment, increased RANKL/OPG ratio may not necessarily predict on-going disease activity (Belibasakis and Bostanci 2012).
1.9 Resolution of inflammation

Periodontal disease may be considered as a failure of resolution pathways to control excessive inflammation. Inadequate resolution and failure to return to homeostasis results in neutrophil mediated destruction and chronic inflammation (Van Dyke and Serhan 2003), with destruction of both extracellular matrix and bone and subsequent scarring and fibrosis (Van Dyke 2007, 2008) which prevent the return to homeostasis. The resolution of inflammation is an active process that activate certain biochemical programs of resolution in which lipoxins, resolvins and protectins play an important role to counter-regulate proinflammatory signals through a number of complex intracellular processes. This process leads to the release of cytokines which stop neutrophil migration to the inflammatory site, attract monocytes that do not release proinflammatory mediators, enhance phagocytosis of bacteria and apoptotic cells by macrophages, direct the movement of phagocytes away from the site via the lymphatics and stimulate the synthesis of antimicrobial agents (Campbell et al. 2007; Serhan et al. 2008). Within this scenario, lipoxins are receptor agonists that promote resolution of inflammation by limiting the migration of neutrophils into sites of inflammation and modulating the phenotype of macrophages to stimulate the uptake of apoptotic polymorphonuclear neutrophils without secreting proinflammatory cytokines (Serhan et al. 1993; Maddox and Serhan 1996; Maddox et al. 1997). Resolvins, which have similar biological activity to lipoxins (Van Dyke 2007; Serhan and Chiang 2008) stimulate the resolution of inflammation by preventing neutrophil penetration, the phagocytosis of apoptotic neutrophils to clear the lesion and enhance the clearing of inflammation within the lesion to promote tissue regeneration (Bannenberg et al. 2005; Hasturk et al. 2007; Schwab et al. 2007).

The resolution of inflammation is a process commonly divided into three sequential phases characterised by inflammation, granulation tissue formation, and matrix formation and remodelling. Initially a neutrophil infiltrate decontaminate the wound by
phagocytosing injured and necrotic tissue. This stage is followed by a decrease in neutrophil and an increase in macrophages which contributes to wound debridement by removing effete red blood cells, neutrophils, and residual tissue debris and, in addition, has a conspicuous role including release of growth factors which support fibroblast proliferation and matrix production, smooth muscle cell proliferation, and endothelial cell proliferation and angiogenesis. IL-1ra, TGF-β play an important role in shutting down the inflammatory process. Other cytokines include IL-4, IL-10 and IL-11. IL-1α and β indirectly induce fibroblast proliferation and collagen synthesis by stimulating production of PDGF, TGF-β and PGE₂. PDGF activates fibroblasts and osteoblasts resulting in the induction of protein synthesis. TGF-β stimulates fibroblasts and inhibits osteoclasts, epithelial cells and most immune cells. It also promotes fibroblast extracellular matrix adhesion. Osteoclast differentiation and activation are inhibited by interferon-γ which acts by the inhibition of IL-1 and TNF-α induced osteoclast activation. The newly formed cell-rich tissue undergoes maturation and remodelling to meet functional demands.

1.9.1 Anti-inflammatory cytokines
The destructive process promoted by the pro-inflammatory cytokines may be counteracted by the regulatory pathways modulated by the anti-inflammatory cytokines. Cytokines implicated in the suppression of tissue destructive cytokines include IL-4, IL-6, IL-10, TGF-β and IL-8. IL-1RA is a member of the IL-1 gene family that binds IL-1 receptors without inducing apparent cell activation. It is produced by monocytes and PMN’s and competes with IL-1 for binding to IL-1 receptor on target cells, thus preventing the formation of the protein necessary for signal transduction and thereby inhibiting IL-1 mediated effects. It is thus an important physiological regulator of IL-1 activity.
IL-10, the prototypical anti-inflammatory cytokine (Pestka et al. 2004) has been reported to be widely expressed in periodontal tissues where it is thought to be associated with lower disease severity (Lappin et al. 2001; Garlet et al. 2004; Garlet et al. 2006). Studies have suggested that IL-10 can act in several ways to control the severity of periodontitis. Yoshimura suggested that the control of inflammatory signalling mediated by IL-10 may involve the inhibition of inflammatory mediator mRNA transcription after TLR or cytokine signalling and that this control could be exerted by the suppressors of cytokine signalling (SOCS), which act to attenuate signal transduction as part of a negative feedback loop to inhibit the response to subsequent stimuli (Yoshimura et al. 2003; Yoshimura et al. 2007). In addition, IL-10 has been reported to interfere directly with production of IFNγ and IL-17 production by T-cells (Naundorf et al. 2009) and therefore, it has been postulated that IL-10 may reduce the inflammatory signalling that leads to inflammatory and Th1 cytokine mRNA transcription which in turn could down regulate downstream pathways under its influence (Hosokawa et al. 2009). Additionally, IL-10 modulates the MMP’s and RANK systems by up-regulation of TIMPS (Garlet et al. 2004; Claudino et al. 2008) and OPG (Zhang and Teng 2006) thereby providing a direct protective role in tissue destruction. IL-10 has also been reported to suppress osteoclastogenesis (Park-Min et al. 2009) and present a direct effect over bone formation (Claudino et al. 2010). Furthermore, IL-10 has been shown to down-regulate IL-1 and TNF-α gene expression in human monocytes (Essner et al. 1989) and in polymorphonuclear neutrophils (Cassatella et al. 1993; Cassatella et al. 1994).

IL-4, a Th2 type cytokine presents marked anti-inflammatory and suppressive properties mediated by its capacity to inhibit the transcription of anti-inflammatory cytokines and IFN-γ (Agnello et al. 2003; Appay et al. 2008; Bluestone et al. 2009). It induces the production of cytokines with similar or complementary suppressive properties such as IL-10 (Pestka et al. 2004) and is able to inhibit production of MMP’s and RANKL and up-regulate TIMP’s and OPG (Ihn et al. 2002), thereby reinforcing its
protective role in periodontal disease pathogenesis (Giannopoulou et al. 2003). IL-4, like IL-10 has been shown to down regulate IL-1 and TNF-α gene expression in human monocytes (Essner et al. 1989) and to inhibit their secretion and that of another proinflammatory cytokine, IL-6 (te Velde et al. 1990). Furthermore, IL-4 has been shown to induce the death by apoptosis of IL-1 or LPS stimulated monocytes but not unstimulated monocytes (Mangan et al. 1992). Human monocytes contribute to both the persistence and resolution of chronic inflammation and the regulation of the production of monocyte mediators may have great value in healing or reducing the immunopathogenesis of chronic inflammation (Mangan et al. 1993). Recent studies have reported increased levels of IL-4 and IL-10 in GCF of healthy controls compared to GCF from patients with rheumatoid arthritis or chronic periodontitis and higher levels of IL-4 and IL-10 in patients with rheumatoid arthritis than in chronic periodontitis possibly due to the regular use of NSAID’s by the rheumatoid arthritis group (Bozkurt et al. 2006; Cetinkaya et al. 2012).

TGF-β is produced locally at the site of resorption of bone and has been shown to initiate new bone formation, an IL-1 inhibitor and acts by reducing the constitutive or induced level of IL-1 receptors (Centrella et al. 1988). IL-8 is produced by a wide variety of cell types including PMN’s, monocytes, macrophages, fibroblasts and keratinocytes in response to microorganisms, mitogens and endogenous mediators such as IL-1 and TNF. One of the main functions of IL-8 is its ability to induce the directional migration of cells including PMN’s, monocytes and T cells thus playing a key role in the accumulation of leukocytes at sites of inflammation (Nicola NA 1994).

As the primary effector cells in acute inflammation, PMN’s synthesize and release inflammatory cytokines and thereby modulate both T and B cell function. IL-11 has been shown to have an anti-inflammatory effect by inhibition of TNF-α and other cytokines (Trepicchio et al. 1996). It indirectly minimizes tissue injury through stimulation of TIMP-1 (Leng and Elias 1997). Subcutaneous injection of recombinant
IL-11 significantly reduced periodontal attachment loss in ligature induced periodontitis in an animal model (Martuscelli et al. 2000). Further studies are needed to evaluate its potential use in treatment of periodontal disease.

In addition to its role in tissue destruction, recent studies have demonstrated the role of some pro-inflammatory cytokines such as TNF-α, IFN-γ in the control of infection. TNF-α plays a critical role in both innate and adaptive immune responses, upregulating antigen presentation and the bactericidal activity of phagocytes (Dinarello 2000). IFN-γ contributes towards leucocyte recruitment and its subsequent activation and is considered to be the main phagocyte activating cytokine by enhancing phagocytosis and antigen uptake and stimulating the production of inflammatory cytokines and chemokines and microbial molecules. Both TNF-α and IFN-γ play an important role in the control of bacterial load as demonstrated by the increased bacterial load and acute phase response presented by TNFp55-KO and IFNγ-KO mice to A. actinomycetemcomitans induced periodontal disease (Garlet et al. 2007; Garlet et al. 2008). Recent evidence have demonstrated the role of Th17/IL-17 axis by itself or along with pro-inflammatory and Th1 cytokines in the mobilization of macrophages and neutrophils against extracellular and intracellular pathogens (Silva 2010). In addition, Th2/B cells are also thought to contribute to host protection against periodontal pathogens by production of antibodies thereby facilitating phagocytosis by opsonisation and enhancement of the phagocytes' bactericidal activities (Guentsch et al. 2009).

Due to several conflicting results as reported above, it was suggested that the host inflammatory immune response present a dual role in that, cytokines although required to control infection, may also generate an inflammatory response and thereby bring about tissue destruction (Garlet 2010). Although the individual effects of cytokines and their response to periodontal pathogens are investigated in highly controlled systems, one must bear in mind that within the periodontal environment these cytokines are in a complex milieu and function in complex networks in the presence of other cytokines.
which can modulate them or be modulated by them in several ways. However, most of the studies published that have reported on the local levels of cytokines in different periodontal conditions have focussed on one cytokine at a time and have used inadequate sample number. The results from these studies may be misleading and therefore limit our progress in understanding the immunopathogenesis of periodontal disease, as we are now aware that there exists links between the innate and adaptive immune systems and that these cytokines have multiple, overlapping and complex functions and involve recruited as well as cells resident within the periodontium.
1.10 Gingival crevicular fluid

Gingival crevicular fluid is a serum transudate that exists in the healthy gingival crevice. In the presence of periodontal diseases, the volume of this fluid increases and it is then considered to be an inflammatory exudate. In the presence of plaque induced inflammation, enlargement of the intercellular spaces of the junctional epithelium (Freedman et al. 1968) as well as partial destruction of the basal membrane occurs (Cergneux et al. 1982), which allows inward movement of bacterial products to create an osmotic gradient. The resulting osmotic pressure draws interstitial fluid from the surrounding capillaries and lymphatics. Consequently, as the production of fluid by the capillaries exceeds its uptake by the lymphatics, oedema results leading to an increase in GCF. As this ultrafiltrate passes from the tissues into the gingival crevice, it accumulates elements of cellular activity from both the bacterial as well as host tissue, which may be collected by non-invasive means by placement of adsorbant paper strips or a capillary tube at the gingival margin. Therefore, GCF contains a rich array of potential biomarkers derived from host and bacterial origin, a number of which may have value as potential diagnostic or prognostic markers of the periodontium in health and disease (Embery and Waddington 1994). Additionally, the very popular use of GCF in the search of biomarkers is due to the easy access to the body fluid, non-invasive method of sampling and the site-specific nature of the sample which allows laboratory investigations of GCF constituents to be linked to clinical assessments at the site of sample collection.

Components of GCF include multiple proteins, including serum proteins, immunoglobulins, enzymes, periodontal tissue-derived proteins, and inflammation-related proteins, cytokines and bacterial proteins (Embery and Waddington 1994; Griffiths 2003; Utito et al. 2003). Albumin, macroglobulin and immunoglobulin are major proteins present in gingival crevicular fluid (Embery and Waddington 1994; Ozmeric 2004). Enzymes, including lysozyme, alkaline phosphatase, MMPs, aspartate
aminotransferase, lactate dehydrogenase and cathepsins, as well as matrix proteins, such as fibronectin, proteoglycans and osteocalcin, have also been identified in gingival crevicular fluid (Embery and Waddington 1994; Eley and Cox 2003; Giannobile et al. 2003; Ozmeric 2004). Many bacterial products, host inflammatory and immune products, connective tissue degradation products and bone resorption products in GCF have been investigated with a view to identify markers that will provide us with information of the cellular events in the underlying tissues of the periodontium.
1.11 Biomarkers for elucidation of pathology

A biomarker is a substance that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to therapeutic intervention. Because fluids like saliva and GCF can be easily collected and contain locally and systemically derived markers of periodontal disease, they may offer a patient specific biomarker assessment for periodontitis and other systemic disease (Ozmeric 2004). Several fluid based tests have been developed using serum, saliva and GCF to assist in the diagnosis and prognosis of the disease. These tests have been reported to detect the presence of periodontopathogens and their host derived enzymes, inflammatory mediators and tissue breakdown products.

1.11.1 Bacterial products in GCF

Bacterial proteases are released in the pocket and can be detected in the GCF (Cox and Eley 1989a). Commercial assays have been developed for the development of bacterial sulphide (Perio 2000), trypsin like protease activity from *P. gingivalis* (Perioscan), non-specific neutral proteases (Periocheck); elastase (Prognostick) and β-glucoronidase (PeriGard). However, there are several disadvantages of using bacteria and their products. In the main, most are not predictive of disease activity. The polymicrobial nature of the disease and the complex nature of the subgingival flora which may vary from site to site and patient to patient make it difficult to select any specific bacterial species to assay as a marker. Additionally, some of these markers may equally be derived from mammalian and bacterial cells may have to be sent away to special labs for analysis and may also have cost implications.
1.11.2 Inflammatory and immune markers

Cytokines, arachidonic metabolites such as PGE$_2$, antibodies, are potential candidates suitable as markers. An early study by Offenbacher et al, has reported PGE$_2$ levels in GCF to have an overall predictive value of 0.92-0.95 (Offenbacher et al. 1993), thereby suggesting that it could be predictive of periodontal disease activity. Cytokines such as IL-1, IL-6, TNF have been evaluated in GCF and show promise. However, as the predictive ability of these cytokines is questionable, further research is needed to investigate the potential of these cytokines as markers. Additionally, these markers are associated with gingival inflammation and therefore could possibly be wrongly associated with disease activity. These issues will be discussed further in Chapter 5 and 6.

1.11.3 Hydrolytic and cytosolic enzymes

Several enzymes have identified as potential markers. Among these, longitudinal studies have demonstrated cathepsin B (Cox and Eley 1992), elastase (Eley and Cox 1990), dipeptidyl peptidase (Eley and Cox 1995) and β-glucuronidase (Lamster 1992) to be predictive of periodontal disease progression. Collagenase (Sorsa et al. 2004), trypptase (Eley and Cox 1990), alkaline phosphatase (Chapple et al. 1994), arylsulfatase (Page 1992), myeloperoxidase (Over et al. 1993) and aspartate aminotransferase (Persson et al. 1990a) have been shown to be associated with disease severity and activity. However, these are not predictive of disease activity. As mentioned above, these markers are also associated with inflammation and therefore could possibly be wrongly associated with disease activity. The above markers have been associated with disease activity but do not predict it. Commercially available tests include Periocheck (collagenase), Prognostik (elastase) and Periogard (AST).
1.11.4 Markers of connective tissue degradation

Collagens, proteoglycans, fibronectin have been investigated for the potential use as markers and will be discussed further in Chapter 5. Carboxyterminal telopeptide (CTP), of type I collagen which makes up 90% of the organic matrix of bone has been detected in GCF in patients with periodontitis as well as in experimental periodontitis in dogs (Talonpoika and Hamalainen 1994; Giannobile et al. 1995). In addition, periodontal treatment reduced the GCF CTP levels to that of healthy controls. However, large variations were observed in the amount of GCF CTP found in individual patients and at individual sites within each patient (Talonpoika and Hamalainen 1994). GAGs were detected in GCF samples from sites with periodontal disease as well as teeth undergoing orthodontic treatment (Embrey et al. 1982; Last et al. 1985). Based on cross-sectional studies, its presence in GCF has been correlated with those clinical conditions in which degradation changes are occurring in the deeper tissues (Last et al. 1985; Waddington et al. 1996). Although a potential marker of disease activity, no longitudinal studies have been carried out to correlate GCF GAG levels to disease activity. Additionally, cellulose acetate electrophoresis is time consuming and technique sensitive, therefore not suitable for chair-side use.

Bone specific proteins such as osteonectin, bone phosphoprotein and osteocalcin have also been investigated. GCF osteonectin and bone phosphoprotein levels have been shown to increase in line with site probing depth (Bowers et al. 1989). However, longitudinal studies on these proteins have not been reported. Studies on osteocalcin levels in GCF have reported that the total amount at diseased sites were significantly higher than those at healthy or gingivitis sites. Nonetheless, its value as a biomarker has been questioned due to the apparent high levels of circulatory osteocalcin and the inability to distinguish between alveolar bone resorption and remodelling of other
skeletal bones within the body (Waddington and Embery 2001). These markers are discussed further in Chapter 5. Although GCF contains a rich array of potential biomarkers, a diagnostic test that will demonstrate high predictive value for disease progression, will have a proven impact on disease incidence and prevalence and is simple, safe and cost-effective, is sought that will aid clinicians in the management of periodontal disease. However, given the complex nature of periodontal disease, it is unlikely that a sole biomarker exists for disease detection and disease prediction (Taba et al. 2005; Loo et al. 2010). Rather, the use of a panel of host biomarkers and periodontal pathogens may further aid diagnosis and prognosis (Ramseier et al. 2009).

### 1.12 Aims

Against this background, the aim of this thesis is to further our understanding of the biological processes involved in periodontal disease pathogenesis that will justify implementation of biomarkers to aid management of patients with periodontal disease. Specifically, this thesis will investigate:

1. An increase in CS levels in GCF of patients with chronic periodontitis as a marker of disease activity: a longitudinal study.
2. The effects of *P. gingivalis* LPS on periodontal ligament cell behaviour, TLR expression and cytokine expression through a series of *in-vitro* studies
3. Altered cytokine profile in the GCF of patients with periodontal diseases: a cross-sectional study.
Chapter 2

An increase in proteoglycan metabolites in GCF represents periods of disease activity: a longitudinal study in patients with chronic periodontitis.

2.1 Introduction

The diagnosis, prognosis and treatment planning of patients presenting with periodontal diseases is currently based on clinical findings generated using a periodontal probe, supplemented by radiographic assessment. After initial cause related therapy, parameters such as probing depths, clinical attachment levels, bleeding on probing, suppuration are monitored, to determine need for further treatment which may include subgingival re-instrumentation with or without adjunctive antimicrobials or periodontal surgery. Several studies have reported that increase in probing depths, high proportions of persisting deep sites and residual suppuration are considered as indicators of a lack of periodontal stability and are predictors of future attachment loss on a subject level (Badersten et al. 1990; Claffey et al. 1990; Kaldahl et al. 1990; Claffey and Egelberg 1995). However, a systematic review to assess the predictive value of residual probing depths (PD), bleeding on probing (BOP) and furcation involvement (FI) in determining further loss of attachment and tooth loss following initial cause related therapy has failed to confirm the validity of most of these clinical parameters for predicting future disease activity (Renvert and Persson 2002).

The diagnostic ability of clinical parameters such as bleeding on probing may be hampered by several factors such as errors arising from variations in probing force or the inflammatory status of the periodontium (Lang et al. 1991; Karayiannis et al. 1992) or due to the suppressive effects of smoking (Dietrich et al. 2004; Johannsen et al. 2014). In addition, pregnancy, systemic conditions, such as uncontrolled diabetes, haematological conditions, autoimmune disorders, infections, medications invariably have an effect on the periodontal tissues (Genco
and Borgnakke 2013; Alani and Seymour 2014; Chapple and Wilson 2014). The diagnostic evaluation therefore, only helps in the identification and quantification of current clinical signs of inflammation, as well as the severity and extent of previous historical damage, but does not identify patients with active tissue destruction at the time of examination or predict future disease progression or response to therapy.

Therefore in recent years, research has focussed on furthering our knowledge of the pathogenesis of periodontitis that will enable us to implement the use of markers of host as well as microbial origin in the management of patients with periodontal disease. Qualitative and quantitative changes of components present in GCF may provide us with valuable information which may be of prognostic and diagnostic value, that will enable clinicians to identify high risk patients, detect disease activity and evaluate response to treatment. Since considerable structural and metabolic change occurs in the periodontium during the active phase of periodontal disease, much research has focussed on the detection of components of extracellular matrix degradation released into GCF as potential markers.

Cross-sectional studies have investigated components of periodontal tissues such as fibronectin (Talonpoika et al. 1993; Huynh et al. 2002; Brajovic et al. 2010), type 1 collagen carboxyterminal telopeptide (Talonpoika and Hamalainen 1994; Al-Shammari et al. 2001; Reinhardt et al. 2010), protein components more closely associated with the extracellular matrix of bone such as osteonectin, bone phosphoprotein (Bowers et al. 1989), osteocalcin (Kunimatsu et al. 1993; Nakashima et al. 1994; Nakashima et al. 1996; Griffiths et al. 1998; Becerik et al. 2011) in GCF from patients with periodontal diseases. Nonetheless, the value of some of these such as fibronectin, osteocalcin has been questioned due to the apparent high levels in the circulation and the inability to distinguish between alveolar bone resorption and remodelling of other skeletal bones within the body (Waddington and Embery 2001). A major limitation with most of these extracellular matrix components is firstly, the difficulty in differentiating between gingivitis and periodontitis. Gingivitis is characterised by soft tissue inflammation and is reversible, unlike periodontitis where there is additional bone loss and is
irreversible. Secondly, these matrix components are not able to differentiate between sites that are in a state of active tissue destruction and therefore need treatment, compared to inactive sites where no treatment is required. As a consequence, no additional information has been obtained by monitoring these markers other than that obtained by the traditional clinical examination. Due to the site specific and episodic nature of the disease, where patterns of disease progression in susceptible patients is varied and unpredictable, it is essential that longitudinal monitoring of the component is carried out to definitively test that the marker level correlates with attachment loss. Such data is not available for these components.

Much research has focussed on the proteoglycans present in the extracellular matrix of the mineralised connective tissue of the periodontium as a possible biomarker of disease activity. Initial studies by Last et al (1985), examined GCF from individual sites of defined conditions variously affecting the periodontium. High levels of sulphated glycosaminoglycans (sGAGs) were reported in GCF from untreated early and advanced periodontitis, but not in sites with chronic gingivitis or sites that were treated surgically or with daily use of chlorhexidine. Of note, hyaluronan, a major component present in soft and mineralised connective tissue in the body and seen in significant proportions in serum, was consistently present in all samples. In addition, sGAG levels were detected in GCF samples from the control situations of active orthodontic tooth movement, onset of traumatic occlusion and the early healing stages of tooth extraction socket (Last et al. 1985). Interestingly, dermatan sulphate, a component of the soft tissues of the periodontium was not detected in any of the samples, thereby indicating that the GAG detected was a component of the alveolar bone and reflect possible changes in the deeper periodontal tissue (Last et al. 1988). These initial findings supported the hypothesis that the elevated levels of sGAGs present in the GCF samples originate from the hard connective tissue and therefore may be a potential marker for bone loss.

Cross sectional studies investigating GAG levels in GCF, reported that the total GAG content in GCF of patients with periodontitis were higher than in patients with gingivitis or healthy sites
(Giannobile et al. 1993) with sGAG levels elevated in sites with advanced periodontitis compared to healthy sites (Okazaki et al. 1995). These studies confirmed the association between sGAG levels and disease severity and activity. Smith et al (1995) investigated sGAG levels before and after periodontal treatment and reported significantly higher levels of sGAGs at diseased sites prior to treatment, correlating with increased pocket depth or attachment levels. Non responding sites had significant levels of sGAGs compared to responding sites post treatment (Smith et al. 1995) further indicating that sGAG levels reduce following disease resolution.

Subsequent studies examined the association between sGAG levels and underlying changes in the alveolar bone by examining GCF from patients undergoing orthodontic treatment and from peri-implant fluid around endosseous implants. In patients undergoing orthodontic treatment, initial studies detected sGAGs in GCF from sites towards which tooth movement was being directed by an orthodontic appliance (Last et al. 1985; Last et al. 1988). Further longitudinal studies reported increased levels of sGAGs in the pre-treatment phase involving rapid movement in patients undergoing orthodontic canine retraction (Samuels et al. 1993) and increased levels of sGAGs in teeth undergoing active tooth movement compared to the retention stages (Baldwin et al. 1999). The variations in GAG levels mentioned above may reflect the tissue resorptive state of the underlying periodontal tissues during the different stages of orthodontic treatment. In a longitudinal study of patients with dental implants, peri-implant fluid was collected at 1-2 weeks, 5-6 weeks and 3 months post insertion and again at 2 weeks and at 4 months after occlusal loading. Higher sGAG levels were detected around failing implants compared with the healing and loading stages of successful implants (Last et al. 1991). In addition, sGAG levels were found to be lower in peri-implant fluid collected from established functional implants than at the stages shortly after surgical exposure or full loading of implants (Last et al. 1995). However, as a serum component, hyaluronan content showed no significant changes. These changes in sGAG levels may reflect bone resorptive activity as well as remodelling of the supporting alveolar bone.
The above clinical studies correlated the presence of sGAGs with various clinical conditions and confirmed its presence with bone remodelling. Subsequent biochemical and immunological studies were carried out to identify these components in GCF and thereby support its use as a marker. Among the GAG’s, chondroitin sulphate (CS) was found to be the principal GAG in cementum and alveolar bone (Bartold et al. 1988; Bartold 1990). Characterisation studies by Waddington and Embery on human alveolar bone, identified chondroitin 4 sulphate (C4S) as the predominant glycosaminoglycan constituent within human alveolar bone along with much lower amounts of chondroitin 6 sulphate (Waddington et al. 1989; Waddington and Embery 1991), with the additional presence of heparin sulphate, dermatan sulphate (DS) and hyaluronan associated with the non-mineralised portions of the matrix. Further analysis of the proteoglycan species demonstrated small molecular weight proteoglycans confirmed to be decorin and biglycan (Waddington et al. 1998).

Chondroitin 4 sulphate proteoglycan with a molecular weight of 55-65 kDa was identified in GCF from sites with advanced periodontal disease and from teeth undergoing orthodontic treatment (Waddington et al. 1994; Waddington et al. 1998). Decorin and biglycan with molecular weight in the range of 200-90 kDa with separate core protein of 50kDa each were demonstrated on match analysis of human alveolar bone proteoglycan (Waddington et al. 1998). The difference observed in the molecular weight is suggestive of it being a degradation product in GCF. In addition, hyaluronan was also detected in the GCF from various clinical conditions. However, comparative analysis failed to confirm the presence of dermatan sulphate, which is a component of the soft tissues of the periodontium. Its absence in GCF may reflect a high metabolic turnover of the soft tissues of the periodontium.

Although extensive basic and clinical research has been carried out to evaluate the use of sGAGs as a potential marker for disease activity, most of the information has been collected from cross-sectional studies. Nonetheless, the data obtained from cross-sectional studies may be misleading due to relating to inadequate clinical indices which are unable to identify...
areas of active tissue destruction and therefore, a need for further longitudinal studies were addressed.

Therefore, a preliminary longitudinal study, to evaluate the relationship of sGAG in GCF to attachment loss was carried out on a group of 10 patients with chronic periodontitis (Waddington et al. 1996). GCF was collected from two deep sites at baseline and then every three months for 21 months. Increased sGAG levels were reported in active sites (showing attachment loss of at least 1.5mm) compared to control sites (which showed no attachment loss for 6 months or more). This study suggested that elevated levels of sGAG may indicate active destruction of the underlying connective tissue of the periodontium (Waddington et al. 1996) and also provided sufficient information to determine sample size for a further longitudinal study.

Against this background, the aim of this chapter was to further validate the use of sGAG as a suitable marker of active tissue destruction by means of a longitudinal study. Furthermore, in establishing its use as a marker, we also wish to investigate if the marker represented a product released continually within the sampling period and thereby further our understanding of the disease pathogenesis.
2.2 Materials and Methods

R & D approval was obtained from the Joint Trust / University Peer & Risk Review Committee. (Project ID: 07/DH/3925- copy provided in Appendix-A). Ethical approval was provided by South East Wales Local Research Ethics Committee (08/WSE03/3- copy provided in enclosed CD). The study was conducted as per Good Clinical Practice regulations and informed consent was obtained from each patient.

2.2.1 Longitudinal monitoring of proteoglycan metabolites in GCF.

The sample size for the longitudinal study was determined with the help of professional statistical advice (Professor Newcombe, Cardiff University) using data from a previous pilot study (Waddington et al, 1996). Within this previous study, GCF was collected from two deep sites from 10 patients every three months over a period of 21 months to determine GAG levels. Attachment levels were also recorded at these visits. Statistical analysis to evaluate presence of marker with disease activity was carried out. 10% of the samples were identified as being active (demonstrating attachment loss of at least 1.5mm) at the point of sampling and 8.75% identified with confidence as being inactive (no attachment loss for a minimum of 6 months). Based on this data, a power analysis was carried out to determine adequate sample size as 50 patients. To account for dropouts during the 21 month period, the sample size was increased to a total of 70 patients. 70 new patients with a clinical diagnosis of chronic periodontitis were recruited from patients referred to the Restorative Clinic at Cardiff University School of Dentistry.
2.2.1.1 Validation of methodology

2.2.1.1.1 Inclusion/Exclusion Criteria

Patients between the age group, 25-55, with clear medical history were recruited to the study. Exclusion criteria included pregnancy, any medical conditions or medications that may have an effect on the periodontal health, intake of any antibiotics or anti-inflammatory drugs in the previous six months or a history of periodontal therapy in the previous six months. Patients were recruited between October 2008 and November 2009. Follow up examinations were completed by October 2011. A clinical examination supplemented with radiographic evaluation was carried out to identify suitable patients with chronic periodontitis and two deep sites were selected for sample collection and longitudinal monitoring. Sample collection and clinical parameters were measured at initial visit, and then again every three months after treatment for a period of 21 months.

2.2.1.1.2 Sample collection

Following isolation of the selected teeth with cotton rolls and high volume suction to prevent contamination with saliva, supragingival plaque was gently removed, the tooth air dried and GCF was collected using small diameter 2μl capillary tubes (Drummond Microcaps, Drummond Scientific Co, Pennsylvania, USA) placed at the gingival margin for a period of 10 minutes (Fig 2.1). Fluid accumulated into the tube by capillary action. When the tube became full or blocked due to plaque debris it was refreshed with a new tube. GCF was collected prior to clinical measurements to ensure collection of GCF reflecting disease activity rather than the exudate released as a response to inflammation caused by probing. Samples visibly contaminated with excessive blood were discarded. GCF volume was determined using the formula:

\[
\text{Volume of GCF} = \frac{\text{Linear distance the fluid collected in the tube}}{32} \times 2 \mu l \quad \text{(total volume of tube)}
\]
The samples were immediately placed in 1.5ml eppendorf tubes and stored at −80°C, under HTA regulations, until further analysis.

2.2.1.3 Measurements of clinical parameters

2.2.1.3.1 Florida Probe

Clinical parameters were recorded using the Florida Probe (Florida Probe with PASHA probe-pressure controlled, automated, standardised handpiece from Florida Probe Corporation, Gainesville, FL, USA). The Florida Probe is equipped to measure to the nearest 0.2mm and utilises a constant probing force of 0.2N. The probe is able to measure automatically the Probing Depth as the distance from the tip of the probe inserted at the base of the pocket to the flange of the probe at the gingival margin. Recession, measured as the distance from the gingival margin to the cement-enamel junction, was recorded on withdrawal of the flange from the gingival margin. The Clinical Attachment Level was calculated at each site as the sum of probing depth and recession. Probing depths and recession were recorded at six points per tooth. Bleeding on probing and suppuration were recorded as present or absent for each site after probing. The probe was calibrated for each individual patient prior to recording indices. All measurements and sample collection were carried out by a single operator to ensure reproducibility, following which the patients underwent a hygiene phase which included oral hygiene instructions and scale and polish. Oral hygiene instructions were repeated until sufficient plaque levels were achieved following which, root surface debridement was carried out for pockets > 4mm under local anaesthesia. Full mouth root surface debridement was completed within 10 days.
2.2.1.1.3.2 Investigator calibration

A one day Florida Probe training session using models was carried out by the local representative following which an intra-examiner calibration was carried out to ensure reproducibility. Probing depths and recession were recorded at mesio-buccal, mid-buccal and disto-buccal points from 2 selected sites from 10 individuals. These measurements were repeated again within 24 hours and statistical analysis to determine Kappa value was carried out to ensure reproducibility.

2.2.1.1.3.3 Longitudinal assessment of periodontal health

On completion of the treatment, patients were recalled at 3 monthly intervals over a 21 month period for maintenance therapy. At each of these recall visits, GCF samples from the 2 designated sites were collected, followed by recording of periodontal indices using the Florida Probe as described above. Sites that demonstrated a clinical attachment loss ≥ 2mm over 3 months were presumed active and sites that remained static over a period of 6 months were considered as stable sites. Oral hygiene instructions were reinforced followed by subgingival debridement of sites which bleed on probing.

2.2.1.1.4 Evaluation of release of sGAG in GCF during collection period.

Current practice for collection of GCF is to use capillary tubes over a timed 10 minute period. However, it is not clear whether the biomarker detected represents a pooled accumulation of the GAG within the gingival pocket or GAG released into the GCF over a timed period of 10 minutes.

To evaluate the release of biomarkers, 15 patients with chronic periodontitis were recruited from patients referred to the Restorative Clinic at Cardiff University School of Dentistry. Inclusion and exclusion criteria were as above (Section 2.2.1.1.1). GCF was collected from 2
sites over a period of 10 mins each. The sampling period of 10 minutes was divided into 3 time points as the first 4 mins, followed by second 3 minutes and the final 3 minutes. GCF was collected using fresh micro-capillary tubes at each time point. Analysis of the GAG component by cellulose acetate electrophoresis was carried out as detailed in section 2.2.1.1.5. below. Intensity levels of the bands were compared over the three time points to detect significant change in levels.

2.2.1.1.5 Cellulose Acetate Electrophoresis (CAE)

Cellulose acetate electrophoresis technique was used to identify the presence of CS in the GCF or serum samples. This technique, originally described by Stanbury and Embery (1977) and further modified by Waddington (1988), separates the GAG components on the basis of their charge density and has therefore been used as a technique for the separation and characterisation of GAG chains.

Cellulose diacetate sheets (Electrafor, Cellulose Diacetate, Shandon Southern Ltd) were marked at midline (7.5cm) to divide the sheet into two equal halves. At 1cm away from the midline (6.5mm), a line of origin was drawn for application of samples. The sheets were immersed in running buffer (0.2M calcium acetate buffer, pH7.2), blotted from excess buffer and positioned centrally (7.5cm) in the electrophoresis apparatus (Shandon low voltage electrophoresis apparatus, Shandon Southern Instrument Ltd, Cheshire, UK) with the line of origin lying towards the cathode. Electrical contact was made using filter paper wicks (Whatman Chromatography Paper 1CHR). Samples were applied onto the electrophoresis sheets along the line of origin positioned 6.5cm from one end. The samples were placed within 1cm of the edge of the sheet to prevent distortion due to edge effect and applied over 0.5cm at 0.5cm intervals to avoid cross contamination. Standards containing commercially available Hyaluronan (HA), Heparan sulphate (HS), Dermatan sulphate (DS), Chondroitin-4-sulphate (C4S) and Chondroitin-6-sulphate (C6S) (Sigma Chemical Co, U.K) at a
concentration of 0.05mg/ml were included on each sheet. Electrophoresis was then carried out at 0.6mA per cm width for 4.5 hr. Following electrophoresis, the cellulose acetate sheets were stained with 0.05% w/v Alican blue 8 GX (Gurr Biological Stain, BDH) in 3% acetic acid, containing 0.05M magnesium chloride, pH 3.9 for 15 min. The sheets were destained with 1% acetic acid, 0.05M magnesium chloride at pH 3.9. The solution was changed several times until all excess stain was removed. The sheets were finally rinsed with water and allowed to air dry. Digital images were captured using Gel Doc (Bio-Rad Laboratories, Hertfordshire, UK), followed by densitometric analysis using Image-Pro Plus Analysis software (Version 6.0, Media Cybernetics, Inc). The ‘black’ (intensity generated when no light passes through the material) and ‘incident’ values (intensity generated when no material was present) on each image was determined so as to calibrate the intensity scale to standard optical density values. The total pixel density of each band was determined by placing a pair of defining lines horizontally at the top and bottom end of the band (Fig 2.2). Concentrations were determined by comparison to the GAG standards. GAG levels were determined for each site over a period of 21 months.

2.2.1.1.6 Assessment of methods for GAG quantification

In order to assess the accuracy of the technique in the quantification of GAGs, standard curves were generated prior to analysis of samples. Cellulose acetate electrophoresis of standards containing commercially available Hyaluron (HA), Heparan sulphate (HS), Dermatan sulphate (DS), Chondroitin-4-sulphate(C4S) and Chondroitin-6-sulphate (C6S) (Sigma Chemical Co, U.K) at concentrations of 0.05mg/ml, 0.04mg/ml, 0.03mg/ml, 0.02mg/ml and 0.01mg/ml were carried out several times to ensure reproducibility and sensitivity of the technique in detecting the GAG component. The sheets were then stained, air dried and scanned using the technique above (Section 2.2.1.1.5). GAG levels were plotted against staining intensity and correlation coefficients were calculated to determine linearity of standard curves.
2.2.1.1.7 Statistical Analysis

Statistical advice was obtained from Prof. R. Newcombe and Prof. S. Herawi (Cardiff University) and analysis carried out using SPSS Statistics 20. The data obtained were evaluated initially in the form of a longitudinal study. Activity at a site was defined as increase in attachment loss of 2mm or greater over a 3 month period. Sites that remained unchanged or showed improvement over a 6 month period or more were regarded as inactive sites and served as controls. The presence of a clear hyaluronan band is indicative of an adequate sample size of the serum based fluid and therefore GCF samples that had hyaluronan levels \( \geq 50\text{ng} \) were included in the analysis (Fig 2.6). Statistical analysis was carried out using one site per patient as the unit of analysis. The sGAG content in GCF at the onset of a period of active tissue destruction was compared statistically with the sGAG concentrations determined at the commencement of a control inactive period. The values obtained from scanning pixel density of the bands was subjected to normality tests using Q-Q plots and histograms as shown in (Fig 2.10 and Fig 2.11) to analyse distribution of data. Due to the non-uniform distribution of the data and to test the possibility that the sites that progressed were worse than those sites that remained static, a non-parametric one tailed unpaired Mann-Whitney test was used making no restrictive assumptions regarding scatter of the data. As per prior clinical assumption, a one-tailed test was considered suitable for the analysis of data as the results were expected to be unidirectional (ie sites that that progressed were worse than the sites that remained static). A one-tailed \( p \)-value of 0.05 was considered to indicate a significant difference in statistical analysis.

The data was also analysed as a cross-sectional study. As above, samples that had levels less than 50ng of hyaluronan were not included in the analysis, regarded as providing an inadequate GCF sample size. Sites were divided into 3 groups based on clinical attachment levels as 3 to 5mm loss of attachment, 6 to 8mm loss of attachment and \( \geq 9\text{mm} \) loss of attachment. A Kruskal-Wallis test (Nonparametric ANOVA) with Dunn's Multiple Comparison
test was used to compare sulphated GAG levels between the three groups. Differences were considered significant when $p$-value was < 0.05. For all samples with HA $\geq 50$ng, sGAG was plotted against attachment loss and correlation assessed by the Spearman rank correlation test.
2.3 Results

2.3.1 Investigator calibration

In order to assess reproducibility, an intra-examiner calibration was carried out by recording probing depths and recession on the mesiobuccal, midbuccal and distobuccal aspects of two selected teeth from 10 individuals. These measurements were repeated again within 24 hours and the statistical analysis was carried out using Kappa statistics.

<table>
<thead>
<tr>
<th>Tooth site designation</th>
<th>K-coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.7</td>
</tr>
<tr>
<td>B</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Table 2.1**: Kappa statistical values obtained from analysis of three sites on two selected teeth from 10 patients. (A Kappa value of 1 indicates perfect agreement whereas a Kappa value of 0 indicates agreement purely due to chance).

In this sample population, analysis revealed k-values of 0.7 for site A and 0.9 for site B, reflecting substantial agreement between the repeat measurements.

2.3.2 Assessment of methods for quantification of GAGs

Electrophoretic separation of a mixture of four standard GAG preparations at concentrations of 0.05mg/ml, 0.04mg/ml, 0.03mg/ml, 0.02mg/ml and 0.01mg/ml revealed clear, distinct faster and slower migrating bands representing hyaluronan, dermatan sulphate, chondrotin-4-sulphate and chondroitin-6-sulphate, thus confirming the sensitivity of the technique in
detecting levels as low as 0.01mg/ml or 20ng/2µl sample (Fig 2.2). Pixel density of scanned bands, against respective GAG standards are shown graphically and calculation of correlation coefficient produced R^2 values of 0.8 for both hyaluronan and the sulphated GAGs indicating linearity in the standard curve and therefore ensure reproducibility of the methodology (Fig 2.3 A and B).

2.3.3 Longitudinal analysis of proteoglycan metabolites in GCF with disease severity

Clear, distinct faster and slower migrating bands representing hyaluronan, dermatan sulphate, chondroitin 4 and chondroitin 6 sulphate were observed in the standards. Electrophoretic separation of the GCF samples revealed hyaluronan and sGAG bands that were comparable with the standards. In addition, slow migrating bands were also observed close to line of origin (Fig 2.4 and 2.5). HA and sGAGs were determined by comparison of the pixel density of the GAG standard separation on the same sheet and was calculated as total sGAG within the whole sample collected over 10 minutes. A clear hyaluronan band was present in active as well as in inactive sites and its presence above 50ng was used as an indicator of an adequate sample size for analysis (Fig 2.6). This HA value was selected on considering HA levels in all samples, where it presented a clear cut-off value, with lower levels of HA were significantly lower (less than 10ng per sample). On examining the attachment loss data alongside sGAG data for each site as a longitudinal analysis of sites, Fig 2.7 presents examples of profiles identifiable with some sites demonstrating disease progression with an increase in attachment loss and increase in GAG levels, whereas some sites remain the same. Sites which demonstrated fluctuation of attachment loss with time (Figs 2.8 and 2.9) were disregarded from the statistical analysis as they confirmed neither disease progression nor quiescence. Only sites that remained static over 6 months or had attachment loss of >2mm over a period of 3 months were included in the analysis (Fig 2.7). A total of 85 samples were included in the analysis. Data was categorised as static and progressive (Fig 2.10 and 2.11) and further
subjected to normality tests using Q-Q plots and histogram. A non-uniform distribution of data was observed, with a higher proportion of the static sites showed negligible sGAG (presumed inactive) compared to the progressive sites, whereas an increased proportion of the samples collected from the progressive sites showed an increase in sGAG levels (presumed active) (Figs 2.10 and 2.11). Subsequent longitudinal analysis of GCF samples using a one way analysis of variance revealed statistically significant differences between the GAG levels in GCF samples obtained from active group compared to control inactive group ($p=0.03$) as shown in Fig 2.12.
Fig 2.1: Collection of GCF samples using microcapillary tube. Selected sites were isolated, ensured plaque free and air dried. 2µl microcapillary tubes were placed at the gingival margin and GCF allowed to accumulate by capillary action.
**Fig 2.2:** Cellulose acetate electrophoresis of GAG standards at a concentration of 0.05mg/ml, 0.04mg/ml, 0.03mg/ml, 0.02mg/ml and 0.01mg/ml, demonstrating clear distinct bands representing Hyaluronan(HA), Dermatan Sulphate(DS), Chondroitin-4-Sulphate(C4S) and Chondroitin-6-Sulphate(C6S). Brackets shown enclosing area of band scanned for densitometric analysis.
Fig 2.3: Graphical representation of analysis of GAG standards demonstrating linearity for both Hyaluronan ($R^2=0.8$) and sulphated GAGs ($R^2=0.8$).
Fig 2.4: Analysis of GCF samples collected over a sequential period of 4, 3 and 3 mins (≈10 mins) demonstrated that the sGAG was detected at all time points investigated, indicating that the sGAG was not a pooled product but released continuously at the point of sampling. Brackets shown enclosing area of band scanned for densitometric analysis.
Fig 2.5: Longitudinal analysis of GCF samples collected from one site (A) in a patient (No.62) at 12 months (562), 15 months (662), 18 months (762) and 21 months (862) representing periods of relative inactivity (lighter bands) at 12 months and activity (represented by darker bands) at 15 months, 18 months and 21 months. Brackets shown enclosing area of band scanned for densitometric analysis. Dark areas preceding HA bands represent presence of non-specific blood proteins.
Fig 2.6: Cumulative distribution graph analysing levels of sGAG and HA in each GCF sample analysed demonstrating that some samples (about 31) had substantially low levels of HA and sGAG compared to other samples (about 85). A clear cut-off in HA levels is apparent in these samples when compared with the remaining 85 samples in the graph above. These were considered as inadequate for sample size and therefore excluded from further analysis.
**Fig 2.7:** Longitudinal analysis (over 21 months) for a selection of patients demonstrating periods of activity represented as an increase in attachment loss (Pt1:0-6mths, pt3:6-12mths) and periods of inactivity/stability represented as no change in attachment levels over 6 months (pt2:12-21mths, pt4:0-6mths).

GAG- , HA- , LOA-
Fig 2.8: Examples of samples excluded from analysis representing sites demonstrating fluctuation of attachment levels (pt6:3-15mths, 8:0-18mths) as sites that failed to demonstrate a high probability for active tissue destruction or presumed inactivity over 6 months, inadequate sample size due to absence of hyaluronan (pt 5:3-12mths, 6:0-9mths, 7:3-12mths) and absence of GAG (pt5:3-12mths, 6:0-9mths, 7:3-12mths, 8:0-9mths).
Fig 2.9: Examples of samples excluded from analysis representing sites demonstrating fluctuation of attachment levels (pt9:6-15mths, 10:0-6mths, 12:0-21mths) as sites that failed to demonstrate a high probability for active tissue destruction or presumed inactivity over 6 months, inadequate sample size due to absence of hyaluronan (pt10:0-6mths, 11:12-21mths, 12:0-21mths) and absence of GAG (pt10:0-6mths, 12:0-21mths). GAG - HA - OA -
Fig 2.10: Q-Q plot and histogram demonstrating non-uniform distribution of data for sites that remained static. Increased samples with negligible GAG (suggesting inactivity) when compared to the progressive sites (Fig 2.11)
Fig 2.11: Q-Q plot and histogram demonstrating non-uniform distribution of data for progressive sites (as demonstrated by an increase in LOA of ≥2mm). Increased samples with an increase in GAG levels (suggesting disease activity) when compared to the static sites (Fig 2.10).
**Fig 2.12**: Longitudinal analysis of data comparing distribution of GAG levels in GCF collected from sites that demonstrated disease activity by an increase in loss of attachment of 2mm or more over 3 months compared to GAG levels in GCF from sites which showed no change over a period of 6 months or demonstrated improvement by gain in attachment (p<0.05).
2.3.4 Cross-sectional analysis of proteoglycan metabolites in GCF with disease severity

Analysis of GCF samples collected at fixed time points (initial 4 minutes, followed by subsequent time periods of 3 minutes each) revealed clear, distinct faster and slower migrating bands representing hyaluronan, dermatan sulphate, chondroitin 4 and chondroitin 6 sulphate in the standards. Additionally, clear hyaluronan bands were observed from both active and inactive sites. As mentioned above in section 2.3.3, samples that contained no distinct hyaluronan band were rejected from the analysis. The final analysis involved 8 samples. Varying but detectable levels of sGAGs were observed in most samples as high and low intensities. However no trends were observed. Detectable levels of sulphated GAGs were present at all time points in some of the samples whereas in some samples the GAG was present only at the first time point. A typical profile is shown in Fig 2.4. In addition, non specific staining was also observed around the line of origin. However, in this group, statistical analysis could not carried out due to an insufficient sample size.

Further, data obtained from 2.3.3 was also subjected to cross-sectional analysis. Out of a total of 716 samples, 498 were rejected as they contained no distinct hyaluronan band and only 218 samples were included in the analysis. All data with sufficient sample size were catagorised with respect to attachment loss. On cross-sectional examination of the data (Fig 2.13), significant differences were noted between the groups that had attachment loss of 6-8mm and ≥9mm (p<0.05). However, no correlation was observed between GAG levels and attachment loss (Fig 2.14).
**Fig.2.13:** Cross-sectional analysis of data comparing GAG levels from sites with loss of attachment of 3-5mm compared to loss of attachment of 6-8mm and >9mm. Significant differences were observed on comparing groups 6-8mm and >9mm (p<0.05). Increased GAG levels at deeper sites suggest increased disease activity at deeper sites.
**Fig: 2.14**: Cross sectional analysis of data demonstrating no correlation between loss of attachment and GAG levels, further confirming the episodic nature of the disease.
Correlation coefficient \( r = 0.1388 \) and \( p \text{ value} = 0.0006 \)
2.4 Discussion

This study investigated the relationship between the levels of sGAG in GCF and sites of active periodontal destruction. Our results demonstrated a significant increase in sGAGs associated with sites that demonstrated continuing signs of disease activity and progressive attachment loss, identified periods of activity and inactivity, with few sites demonstrating disease activity over a 21 month period. Moreover, the sGAG was identified as a product released continuously at the point of sampling. Therefore, the presence of sGAG in GCF could be used as a potential marker of disease activity.

Although extensive research has been carried out to further our understanding of the pathogenesis of periodontal disease with a view to develop improved techniques that may be of diagnostic and prognostic value in the management of periodontal disease, there have been several limitations such as the nature of the disease process itself and inconsistencies in research methodologies that have hindered progress in this field. With regards to periodontal disease progression, several models of disease progression have been proposed in the past with early studies reporting that the common forms of destructive periodontal disease once established, progress slowly and continuously until treatment or tooth loss (Suomi et al. 1971; Sheiham et al. 1986). The limitations of these early studies were that they were cross-sectional in nature, used inadequate sample size and used average values of attachment loss for a given mouth, thereby eliminating intra-oral variation and disease severity. As a consequence, the information obtained from these studies have the potential to be misleading. Additionally, data from cross sectional studies were used to interpret longitudinal changes observed in periodontal disease. Subsequent studies examined loss of attachment at specific sites over a period of time and reported that periodontal destruction is not continuous but progresses in an episodic manner with bursts of activity followed by periods of quiescence and possibly repair (Goodson et al. 1982; Lindhe et al. 1983; Socransky et al. 1984). These studies have used manual probing which cannot reliably detect changes of ≤2.5 mm and therefore would be able to detect only rapid progressive attachment loss of ≥ 2.5mm and not
identify gradual attachment loss of ≤2.5 mm possibly over a longer period of time. With the development of automated probes, it has been demonstrated that using large thresholds, only rapid progressive attachment loss can be detected whereas using smaller thresholds, a higher proportion of gradual attachment loss was also detectable (Jeffcoat and Reddy 1991). This led to the conclusion that progression of periodontal disease included both rapid loss of attachment as well as gradual loss of attachment and that the type of attachment loss would be based on the susceptibility of the patient. However, accurate diagnosis and prognosis has not been possible yet, due to the absence of reliable markers to detect disease activity and identify high risk patients.

These limitations were taken into account when validating the methodology in this study in that, firstly this is the first substantial longitudinal study with a significant sample population that has attempted to assess the relationship between sGAG levels in GCF and periodontal disease activity and therefore has allowed for statistical robustness of the study. Previous studies investigating this relationship have been cross-sectional in nature and have used fewer samples and therefore the information obtained from these studies have been limited.

Secondly, taking into account that the chances of error are high especially when the magnitude of changes are minute (Haffajee et al. 1983; Haffajee and Socransky 1986), in this study, a Florida probe with a precision of 0.2mm was used to standardise probing forces and improve reproducibility. Intra-examiner calibration was carried out to increase confidence in clinical measurements. Furthermore, the release of sGAG was monitored over timed intervals to determine whether the detected GAGs were released continually at the point of sampling or pooled in GCF which helped further our understanding of disease pathogenesis and contributed towards validating the use of sGAG as a marker of disease activity.

With regards to analytical method used, CAE was the technique of choice as it has been shown to be more sensitive than other techniques in detecting GAGs. Although a time consuming technique which requires specialist training, it is able to detect levels as low as 5ng
and yield reproducible results (Embery et al. 2000). Despite the fact that a range of monoclonal and polyclonal antibodies have been developed to a variety of epitopes within the GAG chain of proteoglycan species for the development of immunological assays, there have been difficulties in raising such antibodies with high immunogenicity due to the universal distribution of these GAG chains in both soft and mineralised tissues, in addition to inter-species similarities. Therefore the creation of neo-epitopes by selective digestion with chondroitinases yielding an unsaturated non-reducing terminal as a suitable antigen for the development of antibody has been proposed. However, the use of hydrolytic enzymes may result in disintegration of the proteoglycan and therefore reduce the sensitivity of the technique. Some studies have used ELISA for GAG detection in GCF (Shibutani et al. 1993; Khongkhunthian et al. 2008; Makeoudom et al. 2014) in patients with periodontitis and patients undergoing orthodontic treatment (Intachai et al. 2010; Insee et al. 2014) but the sensitivity of the technique has been reported to be 15-1000ng/ml which is lower that of CAE which is able to detect up to 5ng/ml (Embery et al. 2000).

In the analysis of data, strict parameters were applied to the data for inclusion in the analysis and to identify sites as highly likely to be active or inactive at the time of sampling. The presence of HA of ≥50ng has been shown previously as an effective internal control measure in ascertaining the collection of a sufficient GCF sample size required for analysis (Waddington et al. 1994; Waddington et al. 1996). Although present ubiquitously in all tissues and extracellular fluids, hyaluronan is a critical component of the extracellular matrices of the mineralised as well as well as non-mineralised connective tissue, where it contributes significantly to tissue hydrodynamics, cell signalling, proliferation and migration (Bansal et al. 2010). Hyaluronan is also produced by fibroblasts in the presence of endotoxin; and plays an important anti-inflammatory role through the inhibition of tissue destruction and facilitates healing (Moseley et al. 2002). The anti-inflammatory effect may be due to the action of exogenous hyaluronan as a scavenger by draining prostaglandins, metalloproteinases and other bio-active molecules (Laurent et al. 1995). Due to its anti-inflammatory property,
hyaluronan has been used effectively in the field of medicine in the treatment of osteoarthritis and rheumatoid arthritis. Therefore, the presence of detectable levels of hyaluronan in the GCF samples may indicate its important role in the modulation of wound healing. The high turnover rate observed in the periodontal tissue (Sodek 1976) in addition to being in a constant state of remodelling due to functional forces, may also contribute towards its constant release in the GCF. Therefore, a combination of disease activity, repair and remodelling may contribute to its consistent presence in the GCF. In the analysis, all samples that contained HA levels of <50ng were excluded from the study due to insufficient sample size (Fig 2.6). Only sites that demonstrated a clinical attachment loss ≥ 2mm over 3 months or remained static over a period of 6 months were included in the analysis (Fig 2.7 and 2.8). This resulted in rejection of a major proportion of the sample. The change in sGAG profile over a period of 21 months was notable (Fig 2.7) and possibly reflects periods of activity and quiescence which is characteristic of periodontal disease progression as mentioned previously.

On statistical analysis of the results from the longitudinal study by associating the presence of the biomarker with loss of attachment, large standard deviations were observed (Fig 2.12). This reflects possible variations in disease activity in that, at the point of sampling, the selected sites may be in a state of ongoing activity or at the initial early stage or at the terminal end stage of the disease active period. Further, on normality testing using Q-Q plots and histogram, variations were observed between the groups in that more samples with increased GAG levels were observed in the progressive sites which was indicative of representing disease activity (Fig.2.10 and 2.11). Depending on the level of activity, the amount of sGAG produced may vary, with increased levels of GAG being released during the ongoing phase compared to decreased amounts of sGAG released during the initial or terminal stages of activity. In addition, the level of sGAG may also reflect the severity of the disease with higher levels reflecting rapid destruction and lower levels reflecting gradual destruction. Statistical analysis of the data in a cross sectional manner to evaluate sGAG levels with disease severity, significant differences were noted between the groups with 6-8mm attachment loss and
≥9mm. These findings are supported from alternative clinical studies which have reported that further attachment loss mostly occurred at deeper sites (Grbic et al. 1991; Grbic and Lamster 1992). Therefore, an increase in sGAG levels in sites with attachment loss of ≥9mm may reflect higher disease activity within this group. Furthermore, statistical evaluation of the cross sectional data examining sGAG levels with attachment loss revealed no correlation, which may possibly be due to the varying levels of disease activity among the sites in that at the point of sampling, the site may be in a state of inactivity or in the initial, mid or terminal stage irrespective of the severity of the disease. Moreover, the lack of correlation further corroborates on the limitations of cross-sectional analysis in assessing longitudinal changes as observed in periodontal disease.

In monitoring the release of the marker over a timed period of 10 minutes, variations were observed in GAG levels over the 21 month time period. These variations reflect the amount of GAGs released during these timed period. In some of the patients, detectable GAG levels were present only in the first time point whereas in others, the levels were detected at all time points. These variations may possibly reflect on degree of disease severity whereby, the consistent GAG levels at all time points indicate continuous release of the marker brought about by the ongoing degradative changes at the point of sampling rather than the pooled marker. Irrespective of whether the marker was released on sample collection or pooled, its presence in the collected GCF indicates disease activity and therefore a need for treatment and its absence therefore indicates the site as being inactive. Furthermore, the absence of sGAG in the inactive sites suggests that the sGAG detected in GCF in sites presumed “active” was as a consequence of disease and not turnover and therefore has contributed to further our understanding of the disease process.

In conclusion, the results obtained from this study demonstrate that the increase in sGAGs detected in GCF may be indicative of active destruction of the underlying tissues of the periodontium and therefore the detection of sGAG in GCF may be used as a potential marker of disease activity. The longitudinal design of the study helped further our understanding of
disease pathogenesis by enabling us to make observations of the disease process at regular intervals of 3 months over a 21 month period and identify sites presumed active and inactive. The wide range of values observed on data analysis may possibly suggest that the sites may be at different stages of the disease process which further corroborates on to the several models of disease progression such as gradual loss, single burst, multiple burst, random burst, random walk theories that have been reported in the literature none of which can explain variation in data (Yang et al. 1992). Although the underlying disease process may have evolved continuously over time and it is difficult to interpret exactly at what point the transitions occur, the possibility of identifying an actively degrading site still appears to be relatively high and therefore the presence of sGAG in the sample could be used as a measurement of actual tissue destruction. While this study is not without limitations due to the nature of the disease process and experimental design, the use of strict parameters in study design, analytical methods, statistical and data analysis have helped minimise inaccuracies due to methodology, operator and technical errors as highlighted in previous studies. However the development of an assay system that is relatively easy to perform, sensitive, selective, reproducible and yields quick results is sought that will assist the clinician in managing the patient with periodontal disease.
Chapter 3:

*P. gingivalis* LPS inhibits the reparative and regenerative processes during periodontal disease by influencing periodontal ligament cell behaviour and altering the biological events associated with matrix synthesis.

3.1 Introduction

The previous chapter evaluated the presence of sGAG in GCF samples collected from patients with periodontal disease as a marker of disease activity. The pathological processes leading to matrix degradation and the release of these sGAGs into the GCF, are however unclear. Extensive research has identified that the tissue destruction observed in periodontal disease is largely attributed to local inflammation resulting from interaction between host and microbial factors which alter the host immune response. These microbial factors have an effect on the resident cells resulting in release of pro-inflammatory mediators (Taylor 2010; Kinane et al. 2011). Within this scenario, it has been proposed that the activity of the periodontal ligament (PDL) cells is altered, consequently affecting its important functions such as maintenance of homeostasis and repair by tissue degradation and formation, remodelling, maintaining a high turn-over rate and structural integrity. Although the PDL cells play an important role especially in repair and regeneration, the response of these cells during the inflammatory process has not been extensively investigated and therefore the focus of this chapter is to investigate by means of *in-vitro* studies, the biological effects of *P. gingivalis* LPS on PDL cells, which will further our understanding of the cellular synthesis of proteoglycans within a pathological condition.

Although over 700 different bacterial species have been identified in the oral cavity (Aas et al. 2005; Palmer 2014), only 10 bacterial species as combinations, or on their own, have been implicated in the progression of the disease. The pathogenic role of gram negative anaerobic
bacteria in the severe forms of disease has been extensively investigated and studies have confirmed that *P. gingivalis* can be detected in 86% of disease sites (Yang et al. 2004b) and is the species most highly associated with the chronic forms of periodontitis. Kawada (2004) reported a significant positive correlation between *P. gingivalis* numbers and pocket depth (Kawada et al. 2004). In addition, a reduction of *P. gingivalis* numbers was associated with resolution of disease at the affected site following treatment (Haffajee et al. 1997; Fujise et al. 2002). Additionally, experimental implantation of *P. gingivalis* in animal models induces inflammatory response and periodontal bone loss (Evans et al. 1992a; Hajishengallis and Lambris 2011). These studies correlated the presence of *P. gingivalis* with chronic periodontitis. Further studies demonstrated that *P. gingivalis* possesses bioactive materials such as cytoplasmic membranes, peptidoglycans, outer membrane proteins, lipopolysaccharides (LPS), capsules and fimbriae on their cell surface (Offenbacher 1996) in addition to cysteine proteases which may induce excessive production of cytokines and modulate cytokine networks in the periodontal tissues (Genco and Slots 1984). Among the several virulence factors, *P. gingivalis* LPS was reported to be a potent stimulator of inflammatory cytokine production and bone resorption (Chiang et al. 1999; Nishida et al. 2001). The LPS molecule is an essential constituent of the cell wall of gram-negative bacteria, is amphiphilic in nature, comprising three regions: lipid A which is responsible for its toxicity, the core region and a polysaccharide portion (Gabrielli et al. 2012) as detailed in chapter 1. Studies examining LPS toxicity, demonstrated that although the endotoxic activity of *P. gingivalis* LPS was low compared to that of LPS isolated from that of enterobacteria (Ogawa 1994), *P. gingivalis* LPS was a potent inducer of various biological responses such as bone resorption, polyclonal B-cell activation, inhibition of bone formation and fibroblast proliferation (Mayrand and Holt 1988; Wang et al. 1999b). Therefore, *P. gingivalis* LPS has been considered to be an important pathogenic component in the initiation and development of periodontal disease (Tobias et al. 1997). In addition to the presence of periodontopathic bacteria, other factors such as genetic susceptibility, systemic diseases, environmental factors
such as smoking and stress play an important role in the onset and progression of periodontal disease (Pihlstrom et al. 2005).

The periodontium is composed of the gingiva, periodontal ligament, cementum and alveolar bone and hosts different fibroblast populations with heterogeneity existing within the population of gingival fibroblasts (Bordin et al. 1998; Zhou and Windsor 2007) as well as periodontal ligament fibroblasts (Saito et al. 2002; Tomokiyo et al. 2008) with both populations containing cells with stem-cell characteristics and self-renewal capacity (Fournier et al. 2010; Hynes et al. 2012; Zhang et al. 2012). The periodontal ligament is a unique soft connective tissue positioned between the root surface of the tooth and the inner wall of the alveolar socket, made up highly specialised connective ligament fibres which provide for flexibility during movement, nutrition, maintain homeostasis and repair (Bartold et al. 2000; Shimono et al. 2003). The heterogeneous cell population of the periodontal ligament composed of resident cells such as fibroblasts, osteoblasts, cementoblasts, epithelial cells, endothelial cells, undifferentiated mesenchymal cells and recruited cells such as neutrophils, monocytes, macrophages, provide for maintenance of homeostasis and repair by tissue degradation and formation, alveolar bone remodelling and maintaining a high turn-over rate. The fibroblasts are the predominant cell type and are oriented along collagen fibres present throughout the periodontal ligament. These fibroblasts are responsible for the unusually high collagen turnover in the periodontal ligament compared with other connective (Sodek 1977; McCulloch et al. 2000) tissues in that, they are capable of endocytosis of collagen during degradation, unlike gingival and other mucosal or dermal fibroblasts. They preserve collagen homeostasis in the periodontium according to mechanical challenge which the cells are able to perceive as mechanical signals through extracellular matrix linkages by forming intimate contacts with collagen fibrils and intracellular stress fibers (McCulloch et al. 2000). PDL fibroblasts exhibit different biologic reactions in response to environmental factors such as infections (Scheres et al. 2010) in that, when compared to gingival fibroblasts, PDL fibroblasts differ in their inflammatory response to viable P. gingivalis, suggesting considerable heterogeneity in their
response and that these differences are possible crucial determinants for the susceptibility to develop periodontitis. Similar results were reported on exposure to mechanical challenge (Pender and McCulloch 1991) in that, mechanically induced remodelling is mediated by a complex feedback mechanism involving the synthesis of cytokines such as IL-1, IL-6 and RANKL by cells present in the PDL, which then act in an autocrine and paracrine manner to regulate the expression of transcription factors, cytokines, growth factors, enzymes and structural molecules involved in the proliferation, differentiation and function of mesenchymal and other cell types (Meikle 2006).

PDL cells have an important role in maintaining tissue regeneration (Hughes et al. 2010), express alkaline phosphatase activity and mineralization capacity (Nohutcu et al. 1997; Hou et al. 2000; Lin et al. 2000). These cells exhibit phenotypic characteristics consistent with osteoblast like cells and have the potential to differentiate into osteoblasts and or cementoblasts with the capacity to form mineralized deposits (Somerman et al. 1988; Nojima et al. 1990a; Arceo et al. 1991). This heterogenous cell population within the PDL allow for the unique properties of the PDL which include rapid turnover (Sodek 1977) as well as the ability to withstand the forces brought about by mastication, thereby maintaining dynamic and strong connections between the tooth and bone (Berkovitz 1990; McCulloch et al. 2000). The undifferentiated mesenchymal cells present within the periodontal ligament, also identified by previous studies as ‘progenitor cells’, exhibit some of the classical cytological features of stem cells (McCulloch et al. 1987) and it has been postulated that these mesenchymal cells are recruited and activated following damage to the periodontium, where they undergo terminal differentiation into ligament forming cells or mineral forming cementoblasts, both of which act to secure the connections between the cementum and alveolar bone (Bartold et al. 2006). Experimental studies have demonstrated the continual recruitment of proliferating cells from bone marrow stroma through vascular channels that communicate with the PDL (McCulloch et al. 1987). These cells appear to preferentially migrate to the osteoblast and cementoblast surfaces, consistent with the recruitment of osteoprogenitor cells from stem cells located in
the bone marrow stroma through the PDL and ultimately undergo differentiation, similar to kinetics described in other bony tissues (McCulloch et al. 1987). These studies confirmed that the PDL is constantly repopulated by relatively undifferentiated mesenchymal cells and is a rich source of progenitor cells with the capacity to form new hard and soft tissue. Further studies have reported that the mesenchymal stem cells isolated from the PDL have the capacity to form ligamentous structures resembling Sharpey’s fibres and mineralised tissues similar to bone and cementum in vivo (Seo et al. 2004), with recent work reporting that PDL derived MSCs exhibit a superior capacity to form mature mineralized structures which were histologically similar to mature bone when compared to MSC derived from gingiva or lung (Hynes et al. 2014). Therefore PDL cells play an important role in repair and regeneration by providing a rich source of progenitor cells to facilitate the healing process.

The effect of LPS on the various cell types present within the periodontium such as fibroblasts, osteoblasts, epithelial cells, dendritic cells have been extensively investigated and reported in the literature. However, the effects of LPS on the the progenitor cell population has not been investigated previously. This investigation is crucial, as it is the progenitor cell population which exhibit greater potential to remodel periodontal tissue compared to other MSC’s and provide an important source of cells in periodontal tissue regeneration. Therefore, it was the aim of this present study to investigate the effect of sub-toxic levels of $P. \text{gingivalis}$ effect on cell proliferation and matrix formation using an in vitro culture system. This unique culture system utilises a possible high proportion of progenitor cells derived from the periodontal ligament and is therefore highly relevant for understanding periodontal disease, as it is these cells which are recruited during attempts at periodontal tissue repair.
3.2 Materials and Methods

3.2.1 Extraction of LPS from *P. gingivalis*

At the commencement of this study (2006), only *E. coli* LPS was available commercially. However, the commercially available LPS proved to be contaminated and due to the large quantities required for the study, justification was obtained for the necessity to extract and purify LPS. *Porphyromonas gingivalis* W50 (generous gift from Prof. A Smith, Glasgow Dental School) was grown as continuous culture in 500ml fastidious anaerobe agar (Lab M Ltd, Lancashire, UK) supplemented with 0.1µg/ml hemin (Sigma Chemical Co, Dorset, U.K) and maintained at 37˚C, in an anaerobic atmosphere containing 80%N₂, 10%H₂ and 10% CO₂. The purity of the cultures was verified by Gram staining and sub-culture on blood agar plates. Bacteria were harvested by centrifugation at 10,000g for 15min at 4˚C. The supernatant medium was removed and the bacterial cell pellets were further washed twice with PBS and lyophilised.

The hot-phenol water technique as described by Westphal and Jann (1965) was used to extract the LPS fraction of the lyophilised bacterial cells. 10mg of the lyophilised bacterial cells were first ground using a mortar and pestle to break the pellet into a uniform mix and then resuspended in a centrifuge tube containing equal volumes (15ml each) of double distilled water and 90% phenol (Sigma Chemical Co, Dorset, U.K). The mixtures were placed in a water bath pre-heated at 65-68˚C and shaken vigorously for 20 mins. This resulted in the formation of one homogeneous phase. The mixture was immediately cooled in an ice bath for 5 min and centrifuged at 8,000g for 20 mins at 4˚C. This resulted in the formation of three distinct layers; an upper aqueous layer containing polysaccharides and nucleic acids, a phenol layer containing proteins and a lower insoluble layer containing cell debris. The upper layer was collected and the residual phase was treated with another volume of hot water as above. The upper layers were collected, pooled and dialysed against double-distilled water at 4˚C overnight and centrifuged at 100,000g for 3hrs at 4˚C. The resulting precipitate was washed
twice with double-distilled water and lyophilised. The lyophilised pellet containing the LPS was resuspended in 2mls pyrogen free distilled water and treated with 1μg/ml of DNase (from bovine pancreas; Sigma Chemical Co) and 1μg/ml of RNase (from bovine pancreas; BDH, Poole, UK) and incubated at 30˚C for 90 mins at pH 7.5 to remove nucleic acids. Finally 1μg/ml of Proteinase K (from fungi; Sigma Chemical Co) was added and incubated at 30˚C for 90 mins to remove contaminating bacterial proteins. Following these enzymatic treatments, the solution was heated at 90˚C for 30 mins in a water bath to inactivate the enzymes. This was followed by extensive dialysis against double distilled water at 4˚C and then lyophilized. Stock solutions were prepared in pyrogen free distilled water at concentrations of 1mg/ml.

### 3.2.2 Characterisation of LPS

The LPS from *P. gingivalis* extracted by hot-phenol water technique was further examined to assess its purity. Absence of contamination with nucleic acids was confirmed by electrophoresis of samples using agarose gel stained with ethidium bromide. Removal of contaminating protein was confirmed by SDS PAGE followed by staining with Coomassie Blue and Silver stain. The molecular size profile of the extracted *P. gingivalis* LPS was compared to commercially available *E.coli* LPS (Sigma Chemical Co.), also reported to be extracted using the same technique. LAL assay was carried out for the quantification of endotoxin levels.
3.2.2.1 SDS- Polyacrylamide Gel Electrophoresis

*P. gingivalis* or *E. coli* LPS samples were prepared at concentration of 1mg/ml. To 10 µl of sample, 1 µl of NuPAGE sample reducing agent (500 mM dithiothreitol (DTT) at a 10X concentration, Invitrogen, UK) and 2.5 µl of sample buffer (containing Coomassie G250 and Phenol Red as tracking dyes) was added. 500mls of running buffer was prepared by adding 25mls of 20X NuPAGE SDS Running buffer containing 50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7 (Invitrogen, UK) to 475 mls of deionized water. The antioxidant buffer was prepared by adding 500 µl of NuPAGE antioxidant (Invitrogen, UK) to 200mls of the running buffer.

The pre-cast NuPAGE® 4-12% Novex Bis-Tris Gel (Invitrogen, UK) was removed from gel pouch and the gel cassette rinsed with deionized water. The tape was removed from the bottom of the cassette and the comb gently removed from the cassette. The sample wells were rinsed with 1x NuPAGE SDS running buffer and the gel oriented in the tank as per manufacturer’s instructions. The seal was checked to ensure that there was no leakage. The inner chamber of the tank was filled with the antioxidant buffer and the out chamber with the running buffer. 10µl each of the molecular weight marker (SeeBlue, Invitrogen, UK) and the prepared samples were loaded onto the gel and the gels were run for 35 mins at 120mA to separate the LPS molecule.
3.2.2.2 Staining with Coomassie Brilliant Blue for detection of protein contamination

Following electrophoresis, the gels were submerged in staining solution containing 0.1% Coomassie Blue R250 (w/w), 30% methanol and 5% acetic acid for 30mins on a platform shaker. Destaining was carried out by soaking the gel firstly in 30% methanol and 5% acetic acid for 15mins followed by 7% acetic acid and 5% methanol until the gel background was clear. Presence of proteins were detected as blue bands on a clear background.

3.2.2.3 Staining with Silver for characterisation of LPS

The Color Silver Stain Kit (Pierce, UK) was used for characterisation of LPS. Following electrophoresis, the gel was fixed with 50% ethanol, 5% acetic acid for 4 hours with frequent changes of the fixative buffer. Gels were then washed four times with ultrapure (deionised and distilled) water. Silver working solution (WS) was prepared by adding 10 ml of silver reagent (provided in the kit) to 140ml of water. Reducer aldehyde working solution and reducer base working solution was prepared by adding 10 ml each of reducer aldehyde reagent or reducer base reagent (both provided in the kit) to 65ml of water. Stabiliser base working solution was prepared by adding 10ml of stabiliser base reagent (provided in the kit) to 440ml of water. The gel was then incubated in silver working solution for 30mins followed by water rinse for 20 seconds. Reducer working solution was prepared by combining equal volumes of reducer aldehyde working solution and reducer base working solution immediately before use. The gel was incubated in reducer working solution for 5mins followed by incubation in stabilizer working solution for 40mins. The gel was visualised using Gel Doc (Bio-Rad Laboratories, Hertfordshire, UK) and digital images recorded.
3.2.2.4 Staining with Ethidium Bromide for assessing contamination with nucleic acids

Ethidium Bromide is the most commonly used stain for nucleic acids. It is a large flat molecule which has the capacity to insert itself within the double stranded DNA molecule. This results in the formation of fluorescent complexes which can be viewed under UV light.

2% agarose gels (Sigma, Aldrich) were prepared in 0.5x TBE buffer. The solution was heated and stirred intermittently until the agarose had dissolved to form a clear solution. On slight cooling, 5μl of ethidium bromide solution (Molecular grade 10mg/ml, Promega) was added to the solution and immediately poured into a casting tray and left to set. Once set, the gel along with the casting tray was then placed in an electrophoresis tank containing 0.5x TBE buffer with the comb facing cathode. The comb was removed carefully from the gel. 10μl each of *E. coli* LPS and *P. gingivalis* LPS were loaded into the respective wells along with 5μl of 100bp ladder (Promega) on either side. The gel was run at 80V for approximately 45min until the marker reached the bottom of the gel. The gel was then removed from the casting tray and products visualised on Gel Doc™ scanner (Bio-Rad, Hemel Hempstead, UK) using UV light and digital images recorded.

3.2.2.5 LAL assay

The QCL-1000® assay (Lonza, UK) was used to perform the Limulus Amebocyte Lysate (LAL) assay to estimate levels of LPS in the sample. This assay is based on the biological principle that, in the presence of endotoxin, the proteolytic activity of the proenzyme Factor C found in circulating amebocytes of the horseshoe crab *Limulus polyphemus* is activated. On addition of a chromogenic substrate containing para-nitro aniline (pNA), the enzyme splits the chromophore pNA from the chromogenic substrate resulting in yellow colour that can be quantitated by measuring the absorbance at 405nm and extrapolated against a standard
curve. Standards were prepared at a concentration range of 0.1EU/ml to 1EU/ml. 50µl of samples and standards were pipetted into wells in triplicates in a 96 well plate. 50µl of the LAL reagent provided in the kit was added into each well and samples were incubated at 37°C for 10 minutes. 100 µl of the chromogenic substrate (containing pNA) provided in the kit was added to the samples and standards and incubated at 37°C for an additional 10 min. The enzymatic reaction was stopped by the addition of 50µl of a stop reagent containing 25% v/v glacial acetic acid in water and absorbance measured spectrophotometrically at 405nm. A standard curve was obtained by plotting the absorbance against the corresponding concentrations of standards provided in the kit. The concentration of endotoxin in the sample was determined from the standard curve.

3.2.3 Isolation of periodontal ligament cells

Periodontal ligament (PDL) like cells were obtained as explants from alveolar bone chips. Alveolar bone was dissected from ten week old CD1A mice (culled under schedule 1 for harvest of tissue), by placing incisions at each corner of the mouth and cutting through skin and muscle. All supporting muscle and soft adherent tissues were removed from the alveolar bone. Molars and incisors were removed and the alveolar bone proper was washed three times with 0.1 M phosphate buffered saline (PBS, pH 7.4). The alveolar bone was then split longitudinally into two halves and placed in 6 well plates and cultured in alpha-modification Minimum Essential Medium (αMEM) pre-supplemented with ribonucleosides and deoxyribonucleosides (Invitrogen, UK), containing antibiotics (100 units/mL penicillin G sodium, 0.1 µg/mL streptomycin sulphate and 0.25 µg/mL amphotericin; Invitrogen, UK) at 37°C and 5% CO₂. Migrating cells from bone chips were observed by day 5 and confluency attained at 3-4 weeks. Existing media was replaced with fresh media once every 48 hours.
On attaining confluency, the alveolar bone sections were removed, confluent cells were washed with PBS and 1.5ml accutase (0.5 mM EDTA, Sigma-Aldrich, UK) was added to each well, incubated at 37°C, 5% CO₂ for 5 min to detach the cells from the plate. The cells were then collected into a 15 ml centrifuge tube and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml of media and counted using a haemocytometer. The cells were then reseeded in appropriate volume of working media to provide a cell density of 1×10⁴ cells/cm² in 6 well plates and expanded. Cells of the third passage were used for experiments.

### 3.2.4 Characterization of periodontal ligament cells

The PDL cells were evaluated by immunocytochemical staining and characterized according to the expression of cell surface markers CD105 (Endoglin), CD90 (Thy1), CD73 and OPN. The presence of CD105, CD90 and CD73 positive cells allows for characterization of mesenchymal stem cells whereas, the presence of OPN positive cells allows for characterization of osteoprogenitor cells. PDL cells from passage 3 were reseeded at a density of 1×10⁴ cells/cm² in 8 well chamber slides (BD Biosciences) and incubated at 37°C, 5% CO₂ for 24 hours in culture conditions as described in section 3.2.3 above. The cells were fixed in freshly prepared paraformaldehyde (4%) for 30 mins and then washed twice with PBS. In order to avoid non-specific staining, the cells were incubated in blocking buffer (1% normal horse serum in TBS) at room temperature for 1 hr. Primary antibody (anti CD105, anti CD90, anti CD73 and anti OPN goat polyclonal antibodies, Santa Cruz Biotechnology Inc, Santa Cruz, USA) diluted in 1% BSA in PBS was prepared at a concentration of 1:50. Blocking buffer was discarded and the cells were incubated in primary antibody at 4 °C overnight. Goat IgG was used for isotype control and negative control was obtained by the omission of primary antibody. The cells were then washed 3 times in PBS for 5 mins and then exposed to rabbit anti-goat IgG FITC conjugated secondary antibody (Santa Cruz Biotechnology Inc, Santa
Cruz, USA) diluted in 1% BSA/PBS solution at a concentration of 1:250 and incubated for 90 mins at room temperature in the dark. The cells were then washed 3 times in PBS for 5 mins each time. The slide was placed in 70% acetone for 10 minutes and the slide tool used to dislodge the chamber from the slide. The edges were carefully blotted to remove excess solution and a droplet of mounting medium for fluorescence microscopy viewing containing Dapi (VECTASHIELD, Vector Laboratories Inc, Burlingame, USA) was added onto the slide. A large coverslip was mounted with the cells facing the microscope slide (Polysine slides, Thermos scientific, UK). Dapi or 4′, 6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to the A-T rich regions in the DNA and helps visualise the nuclei whereas FITC (Fluorescein isothiocyanate) is an amine-reactive derivatives of the fluorescein dye, functionalized with an isothiocyanate reactive group in its structure which is reactive towards primary amine groups on proteins. The cells were then viewed under an Olympus AX70 fluorescent microscope and images were captured using a Nikon digital camera DXM 1200.

### 3.2.5. Determination of seeding density using MTS assay

To ensure that cells used in experiments were in the exponential growth phase, MTS assay was used to determine appropriate seeding density. CellTiter 96 AQueous One solution Cell proliferation assay-MTS assay, (Promega, UK) is a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity or chemosensitivity assays. The principle behind this technique is dependent on the capacity of living cells to reduce tetrazolium salt {3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide} to a formazan crystal in their metabolizing mitochondria. In this assay, the number of viable cells at specific time points, were assessed delivering information regarding cell expansion over time. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.
PDL cells obtained from third passage were plated at concentrations of $6 \times 10^3$, $1 \times 10^4$, $2 \times 10^4$, $4 \times 10^4$, $6 \times 10^4$, $8 \times 10^4$, $1 \times 10^5$ in duplicates in a 12 well plate and left to adhere for 24 hours. Cell viability was assessed by adding $200\mu$l of MTS reagent to each well and the cells incubated for 4 hrs. $100\mu$l of supernatant was transferred into a 96 well plate. Absorbance was recorded at 490nm with a 96-well plate reader (Bio-Tek Instruments Ltd).

### 3.2.6 Determination of sub-toxic levels of LPS on cell viability

Staining with Trypan blue dye was carried out to examine the cytotoxic level of LPS on PDL cells. This test is based on the principle that viable cells possess an intact cell membrane and therefore are able to exclude dyes such as Trypan Blue and therefore appear clear unlike non-viable cells which appear stained.

PDL cells obtained from third passage were reseeded to provide a cell density of $1 \times 10^4$ cells/$\text{cm}^2$ in 12 well plates. The cells were left overnight to adhere. On day 0, the supernatant was removed and replaced with culture media supplemented with *P. gingivalis* LPS at a range of concentrations (50ng-500ng). After 24 hours, the media was removed, cells washed three times with PBS and then incubated with 1ml of 0.4% trypan blue stain (Sigma-Aldrich) for 1 minute. The stain was pipetted out and the cells washed once with PBS. The numbers of blue stained and non-stained cells from five fields of view were immediately counted under an inverted microscope and the percentage viability was determined.
3.2.7 Effect of subtoxic levels of LPS on cell proliferation

Following viability count on cells stimulated with different concentrations of LPS, a survival rate of less than 95% was noted for cells stimulated with LPS concentrations of greater than 200ng/ml. Therefore, further experiment were carried out using LPS of concentrations 50ng/ml and 100ng/ml. Cells obtained from the third passage were plated at concentrations of 1x10⁴ cells/cm² in 12 well plates. The cells were left overnight to adhere. The media was then replaced with media containing LPS at concentrations of 50ng/ml and 100ng/ml, each LPS concentration in triplicate. Media not supplemented with LPS was used as control.

On day 0, 200µl MTS reagent was added to 800µl media in each well and the plate incubated at 37°C for 4 hours. 100µl of sample was subsequently transferred to a 96 well plate and absorbance read at 490nm. The procedure was repeated on days 1, 2, 3, and 4 to monitor the expansion of cell numbers with time in culture media. Media was replaced every 24 hours with fresh media containing LPS at concentrations of 50-100ng/ml respectively. Standard deviations were determined for all repeated analysis (n=3) to determine scatter of the data. In order to compare expansion of cells with time at LPS concentrations of 50ng, 100ng and control, a one way ANOVA test was used making no restrictive assumption regarding scatter of data, using Graphpad InStat computer software package. The correlation coefficient to assess linearity between cell number and absorbance at 490nm, were also determined using Graphpad InStat computer software package (Instat Package; GraphPad Software, San Diego, CA, USA).
3.2.8 Effect of subtoxic levels of LPS on alkaline phosphatase activity

The alkaline phosphatase enzymes are a specialised group of glycoproteins usually associated with bone matrix, detected in mineralised matrices and linked with external membranes of osteoblasts (Robey, 1993). At present there are no specific markers for periodontal ligament cells, however several studies have demonstrated that a population of cells capable of expressing alkaline phosphatase activity exist in the periodontal ligament (Lekic et al., 2000; Saito et al., 2002).

The alkaline phosphatase assay is based on the biochemical principle that in the presence of alkaline phosphatase, the chromogenic substrate p-nitrophenylphosphate is hydrolysed to form p-nitrophenol (pNP) which is yellow color and can be measured at 410nm on a spectrophotometer.

PDL cells were cultured as above (section 3.2.3) and seeded at 1x10^4 cells/cm^2 in 12 well plates. Cells were allowed to settle for 24 hours after which (day 0) the media was replaced with media containing LPS at concentrations of 50ng/ml and 100ng/ml respectively, each LPS concentration in triplicate. Media without LPS was used as control. On days 2, 4, 6 and 8, the media was removed and the remaining adherent cells were treated with 2.5 ml of 0.1% Triton X-100 buffer (pH 7.2) for 15 min at 37°C. The wells were scraped with a cell scraper to free adherent cells. The supernatant containing solubilised cell components were removed and buffer-exchanged using PD-10 columns (Amersham Life Sciences, Buckinghamshire, UK) as follows. The PD10 columns were equilibrated with 25ml elution buffer (1M diethanolamine, 0.5mM magnesium chloride, pH 9.8) following which, 2.5 ml of sample treated with Triton-X was allowed to run into the column. Once the whole of the sample had run through the column, 3.5ml of elution buffer was run through the column to elute the protein fraction and the buffer containing the protein fraction was collected. 10μl of 150mM p-nitrophenyl phosphate (Sigma-Aldrich) was mixed with 90μl of sample obtained from elution. Triplicates of each sample were prepared and the plate incubated at 37°C for 30min and absorbance
recorded at 405nm with a 96-well plate reader (BioTek Instruments Ltd). The activity of alkaline phosphatase present was calculated by applying the mean absorbance readings to the equation:

$$\text{ALP activity (units)} = \frac{\text{Abs/min} \times \text{TV}}{18.45 \times \text{LP} \times \text{SV}}$$

Where  
TV = Total vol (ml)  
SV = Sample volume (ml)  
18.45 = Millimolar absorptivity of p-nitrophenol at 405nm  
LP = Lightpath (1-cm).

The assay was performed in triplicate and cell counts were carried out from 5 different fields per well. The mean activity of alkaline phosphatase (activity units/10^4 cells) for each time point was calculated.

### 3.2.9 Effect of sub-toxic levels of LPS on matrix formation

To investigate the effect of sub-toxic levels of LPS on matrix formation, the expression of bone markers were examined at mRNA level and at protein levels. The specific markers examined were osteopontin, BSP, osteonectin, osteocalcin, alkaline phosphatase, decorin and biglycan. RT-PCR was carried out to examine gene expression at mRNA level and Western blot carried out to examine synthesis of protein.
3.2.9.1 RT PCR

3.2.9.1.1 RNA extraction

PDL cells were seeded at $1 \times 10^4$ cells/cm$^2$ in 12 well plates and left overnight to adhere. The cells were then stimulated with 0, 50ng and 100ng/ml *P. gingivalis* LPS, each condition in triplicate. Media was replaced with fresh media every 48 hrs. On days 4, 8 and 12, the media was pipetted out and the cells washed twice with PBS. Using RNeasy™ mini kit (Qiagen, Crawley, UK), total RNA was extracted from the periodontal ligament cells. The kit consists of RLT lysis buffer, RW1 and RWE wash buffers and spin columns containing membranes which bind RNA. 10µl β-mercaptoethanol was added to 1ml RLT Buffer (containing 25% guanidium thiosulphate) to facilitate complete inactivation of RNase activity. 100 µl of this buffer was added to the cells and the cell lysate was mixed and pipetted directly into a QIA shredder spin column placed in a 2ml collection tube and centrifuged for 2min at 10,000g to facilitate homogenization. An equal volume of 70% ethanol (Qiagen Ltd) was added to the homogenised lysate and mixed by pipetting to promote selective binding of the RNA to the column membrane. The spin columns were centrifuged at 8000g and the flow through discarded. 350µl of buffer RW1 (containing 2.5 -10% guanidium thiosulphate, 2.5-10% ethanol) was added to the spin columns, centrifuged for 15 seconds and the flow through discarded. 10µl of DNase enzyme (Qiagen) was added to each column to eliminate contamination with genomic DNA and incubated at room temperature for 15mins prior to a repeat wash with buffer RW1. 500 µl of Buffer RPE was added to the column and centrifuged at 8000g for 15secs and the flow through discarded, followed by a repeat wash using buffer RPE and centrifuged at 12,000g to dry the RNeasy membrane. The RNeasy column was then transferred to 1.5ml collection tube and 30µl of RNase free water (Qiagen Ltd) added to the column membrane and centrifuged at 8,000g for 1min to elute the RNA. The tubes containing the RNA was placed immediately on ice.
3.2.9.1.2 Quantification of RNA

RNA was quantified using Nanovue™ spectrophotometer (GE Healthcare). Purity of RNA was assessed by examining the A 260:A280. RNase free water was first used to obtain a blank reading. 2 μl of the sample was placed onto the sample plate and absorbance values were recorded at 260nm. An absorbance of 1OD is equivalent to approximately 40 μg/ml RNA. Concentration of RNA was calculated by the following equation:

A260 X RNA factor (40) X Dilution factor = Total RNA μg/ml

The absorbance at 260:280nm of all samples were < 2.0 indicating RNA purity.

3.2.9.1.3 Reverse transcription of total RNA

To 1 μg RNA sample, 1 μl of random primer (Promega) was added and final volume made up to 15 μl with nuclease free DEPC water and incubated at 70°C for 5mins in PCR machine. A master mix made up of 55μl 5x reaction buffer (250mM Tris-HCl at pH 8.3, 375mM KCl, 15mM MgCl₂, 50mM DTT),13.75μl 10mM PCR nucleotide mix (dNTP’s), 6.6 μl of 40U/ml RNase inhibitor, 11μl of 200U/μl reverse transcriptase and 23.65 μl DEPC water was prepared. 10 μl of the Master mix was added to 15 μl of RNA/random primer mix. A RT negative control was prepared by excluding RNA from the above components and nuclease free water was used as negative control. Reactions were run on a G-storm™ GS1 Thermal cycler (Genetic Research Instrumentation Ltd, UK) and run at 37°C for 1 hour after which the samples were cooled on ice and stored at -20°C until used for PCR reaction. All reagents used obtained from Promega, Southampton, UK.
3.2.9.1.4 Polymerase chain reaction

A master mix was prepared by adding 55μl of 5x Green GoTaq™ Flexi Buffer (Promega), 5.5 μl 10mM stock nucleotide mix (dNTP’s), 13.75μl (F) Primer and 13.75μl (R) Primer (Table 4.1), 11μl of 3mM MgCl₂, 162.25 μl PCR grade water and 2.75 μl Taq DNA polymerase (Promega). 24μl of Master-mix was pipette into separate 0.25ml PCR tubes and 1μl cDNA was added to each tube to make up a final volume of 25 μl. Nuclease free water was used as negative control. Reactions were run on a G-storm™ GS1 Thermal cycler (Genetic Research Instrumentation Ltd, UK) with an initial denaturing step of 95°C for 4mins, followed by 35 cycles of a 1min 95°C denaturing step, 1 min 62°C annealing step and 1 min 72°C extension step. A final extension step at 72°C was run for 10mins to end the reaction. The reaction products were cooled down to 4°C and then visualised.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteonectin</td>
<td>F: 5’GGG GCC AGG GTG TCT GGG TAG CAC ACA GCC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TGG GAG CAG GGC AGC TGG TGG GGT CCT G-3’</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>F: 5’ CAC TGC GCT CCT TAG GGC TGC CTC CGC TCG C-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CAG TGG CAG TGC CCG CGC TGT CAG GGA C-3’</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>F: 5’GGA GTC CGA TGA GGC TAT CAA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’TCC GAC TGC TCA GTG CTC TC C-3’</td>
</tr>
<tr>
<td>Decorin</td>
<td>F: 5’ GTG GGT GTC AGC TGG ATG CGC TCA CGT CGC AG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ AGG TTG TGT CGG GTG GAA AAT CCC AGG GCA-3’</td>
</tr>
<tr>
<td>Biglycan</td>
<td>F: 5’ AGC GGG CTC CGC AAG ATG AAG TGC ATT G-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ TGC ACT TCC CAG TAG GGC ACA GGG TTG T-3’</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>F: 5’ AGT CCC ACA CAG CAG CTT GGC CCA GAC CTA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ATT GAC CTG CAC GTC TAG CCC TCT GCA GGT -3’</td>
</tr>
<tr>
<td>BSP</td>
<td>F: 5’ CTG CTT TAA TCT TGC TCTG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ CCA TCT CCA TTT TCT TCC-3’</td>
</tr>
<tr>
<td>B-Actin</td>
<td>F: 5’ TGA AGA TCA AGA TCA TTG CTCC TCC -3’</td>
</tr>
<tr>
<td>(House keeping gene)</td>
<td>R: 5’ CTA GAA GCA TTT GCG GTG GAC GATG -3’</td>
</tr>
</tbody>
</table>

Table 3.1: Primer sequences used in the PCR reactions. Primers were designed using Primer Blast to ensure specificity for the intended amplification targets.
3.2.9.1.5 Visualisation of PCR products on Agarose gels

PCR products were run on 2% agarose gels (Sigma, Aldrich) as described in section 3.2.2.4 and visualised on Gel Doc™ scanner (Bio-Rad, Hemel Hempstead, UK) using UV light and digital images recorded.

3.2.9.2 Western blot analysis

3.2.9.2.1 Protein extraction

Periodontal ligament cells from second passage were reseeded at a concentration of 1x10^4 cells/cm^2. After 24 hours, cells were stimulated with P. gingivalis LPS at concentrations of 50ng and 100ng/ml, each condition in triplicate. On days 4, 8 and 12, non-collagenous proteins were extracted using extraction buffer. One Complete™ protease inhibitor cocktail tablet (Roche, Hertfordshire, UK) was dissolved in 50ml of the extraction buffer (4M Guanidine-HCl, 0.03M EDTA and 0.05M Tris HCl) as per manufacturer's instructions, immediately prior to use. The cells cultured in 12 well plates were washed twice for 5mins with PBS. 1ml of extraction buffer was added to each well and left for 48hrs at 4°C with constant agitation. Extracts were collected and centrifuged at 1,000g for 5mins. The resulting supernatents were collected and desalted using Centriprep spin columns (Millipore, Billerica, USA) prior to lyophilisation and resuspension in 2mls of distilled water. All samples were homogenised by sonification and stored at -20°C.
3.2.9.2.2 Evaluation of protein content using BCA assay

The BCA protein assay kit (Pierce, Northumberland, UK) was used to determine the protein concentration in the samples. This assay is based on the principle that Cu$^{+2}$ is reduced to Cu$^{+1}$ in the presence of protein in an alkaline medium (biuret reaction) followed by chelation of the cuprous ion by the bicinchoninic acid resulting in the formation of a purple product which can be measured by absorbance thereby indicating the relative protein concentration in the sample.

Standards provided in the kit were prepared to a concentration range of 125μg/ml to 2,000 μg/ml. 25 μl of samples or standards were placed in triplicate wells of a 96 well plate.

The BCA reagent was prepared by combining 12 mls of reagent A (a sodium bicinchoninate solution) with 240 mls of reagent B (a cupric sulphate solution). 200μl of the combined sodium bicinchoninate/cupric sulphate solution was added to each well containing either sample or standard and incubated at 37˚C for 30 mins. Absorbance at 570nm was read using a Microplate reader (BioTek Instruments Ltd). The protein concentration of the samples was determined from the standard curve generated by the absorbance values of the BSA standards plotted against protein concentration. Protein samples were then diluted to an equal concentration of 2.5 μg/μl for further experiments.

3.2.9.2.3 Protein separation using SDS-PAGE

The molecular size profile of the protein was examined by SDS-PAGE using the Phast System (Amersham Biosciences, Buckingshire, UK). The Phast System is a semi-automated horizontal electrophoresis system, utilising preformed gels and buffer strips with voltage and sample application controlled by a microprocessor. Protein samples were diluted 1:1 with a protein sample buffer containing 26% (v/v) 0.5M Tris HCl, 21%(v/v) glycerol, 42% (v/v) of 10% ( w/v) SDS in distilled water, 10%(v/v) of 2-β-mercaptoethanol and 1%(v/v) of 0.5%(w/v)
bromophenol blue in distilled water (final protein concentration of 2.5μg/µl). Samples were heated to 100˚C for 5mins and the proteins were separated on 4-15% gradient polyacrylamide gels (GE Healthcare).

70μl of double-distilled water was placed on the separation bed cooled to 15˚C and the gel (4-15% gradient polyacrylamide gels, GE Healthcare) was placed on the bed to give a thin film of water between the gel and the bed. The performed buffer strips (GE Healthcare) were inserted into the appropriate anode and cathode compartments and placed onto the gels. Molecular weight marker (SeeBlue™ Plus 2 pre-stained marker, Invitrogen) and samples were first drawn into a six lane, 4μl application comb by capillary action, before being placed in the electrophoresis apparatus and applied to the gel. The separation programme used involved two initial steps: an initial low electric output of 100V, 1.0MA, 1.0W, 4.0Vh, which drew the samples through the stacking gel, followed by an increased electrical output of 250V, 10.0MA, 3W, 66Vh for separation of the molecular components.

3.2.9.2.4 Western Blotting

On completion of separation, the gel was removed from the separation unit and placed in transfer buffer (0.03M Tris Base, 0.2M glycine, 10% methanol, 1% SDS). The anode base plate was cleaned by wiping with transfer buffer and the electrophoresis apparatus was placed over it. The transfer sandwich was built up by placing 3 filter papers (chrom1) wetted in transfer buffer, one on top of the other taking care to exclude air bubbles. Using the gel separating apparatus, the gel was separated from its plastic backing and placed on a paper towel with the polyacrylamide side facing up. Next, a nitrocellulose sheet (ECL hydrobond, GE Healthcare) wetted in transfer buffer was placed carefully over the gel without trapping any air bubbles. The plastic backing was removed from the gel and the gel along with the nitrocellulose sheet was placed over the wet filter papers with the nitrocellulose sheet positioned below the gel. 3 additional sheets of filter papers wetted in transfer buffer were
placed over the gel. The cathode was placed over the sandwich and protein was electro blotted onto the nitrocellulose sheet for 30 min at 15V, constant voltage.

On completion of transfer, the nitrocellulose membrane was separated and incubated in 5% milk (low fat dried skimmed, Marvel) in wash buffer (0.01M Tris HCl pH 7.5, 0.1 M NaCl, 0.1% Tween-20) for one hour to block non-specific protein binding sites. Primary antibodies were diluted 1:50 in 5% milk (Marvel) and applied to the membrane for 1 hr at room temperature. The nitrocellulose membrane was then washed with 4 changes of wash buffer with each wash of a duration of 5 min. Secondary antibody in a dilution of 1: 10,000 in 5% milk was applied to the blot for 1 hr at room temperature. The nitrocellulose membrane was then washed x3 with wash buffer and the immuno-reactive bands were visualised by using BCIP/NBT color development substrate. The combination of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyl phosphate p-toluidine salt) is used for the colorimetric detection of alkaline phosphatase activity and yields an intense, insoluble black-purple precipitate in the presence of alkaline phosphatase. The substrate was prepared in 5ml of alkaline phosphatase buffer (100mM Tris HCl at pH-9.0, 150mM NaCl, 1mM MgCl₂) by adding 33µl of NBT, followed by 16.5µl of BCIP. The nitrocellulose membrane was left in the substrate on a platform shaker with constant agitation till the formation of the blue black substrate and then washed x3 in wash buffer and blotted dry.
<table>
<thead>
<tr>
<th>1º Antibody and dilution used</th>
<th>Antibody source</th>
<th>ALP conjugated 2º antibody and dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti mouse Decorin 1:50</td>
<td>Larry Fisher LF-113</td>
<td>Goat anti rabbit IgG (Sigma) 1:10,000</td>
</tr>
<tr>
<td>Rabbit anti mouse Biglycan 1:50</td>
<td>Larry Fisher LF-104</td>
<td>Goat anti rabbit IgG (Sigma) 1:10,000</td>
</tr>
<tr>
<td>Goat anti mouse Osteonectin (SPARC) 1:50</td>
<td>Santa Cruz Biotech H-14</td>
<td>Rabbit anti goat IgG (Sigma) 1:10,000</td>
</tr>
<tr>
<td>Goat anti mouse Osteopontin 1:50</td>
<td>Santa Cruz Biotech P-18</td>
<td>Rabbit anti goat IgG (Sigma) 1:10,000</td>
</tr>
<tr>
<td>Rabbit anti mouse Osteocalcin 1:50</td>
<td>Santa Cruz Biotech FL-95</td>
<td>Goat anti rabbit IgG (Sigma) 1:10,000</td>
</tr>
<tr>
<td>Rabbit anti mouse BSP 1:50</td>
<td>Larry Fisher LF-87</td>
<td>Goat anti rabbit IgG (Sigma) 1:10,000</td>
</tr>
</tbody>
</table>

**Table 3.2:** 1º and 2º Antibodies used in Western Blot and their dilutions.
3.3 Results

3.3.1 Characterisation of *P. gingivalis* LPS

Electrophoretic separation of *P. gingivalis* LPS extracted using hot phenol water technique and commercially available *E. coli* LPS (Sigma, UK) was carried out by SDS-PAGE followed by silver staining. The classical laddering heterogenous pattern of LPS (Fig 3.1), which is attributed to the variability of the O-specific oligosaccharide moiety (Tsai and Frasch 1982) was observed for *P. gingivalis* LPS, similar to that obtained from *E. coli* although lower molecular weight fragments were also observed in *P. gingivalis* samples.

Staining of SDS-PAGE gels with Coomassie Blue was carried out to assess contamination with protein in the *P. gingivalis* and *E. coli* LPS samples. Successful removal of contaminating protein was confirmed as indicated by the absence of staining in the purified samples as shown in Fig. 3.3. Similarly, *P. gingivalis* and *E. coli* LPS were assessed for contamination with nucleic acid on agarose gel electrophoresis followed by staining with ethidium bromide. Absence of streaks in the purified samples (lane 1 and 3 in Fig 3.2) confirmed successful purification from nucleic acid contamination. Significant amount of nuclear material were present in the non-purified samples as shown in Fig.3.2 (lane 2 and 4)
Fig 3.1: LPS fraction was extracted by hot phenol method and subjected to SDS-PAGE followed by silver staining.
Lane 1 - Unpurified *E. coli* LPS sample with streaks indicating contamination with protein.
Lane 2 - Unpurified *P. gingivalis* LPS sample demonstrating distinct laddering pattern of LPS with streaks indicating protein contamination.
Lane 3 - Purified *P. gingivalis* sample demonstrating distinct laddering pattern with absence of streaks.
Lane 4 - Sample of supernatant obtained during hot-phenol extraction demonstrating heavy streaks indicating contamination.
Fig 3.2: Agarose gel stained with ethidium bromide demonstrating streaks in the unpurified LPS samples (lane 2 and 4) indicating contamination with nuclear material.

Fig 3.3: SDS-PAGE stained with coomassie blue demonstrating dark band in lane 5 indicating contamination with protein in the supernatant obtained from hot phenol extraction.
3.3.2 Isolation and characterisation of PDL cells

The PDL derived cells demonstrated a spindle shape fibroblast like morphology with formation of colonies (Fig 3.4). The presence of MSC’s were evaluated by the presence of cell surface markers CD105 (Endoglin), CD90 (Thy1), CD73 and the presence of osteoprogenitor cells were evaluated by the presence of surface marker OPN by immunocytochemical staining, with a majority of the cell population staining positive to the markers investigated (Fig 3.5).
Fig 3.4: Representative light microscopy images of PDL cell population obtained using explant technique. Cells were initially cultured as explants in culture media until confluent and then passaged further (x3). Typical appearance of cells obtained from passage 3, on day 4 (A) and day 8 (B) demonstrating proliferating PDL cells with spindle shape fibroblast like cell morphology and formation of colonies (indicated by arrow).
Fig 3.5: PDL cells obtained from passage 3 were cultured in chamber slides, fixed with 4% paraformaldehyde and treated with antibody (anti-CD105/anti CD 90/anti CD 73 or anti-OPN antibody) and then visualised with FITC conjugated antibody (stained green) and DAPI (nuclei stained blue). Representative fluorescence microscopy images depicting immunocytochemical staining of the PDL cells demonstrating the presence of A-CD105 (Endoglin), B-CD90 (Thy1), C-CD73 and D-OPN positive cells in the cultured PDL cell population indicating the presence of MSC’s and osteoprogenitor cells.
**Fig 3.6:** Typical examples of isotype control (A) and negative control (B). No staining observed on immunocytochemical staining indicating that the staining observed in Fig 3.5 was not caused by non-specific interactions of immunoglobulin molecules with the sample.
3.3.3 LAL Assay

LAL assay was carried out to estimate levels of LPS in the samples. A standard curve was obtained by plotting the absorbance against the corresponding concentrations of standards provided in the kit as shown in Fig 3.7 and the concentration of endotoxin in the samples was determined from the standard curve (1mg=10,346EU).

3.3.4 Evaluation of seeding density by MTS assay

PDL cells were plated at concentrations of 6x10^3, 1x10^4, 2x10^4, 4x10^4, 6x10^4, 8x10^4, 1x10^5 in duplicates in a 12 well plate and MTS assay carried out as detailed in section 3.2.5. A standard curve was obtained by plotting the absorbance against the corresponding concentrations of cells as shown in Fig 3.8. The correlation coefficient (r) was calculated using Instat Graphpad software package to determine association between seeding density and absorbance. A r value of 0.9688 was obtained indicating a linear response. A seeding density of 1x10,000 was determined as appropriate for further experiments.
Fig 3.7: LAL assay was carried out to determine endotoxin levels in sample. Proteolytic activity was activated in the presence of endotoxin, which resulted in yellow color on addition of chromogenic substrate that was quantitated by measuring the absorbance at 405nm and extrapolated against a standard curve (1mg=10,346EU).
**Fig 3.8:** PDL cells were plated at various seeding densities and cell proliferation assessed by MTS assay showed a linear increase in absorbance with increasing cell seeding density at 490nm ($R=0.9688$). A seeding density of 10,000 cells/cm$^2$ was determined as appropriate for further experiments.
3.3.5 Effect of *P. gingivalis* LPS on cell viability

The influence of *P. gingivalis* LPS on PDL cell viability was assessed by trypan blue staining. Viable (unstained) and non-viable cells (blue stained cells) were counted from 5 different fields, from which the percentage of viable cells were calculated, where a cell viability count greater than 95% was acceptable. A dose dependent decrease in cell viability was observed as shown in Fig.3.9. Cells cultured in the presence of LPS at a concentration of 50ng/ml and 100ng/ml demonstrated a viability between 80-90% whereas cells cultured in the presence of 500ng/ml LPS demonstrated a cell viability of <50%.
Fig 3.9: Toxic and sub-toxic levels were determined by monitoring cell viability by trypan blue staining. PDL cells obtained from passage 3 were exposed to various concentrations of LPS and the % of viable and non-viable cells determined. The data represent mean ± SD of triplicate experiments. With increasing levels of LPS, the cell viability reduced to <50% for cells exposed to 500ng/ml LPS (**p<0.01 at 100ng/ml and ***p<0.001 at 200ng/ml and 500ng/ml).
3.3.6 Effect of sub-toxic levels of LPS on cell behaviour.

The influence of *P. gingivalis* LPS at concentrations of 50ng/ml and 100ng/ml on PDL cell expansion over the first 4 days post reseeding was monitored by means of MTS assay (Fig 3.10). Significant differences (*p*<0.05) were observed between LPS supplemented and unsupplemented cells on days 1, 2 and 3 with the highest stimulatory effect observed on day 3 (*p*<0.001). However, these differences were not dose dependent.

Values are presented as means ± S.D. *p*-values were calculated using one-way analysis of variance with the Tukey post-correction test if the *p*-value was < 0.05 (Instat Package; GraphPad Software, San Diego, CA, USA), to determine the direct differences in cell numbers and absorbance between *P. gingivalis* LPS-supplemented and unsupplemented cells at each time points analysed.
**Fig 3.10:** Cell proliferation of PDL cells was assessed by MTS assay. PDL cells from passage 3 were cultured in media containing *P. gingivalis* LPS (50 or 100ng/ml) and MTS assay carried out at various time points. The data represent mean ± SD of triplicate assays. Cell proliferation was significantly increased when compared to unsupplemented cells at day 1 (*p*<0.05), day 2 (**p*<0.01) and day 3 (**p*<0.001).
3.3.7 **Effect of sub-toxic levels of LPS on alkaline phosphatase activity.**

Fig 3.11 demonstrates the effect of LPS at concentrations of 50ng/ml and 100ng/ml on membrane bound alkaline phosphatase activity. PDL cells were cultured in the presence of LPS at concentrations of 50ng/ml and 100ng/ml and the alkaline phosphatase activity was monitored by the hydrolysis of p-nitrophenol at specific time points (days 2, 4, 6 and 8). Cells cultured in the presence of 50ng/ml and 100ng/ml LPS, significantly inhibited (p<0.001) alkaline phosphatase activity over days 2 and 4 as shown in Fig. 3.11. Significant differences between LPS supplemented and unsupplemented cells (p<0.05) were observed upto day 6.
Differentiation potential of PDL cells was determined by monitoring alkaline phosphatase activity. PDL cells from passage 3 were cultured in media containing *P. gingivalis* LPS (50 or 100ng/ml) and alkaline phosphatase activity was measured at various time points. The data represent mean ± SD of triplicate assays. PDL cells cultured in media containing *P. gingivalis* LPS (50 or 100ng/ml) showed significant reduction in alkaline phosphatase activity when compared to unsupplemented cells on days 2, 4 and 6 (**p<0.01 and ***p<0.001).
3.3.8 Effect of LPS on matrix formation

a) Protein quantification

BCA assay was carried out to quantify protein levels in the samples. A standard curve was generated by plotting the absorbance against the corresponding concentrations of standards provided in the kit as shown in Fig 3.12 and the concentration of the protein in the sample was determined from the standard curve.

b) mRNA level

Expression of markers characteristic of the osteogenic phenotype were assessed at gene and protein levels. Fig 3.13 shows a steady decline in alkaline phosphatase mRNA levels from day 4 to day 12. Expression of decorin was weak at day 4 compared to day 12, whereas biglycan was expressed at all time points. Osteopontin, osteonectin, osteocalcin and BSP mRNA levels were expressed at all time points.

The techniques used in this study (RT-PCR) are semi-quantitative and therefore the absolute levels of mRNA expression between different conditions and time points was not possible. The densitometric analysis was carried out to demonstrate trends in levels and on analysis, a reduction in biglycan expression was observed on day 8 (Fig 3.14).
c) Protein synthesis

Western Blot immunocharacterization of osteopontin, osteonectin, osteocalcin, decorin, biglycan and BSP isolated from the extracellular matrix surrounding cells incubated in the presence or absence of *P. gingivalis* at different concentrations is shown below. Faint bands are present on day 4 and 8 with the presence of (Fig 3.15). Presence of streaks may represent the presence of low molecular weight proteins representing degradation products or may be due to altered phosphorylation or post translational modification of OPN. On day 12, an increase in osteopontin synthesis was observed on stimulation with 100ng LPS suggesting persistence of OPN which may inhibit mineralization. Similarly, osteocalcin (Fig 3.16) and osteonectin (Fig 3.17) were also detected. A dose dependent difference was not observed. On the other hand, a dose dependent difference was observed for biglycan on day 12 (Fig 3.18). Decorin (Fig 3.19) and BSP (Fig 3.20) were weakly expressed at all time points observed.
Fig 3.12: BCA assay was carried out to quantify protein levels in the samples.

\[ y = 0.1781x - 0.0387 \]

\[ R^2 = 0.9335 \]
**Fig 3.13:** PDL cells were cultured in media containing LPS (50ng and 100ng/ml) and gene expression of alkaline phosphatase, decorin, biglycan, osteopontin, osteonectin, osteocalcin and BSP were assessed by RT-PCR on days 4, 8 and 12. A steady decline in alkaline phosphatase mRNA is observed from day 4 to day 12. Weak expression of Decorin and BSP mRNA are indicated on day 4 and 8 whereas biglycan and osteopontin mRNA are expressed consistently on all days. Osteonectin and osteocalcin mRNA expression appear progressively weaker from day 4-12.
Fig 3.14: Densitometric analysis of gene expression of alkaline phosphatase, decorin, biglycan, osteopontin, osteonectin, osteocalcin and BSP investigated by RT-PCR on days 4, 8 and 12 show a trend towards decreased alkaline phosphatase, osteonectin, decorin and biglycan mRNA expression.
Fig 3.15: Western Blot analysis of protein extracted from ECM surrounding PDL cells stimulated with *P. gingivalis* LPS (50ng/ml and 100ng/ml) at various time points (days 4, 8 and 12 post seeding) demonstrating presence of OPN (Mol wt - 44kDa) on day 4 and 8. Presence of streaks may indicate the presence of degradation products indicating delayed/impaired matrix formation.
Fig 3.16: Western Blot analysis of protein extracted from ECM surrounding PDL cells stimulated with *P. gingivalis* LPS (50ng/ml and 100ng/ml) at various time points (days 4, 8 and 12 post seeding) demonstrating expression of osteocalcin (Mol wt -5.8kDa) on days 4 and 8. Absence on day 12 may be suggestive of impaired/ delayed matrix formation.
Fig 3.17: Western Blot analysis of protein extracted from ECM surrounding PDL cells stimulated with *P. gingivalis* LPS (50ng/ml and 100ng/ml) at various time points (days 4, 8 and 12 post seeding) to investigate the presence of osteonectin (Mol wt - 40 kDa). Faint expression of osteonectin observed on day 4 with streaks present at all concentrations and time points which may represent degradation products.
Western Blot analysis of protein extracted from ECM surrounding PDL cells stimulated with P. gingivalis LPS (50ng/ml and 100ng/ml) at various time points (days 4, 8 and 12 post seeding) demonstrating faint expression of biglycan (Mol wt - 45 kDa) on day 4. Streaks present at all concentrations and time points may represent degradation products.
**Fig 3.19:** Western Blot analysis of protein extracted from ECM surrounding PDL cells stimulated with *P. gingivalis* LPS (50ng/ml and 100ng/ml) at various time points (days 4, 8 and 12 post seeding) to investigate presence of decorin (Mol wt - 50 kDa). Faint streaks present at all concentrations and time points may represent degradation products.
**Fig 3.20:** Western Blot analysis of protein extracted from ECM surrounding PDL cells stimulated with *P. gingivalis* LPS (50ng/ml and 100ng/ml) at various time points (days 4, 8 and 12 post seeding) to investigate the presence of BSP (Mol wt -60-80kDa). Streaks present at all concentrations and time points may represent degradation products.
3.4 Discussion

In the last decade, extensive research has identified the presence of mesenchymal stem cell population within the periodontal ligament with a capacity to form physiological bone and connective tissue. Albeit, limited repair/regeneration is observed in patients undergoing periodontal treatment, the reasons for which are less understood. One could speculate that the presence of virulence factors such as LPS may alter cell behaviour of the progenitor cell population, but this has not been investigated previously. Therefore, this present study investigated the influence of sub-toxic levels of *P. gingivalis* LPS on periodontal ligament cell behaviour in terms of proliferation, differentiation and matrix formation with a view to further our understanding of the response of these cells within a pathological situation.

The behaviour of the progenitor cell population thus obtained was further assessed on exposure to sub-toxic levels of *P. gingivalis* LPS and the results obtained from this study provided strong evidence that *P. gingivalis* LPS has the capacity to increase the proliferative activity of the PDL cells but decrease the differentiation and matrix formation potential of these cells. A reduction in differentiation potential was evidenced by a decrease in the alkaline phosphatase activity and an alteration in matrix production was demonstrated by examining mRNA expression profile of decorin, biglycan, osteocalcin, osteonectin and osteopontin in addition to western blot analysis examining protein expression. The effects of LPS on matrix formation has not been investigated previously to include a wide range of matrix proteins and therefore this study reports for the first time that in the presence of virulence factors such as *P. gingivalis* LPS, the biological events associated with the synthesis of a matrix may be altered and consequently influence the potential of the tissues in protecting the alveolar bone from resorption, the preservation of the Sharpey’s fibres and thus influence homeostasis. PDL tissue has been reported to have the highest turnover rate when compared to other connective tissues in the body. Therefore, an imbalance between tissue formation and degradation is
likely to render the tissue more susceptible to rapid breakdown, providing an explanation for its high susceptibility and reduced resistance to combat infection.

At the time of study (2006), only *E. coli* LPS was available commercially which appeared to be contaminated with protein and nucleic acid (Fig 3.3). It has been reported that contaminating components, specifically bacterial proteins and nucleic acids may have the capacity to influence cell behaviour. In addition, structural differences in the core molecule along with variations in length and phosphorylation of the lipid A component have been identified within LPS of *P. gingivalis* species and reported to have an influence on cell behaviour. Therefore, justification was obtained for the necessity to extract and purify *P. gingivalis* LPS and thereby provide a more clinical relevant scenario with which to investigate the effect of LPS on PDL cell behaviour *in vitro*.

LPS was extracted from bacterial cell walls using the hot phenol-water differential extraction technique and the characterisation of *P. gingivalis* LPS demonstrated absence of contamination with bacterial proteins and nucleic acids. The classical heterogenous laddering pattern of *P. gingivalis* LPS as reported in other studies (Chen et al. 1990; Kadono et al. 1999; Roberts et al. 2008) was observed on SDS-PAGE of the LPS samples, which is attributable to the variability of the O-specific oligosaccharide side chain (Tsai and Frasch 1982).

As mentioned previously in Chapter 1, the heterogeneous population present in the PDL also includes a population of stem/progenitor cell population which originate from the ectomesenchymal cranial neural crest cells which possess the capacity to differentiate into periodontal ligament fibroblasts, cementoblasts and osteoblasts (Sodek and McKee 2000; Bartold et al. 2006; Hynes et al. 2012) and it is this progenitor cell population that have been speculated to play an active role in repair and regeneration. Therefore, to confirm the presence of a progenitor cell population within the cultured cells, the presence of cell surface markers CD105, CD90 and CD73 were evaluated by immunocytochemical staining. Additionally, the presence of OPN positive cells was investigated to confirm the presence of osteoprogenitor cell population. The results demonstrate that the PDL population presented
with a typical fibroblastic morphology with a majority of the cell population staining positive to cell surface markers CD105, CD90, CD73 and OPN. The model system used in this study is therefore highly relevant in understanding the pathological processes involved in periodontal disease, as this technique utilises a possible high proportion of progenitor cells similar to that recruited during the repair process. In order to maintain the rich phenotypic and functional heterogeneity of the cells characteristic of the original tissue, primary PDL cell cultures of early passage were used for all experiments.

On exposure to sub-toxic levels of LPS, an increase in cell growth was observed, followed by a decrease in alkaline phosphatase activity over the time period investigated. The mRNA expression profile supported the results obtained from examining the ALP activity of the PDL cells in that, a noticeably weaker mRNA expression of alkaline phosphatase was observed with an increase in time, which further indicated that \textit{P. gingivalis} LPS may inhibit the osteoblastic differentiation of PDL cells. ALP is a marker of the osteoblastic phenotype and is secreted during mid-stage differentiation (Weinreb et al. 1990), which is when mineralisation is initiated (Aubin et al. 1995). Early characterisation studies have identified histochemically, that PDL fibroblasts stain intensively for ALP and biochemical analysis demonstrated an increase in basal ALP activity in culture over time (Basdra and Komposch 1997). Further studies reported PDL cells exhibited phenotypic characteristics consistent with osteoblast like cells and that such cells have the potential to differentiate into osteoblasts and or cementoblasts with the capacity to form mineralized deposits particularly in the maintenance and repair of the Sharpy’s inserts interfacing with the alveolar and cementum surfaces (Somerman et al. 1988; Nojima et al. 1990a; Arceo et al. 1991). During the inactive or repair stages of periodontal disease, repair is facilitated by the recruitment of the progenitor cell population present in the PDL adjacent to the alveolar bone, in addition to growth factors such as bone morphogenic proteins, transforming growth factor β, platelet derived growth factor, which are released as part of the repair process, to stimulate the proliferation and differentiation of the progenitor cells to form osteoblasts and fibroblasts that are capable of the eventual synthesis of the mineralised matrix (Bartold and Narayanan 2006; Hughes et al.
2006). It is therefore highly likely that the cells contributing towards alkaline phosphatase activity are pre-osteoblastic in nature. Other studies have evaluated the effect of LPS on the behaviour of osteoblast cells and reported an increase in the proliferative capacity demonstrated by these cells in response to stimulation with subtoxic levels of LPS and appeared to impede the formation of the osteoblast phenotype as evidenced by a reduction in ALP activity (Roberts et al. 2008; Kato et al. 2014). In the development of the osteogenic phenotype, three distinct phases have been reported by Roberts et al (2008), which involve an initial active cell growth, extracellular matrix synthesis and maturation followed by mineralisation. On exposure to subtoxic levels of LPS, an increase in cell growth was observed, a decrease in alkaline phosphatase activity which is suggestive of an impairment in the development of the osteogenic phenotype and a concomitant decrease in mineral deposition (Roberts et al. 2008). Based on the above findings, one could speculate that within the heterogenous cell population, LPS may increase the cell growth of one or more subsets of the mesenchymal cell population, which may have a detrimental effect on the other subsets. Therefore, the decrease in ALP activity observed in this study, may possibly reflect a decrease in the number of osteogenic precursor cells brought about by an increase in the other progenitor cell population in response to LPS stimulation.

The ECM proteins (osteopontin, osteonectin, osteocalcin, BSP, decorin and biglycan) play an important role in the processes modulating mineralization and remodelling. Therefore mRNA and protein expression profiles for specific markers (osteopontin, osteonectin, osteocalcin, BSP, decorin and biglycan) were investigated for the first time in this study to assess the influence of LPS on the functional ability of PDL cells in the production of matrix, as this has not been investigated by previous studies. Although differences were observed in the intensities of the various product bands, it is not possible to directly compare absolute differences in mRNA expression between stimulated and unstimulated cells as RT-PCR is at best a semi-quantitative method. However, this technique makes it possible to examine a broad range of markers and identify key markers and observe general trends which could then
be further investigated with advanced techniques such as Q-PCR that allows for direct measurement of differences in the levels of mRNA expression between stimulated and unstimulated or between cells stimulated with varying concentrations of LPS. Osteopontin (OPN), a marker of the osteogenic phenotype which is expressed in the early stages of osteoblast differentiation (Hughes et al. 2006), was strongly expressed at all time points. OPN has a wide array of functions which include stimulation of cellular signalling pathways, regulation of cell proliferation and phagocytic activity and can both promote and participate in cell migration in addition to the enhancement of cell survival by inhibiting apoptosis in addition to its primary function of facilitating recovery after injury or infection whereby an increase in its expression is observed (Sodek et al. 2000). Therefore, in this study, the continued expression of OPN at both mRNA and protein levels up to day 12 may indicate the presence of cell population in attempting to facilitate repair, as osteopontin is typically expressed in the early developmental stage by osteogenic cells (Sodek et al. 2000; Hughes et al. 2006). With regards to the wound healing process, osteopontin has several complex functions which include a chemo-attractant and anti-apoptotic signal for macrophages, neutrophils, T-cells, fibroblasts and endothelial cells, in addition to osteoblasts progenitor cells via CD44 interactions (Ashkar et al. 2000; Denhardt et al. 2001; Wang and Denhardt 2008). Therefore, the continued persistence of OPN up to day 12 may also indicate a potential role in the recruitment of cells of immune and inflammatory origin in-vivo, thereby extending the inflammatory response by the secretion of cytokines and other inflammatory mediators, consequently leading to a delay in the repair process. OPN induces the expression of MMP-2 and 9 which play an important role in matrix degradation (Weber et al. 2002; Philip and Kundu 2003) and is an inhibitor of mineralization (McKee et al. 2011; Yuan et al. 2014) further suggesting that its persistent presence in this study may be suggestive of impaired matrix formation. Post translational changes, variable phosphorylation and the presence of degradation products may contribute to the streaking present on W.B analysis. Osteocalcin, an osteoblast specific protein which is considered to be an indicator of a mature osteoblast phenotype and has been proposed to halt the process of bone formation and begin
the process of bone remodelling, thereby participating in the final stages of bone maturation (Boskey 1992; Hughes et al. 2006). In this study, the mRNA expression of osteocalcin decreased with an increase in time which was further supported by an absence of protein expression by day 12, in addition to the presence of degradation products as a result of post-translational modification as represented by streaks on W.B. Therefore in this study, exposure to *P. gingivalis* inhibited osteocalcin production thus inhibiting the differentiation of PDL cells to mature osteoblasts and consequently altered matrix synthesis.

Osteonectin, is a matrix-associated glycoprotein that influences a variety of cellular activities by binding to several proteins of the extracellular matrix (ECM), affect ECM protein expression, alter cell shape, reduce cellular adhesion, influence migration, modulate growth factor-induced cell proliferation and angiogenesis (Brekken and Sage 2000). In addition, it also influences cell interactions with the extracellular milieu during embryonic development and in response to tissue injury (Phan et al. 2007). A decrease in expression of osteonectin with an increase in time period as demonstrated in this study at both mRNA and protein level in addition to the presence of degradation products may suggest an altered ability of the tissues in facilitating repair.

Decorin and biglycan play an important role in cellular signalling, formation of matrix and mineralisation (Waddington et al. 2003a) and were therefore investigated in this study. The mRNA expression profile of biglycan was consistent at all time points investigated whereas decorin was weakly expressed at both mRNA and protein levels. However, at protein levels significant differences were noted for biglycan; an increase in protein synthesis was noted for cells stimulated with 100ng/ml LPS compared to 50ng whereas decreased protein synthesis was observed for the unstimulated cells. As the biglycan synthesis is normally high in the early stages of culture, which are associated with cell proliferation and early differentiation of progenitor cells (Waddington et al. 2003a), the results from this study indicate that the normal high levels were suppressed (day 4) in the early stages involving cell proliferation and differentiation after which it appears by day 12. In considering the repair process, these results are significant as biglycan binds to growth factors such as TGFβ and TNFα, sequestering them
to the matrix and thereby influence cellular activity indirectly (Hildebrand et al. 1994; Tufvesson and Westergren-Thorsson 2002; Bi et al. 2005), modulate BMP 2 induced osteoblast differentiation (Mochida et al. 2006), promote collagen fibrillogenesis (Vogel et al. 1984; Vogel and Trotter 1987; Sugars et al. 2003) and regulate the deposition of mineral in bone (Boskey et al. 1997). Low expression of decorin in this study may lead to a detrimental effect on the collagen matrix as decorin has been shown to bind to collagen and influence collagen fibril formation which ultimately provides the provisional matrix for mineral deposition (Vogel et al. 1984; Vogel and Trotter 1987; Sugars et al. 2003).

In conclusion, this study provides a more detailed assessment of the biological effects of sub-toxic levels of P. gingivalis LPS on PDL cell population and has provided evidence to further our knowledge on the events that take place during repair and regeneration. It is evident that complex interactions occur in the active and quiescent stages of periodontal disease due to imbalances in the metabolism and normal cellular activities of the cells present within the periodontium. Our results demonstrate that low levels of virulence factors such as LPS, have the potential to alter the growth and repair potential of cells involved in the repair process, which may further suppress the role of these cells in the repair and remodelling of tissue lost as a consequence of the disease process. Interestingly, since completion of this study, a recent study has endorsed this work in that, an increase in cell proliferation with a concurrent decrease in alkaline phosphatase activity was reported on exposing human PDL cells to P. gingivalis LPS (Kato et al. 2014). The clinical implications of these results may be that the delayed or impaired healing seen in non-responding deep sites despite extensive treatment, may be due to the presence of virulence factors such as LPS which is capable of affecting a whole range of biological events associated with matrix formation followed by mineralisation and therefore affect the ability of the tissues in attempting remodelling and repair. An imbalance in tissue turnover/remodelling rates may render the tissue susceptible to rapid destruction. Furthermore, these results implicate that as the biological processes associated with matrix synthesis is altered in periodontal disease, the contribution of the PDL towards the matrix degradation products collected in GCF is negligible, further confirming that the
degradation products detected in GCF are as a consequence of tissue destruction and not as a result of repair or remodelling. Therefore these results also contributed towards further evaluating proteoglycan as marker of disease activity in biomarker development.
Chapter 4

*P. gingivalis* LPS upregulates TLR2 and CD14 on periodontal ligament cells.

4.1 Introduction

The biological events associated with matrix synthesis have been proposed to be altered as a consequence to exposure to virulence factors such as LPS as detailed in the previous chapter. These alterations are driven by cytokines which are released as a consequence of the host-microbial interaction which result in amplifying the inflammatory response resulting in destruction of the soft and hard tissues and ultimately tooth loss. The response of the host immune response to invasion by pathogens is initiated by the recognition of conserved pathogen associated molecular patterns (PAMPs) through an array of receptors known as pattern recognition receptors (PRRs) which include the Toll like receptors (TLRs). The engagement of these receptors with their ligands, sets off a cascade of inflammatory reactions which, if not balanced may exacerbate chronic inflammatory processes such as periodontal diseases (Mahanonda and Pichyangkul 2007).

The TLRs are predominantly expressed on the cells of the innate immune system which mainly involve the neutrophils, monocytes, macrophages and dendritic cells. Different TLR expression is observed by these cells, thereby allowing them to induce a wide variety of immune responses to these specific pathogens. CD14 is a glycoprotein that forms a complex with LPS and LPS-binding protein (LBP), playing a role as a co-receptor for TLRs. Binding of the LPS/LBP/CD14 complex to TLRs triggers the downstream events in LPS signalling,
resulting in the activation of nuclear factor-kappa B followed by the transcription of various pro-inflammatory cytokine genes (Akashi-Takamura and Miyake 2008). As well as its role in LPS-mediated signalling, CD14 also plays a role in the recognition of PAMPs from mycobacteria and viruses and participates in signalling events involving TLR2 (Cleveland et al. 1996).

Since the periodontium is constantly exposed to bacteria, it has been suggested that TLR detection and activation may play an important role in the maintenance of periodontal health. The cells of the periodontium express different types of TLRs (Table 5.1) and thereby facilitate active participation in the first line of defence in maintaining periodontal health. Gingival epithelial cells recognise and continually interact with various microorganisms present within the oral cavity as well as those present within the biofilm on the tooth surface with the help of receptors TLR 2, 3, 4, 5, 6 and 9 (Kusumoto et al. 2004). This results in activating innate immune responses involving the release of antibacterial β-defensins cathelicidin and calprotectin as well as neutrophil chemoattractant IL-8 (Weinberg et al. 1998) resulting in limiting microbial invasion and breaching of the epithelial barrier, thereby maintaining gingival health. Tissue destruction results in disruption and penetration of the epithelial barrier by invasive bacteria or their products resulting in activation of TLRs in cells present in the deeper tissues such as macrophages, fibroblasts, osteoblasts, osteoclasts and antigen presenting cells. On stimulation, these cells produce various pro-inflammatory cytokines leading to inflammation and activation of the immune cells, thereby amplifying the inflammatory response leading to destruction of connective tissue and bone.
<table>
<thead>
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<th>Cell type</th>
<th>TLR</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>Langerhans cells/Tissue dendritic cell</td>
<td>TLR 1,2,3,4,5,6,8,10</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>TLR 2,3,4,5,6,9</td>
</tr>
<tr>
<td>Gingival fibroblast</td>
<td>TLR 2,4,9</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>TLR 1,3,4,5</td>
</tr>
<tr>
<td>Perodontal ligament fibroblast</td>
<td>TLR 2,4</td>
</tr>
<tr>
<td>Cementoblasts</td>
<td>TLR 2,4</td>
</tr>
<tr>
<td>Osteoblast</td>
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</tr>
<tr>
<td>Osteoclast</td>
<td>TLR 1,2,3,4,5,6,7,8,9</td>
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**Table 4.1** mRNA expression of TLRs on different cell types within the periodontium (Mahanonda and Pichyangkul 2007)

On sustained exposure to bacterial structures such as LPS, the oral mucosa develops tolerance (Muthukuru et al. 2005) in an attempt to regulate local immune responses. The underlying mechanism of this tolerance may be attributed to the down regulation of TLR expression and inhibition of intracellular signalling. However, chronic stimulation may result in over production of inflammatory mediators thereby contributing towards tissue destruction such as seen in severe forms of the disease. Therefore, studies have investigated the effect of bacteria and their products on TLR activation on various cell types with a view to understand how TLR stimulation determines the outcome of immune response.

Among the TLRs, TLR2 and TLR4 have been reported to be involved in the recognition of *P. gingivalis* LPS (Hirschfeld et al. 2001; Darveau et al. 2004). TLR4 is the principal signal transducer for most types of LPS (Darveau et al. 2004; Hajishengallis et al.
2006), while TLR2 is a signal transducer for other bacterial components, such as peptidoglycan and lipoprotein (Takeuchi et al. 1999). However, *P. gingivalis* LPS is unusual, in that it has been reported to be an agonist for both TLR-2 and TLR-4 (Hajishengallis et al. 2002; Darveau et al. 2004; Zhou et al. 2005).

Studies examining the expression of TLRs by fibroblasts have reported that human gingival fibroblasts (HGF’s) constitutively express mRNA of TLR2 (Tabeta et al. 2000), TLR4 (Wang et al. 2000) as well as receptor related molecules such as CD14 and MyD88 (Hiraoka et al. 1998; Tabeta et al. 2000). DNA microarray analysis demonstrated that expressed levels of TLR2, TLR4 and CD14 in human gingival fibroblasts were higher in patients with periodontitis compared to healthy controls (Wang et al. 2003). Additionally, in vitro stimulation of human gingival fibroblasts showed increased expression of TLR2, TLR4, CD14 and MD-2 (Tabeta et al. 2000). A recent study reported *P. gingivalis* infection induces TLR2 and TLR9 up regulation in chronic periodontitis and that the *P. gingivalis*-induced TLR2 expression in HGFs was partly dependent on TNF-α and may lead to sensitization of HGFs to bacterial components encountered in the periodontal micro-environment (Wara-Aswapati et al. 2012). These studies suggest that *P. gingivalis* LPS may play an important role in the up regulation of TLR2, TLR4, CD14 in periodontal disease. Studies examining expression of TLRs by epithelial cells in gingival biopsies have reported expression of TLR2, TLR6 and TLR9 (Kusumoto et al. 2004). Low levels of TLR4 have also been reported with an increased expression on treatment with IFN-γ (Uehara et al. 2002). An abundance of TLR2 positive cells were observed in connective tissue subjacent to pocket epithelium (Mori et al. 2003). Therefore, TLR2 expression by epithelial cells may be important, given its strategic position in the outermost layer where it is continually exposed to microorganisms. In addition, TLR3 and TLR9 (Kusumoto et al. 2004) have also been detected on epithelial cells thereby reflecting the ability of the epithelial cells to respond to both viral and nucleic acids.

Besides gingival fibroblasts and epithelial cells, little information is available on TLR signalling in other cells of the periodontium. On comparison of human gingival fibroblasts with periodontal ligament fibroblasts from the same donor, a low CD14 and high TLR2
expression in periodontal fibroblasts was reported suggestive of different functions of both cell types in response to plaque bacteria (Kusumoto et al. 2004). Hatakeyama et al, investigated the effects of PAMPs on fibroblasts of gingival and periodontal origin and reported that on stimulation with PAMPs, gingival fibroblasts produce pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, leading to tissue destruction and bone loss whereas periodontal ligament fibroblasts produce proteinases on TLR stimulation resulting in direct degradation of the periodontal tissues (Hatakeyama et al. 2003).

The effect of *P. gingivalis* LPS on a murine cementoblast cell line demonstrated that they express mRNA for TLR2, TLR4, CD14, MD-2 and participate in the inflammatory process by activation of the TLR2 pathway (Nociti et al. 2004; Nemoto et al. 2006). Interestingly, OPG was constitutively expressed by cementoblasts, and was not significantly altered by *P. gingivalis* LPS stimulation thereby suggesting a protective role in physiological as well as pathological conditions (Nemoto et al. 2006). Osteoclasts from mouse bone marrow cells were shown to express mRNA for TLR2, TLR4 and CD14 in response to stimulation with LPS (Itoh et al. 2003) and primary mouse osteoblasts have been reported to respond to LPS stimulation by RANKL expression and osteoclast differentiation through TLR2 and TLR4 (Kikuchi et al. 2001). A study on human osteoblastic cell line SaOS-2, has reported mRNA expression of TLR1, TLR4, TLR6, TLR9, MD-2, CD14 and MyD88 but failed to express mRNA for TLR2. In considering the endothelial cells, these cells express mRNA for TLR1, TLR3, TLR5 but little or no mRNA for TLR2 and therefore respond to only TLR4 ligands such as *E. coli* LPS (Faure et al. 2000). Although from the above studies it is clear that these receptors play a crucial role in the maintenance of periodontal health as well as progression of disease, there are huge gaps in our understanding of the underlying mechanisms in this process. In addition, the role of these molecules in the repair stages of disease has not been addressed.

The engagement of the receptors with their ligands results in activation of downstream signalling and production of cytokines which play an important role in the containment or propagation of inflammation. Chronic inflammation as seen in periodontal disease results in prolonged expression of inflammatory cytokines leading to a general delay in healing of the
periodontal pocket and ultimately stimulation of bone resorption by osteoclasts. These cytokines are multifunctional and have a variety of roles depending on the context of their expression. In addition to its direct effect on the surrounding tissues, these cytokines function in networks and influence the repair process indirectly through the modulation of other cytokines. At present, the strongest evidence for cytokines functioning in networks in periodontal pathogenesis exists for IL-1β, TNF-α, IL-6 and RANK/RANKL/OPG (Preshaw and Taylor 2011). These inflammatory mediators function synergistically to influence the wound healing process at various levels and their function is also dependent on the concurrent expression of other cytokines. Their role includes mediating osteoblast and fibroblast differentiation and matrix production to controlling bone remodelling through the modulation of osteoclast function (Preshaw and Taylor 2011). In addition, a variety of growth factors such as TGF-β are also heavily involved in the regulation of cell differentiation and function within the periodontium. Among the pro-inflammatory cytokines, IL-1β has been shown to increase osteoblast proliferation and differentiation (Lange et al. 2010), to increase bone mineralisation (Ding et al. 2009) and inhibit osteoblast production of bone matrix (Zhang et al. 1996). On the other hand, TNF-α has a broadly suppressive effect on osteoblast proliferation, differentiation and production of matrix components (Rosenquist et al. 1996; Abbas et al. 2003). IL-6 is a multifunctional cytokine that can activate target genes involved in proliferation, differentiation, survival and apoptosis, notable in a variety of cells (Kishimoto et al. 1995; Eulenfeld et al. 2012). Furthermore, IL-6 has been reported to be an important regulator in bone remodelling and an adequate amount of this cytokine is crucial for bone homoeostasis (Li et al. 2008). Its reported effects on cells involved in the repair process are however controversial. Some studies have reported that IL-6 has no effects on osteoblasts unless soluble IL-6 receptor is added (Littlewood et al. 1991; Bellido et al. 1996), which is contradictory to the fact that both IL-6 and IL-6R are expressed in bone marrow osteoblasts in vivo (Wognum et al. 1993; Hoyland et al. 1994; Langub et al. 1996). However, another study has reported the expression of IL-6 receptor increased during in vitro differentiation of osteoblast cells and by signalling through its receptor, IL-6 acted as a differentiation accelerator in pre-osteoblasts and an
apoptosis initiator in mature cells, thereby suggesting that IL-6 plays an important role in controlling the function and life span of osteoblasts (Li et al. 2008).

The regulation of these cytokines is equally complex. IL-1β is a key regulator of the inflammatory process and is known to modulate osteoblast production of TNF-α and IL-6 (Hughes et al. 2006). In addition, IL-1β, TNF-α and IL-6 can all upregulate osteoblast expression of RANKL leading to a potent stimulation of bone resorption through RANKL/RANK mediated osteoclast activation (Boyle et al. 2003; Lerner 2004). IL-1β and TNF-α also influence the negative feedback control of osteoclast activation by stimulating osteoblast production of OPG which competitively binds to RANKL preventing it from activating RANK (Brandstrom et al. 1998). These inflammatory cytokines are closely involved in the maintenance of a RANKL/OPG ratio which directly regulates bone resorption.

In addition to the inflammatory cytokines, growth factors such as TGF-β play an important role in the healing process. TGFβ1 is a crucial component of a complex regulatory cascade which is involved in the initial recruitment of osteoblast progenitor cells (Centrella et al. 1994; Hughes et al. 2006). The specific activity of TGFβ1 is contextual and may vary based on its ratio of expression with other growth factors but it has been shown to specifically drive the early phases of the osteoblast differentiation pathway, particularly in terms of driving the proliferation of osteoblast progenitor cells (Centrella et al. 1994). TGF-β can inhibit osteoclastogenesis, depending on the presence of osteoblasts, possibly by upregulating OPG production and thereby inhibiting RANKL/RANK signalling in osteoclasts and their precursors (Takai et al. 1998; Yan et al. 2001). Additionally, TGFβ1 enhances OPG expression, making it an inhibitor of osteoclast activation and therefore bone resorption (Boyle et al. 2003). TGFβ1 has also been reported to suppress the TNF-α potentiated production of IFN-γ by dendritic precursor cells (Koutoulaki et al. 2010) thereby suggesting that it may have anti-inflammatory properties. Although the role of cytokines and growth factors has been extensively investigated in pathological and healthy conditions, their role in the healing process is less understood. It is therefore clearly important to consider the role of virulence factors such as LPS on the wound
healing process due to the wide range of impact it has on the cell population of the periodontium

Against this background, the aim of this chapter is to investigate the effect of sub-toxic levels of LPS on expression of receptors (TLR2, TLR4 and CD14) and inflammatory cytokines in PDL cells at both mRNA and protein level using the same in vitro model described in the previous chapter. Additionally, due to the involvement of osteoblasts in the regulation of osteoclast activity and the obvious relevance of this to the process of regeneration in the healing process, the expression of RANKL and OPG will also be investigated. Due to the central role that TGF-β plays in the regulation of the osteoblast differentiation pathway, its role in the inhibition of osteoclast activation and its possible anti-inflammatory properties, expression of TGF-β will also be investigated. Investigating the expression of these molecules by PDL cells in response to stimulation with sub-toxic levels of LPS will help further our understanding of cell behaviour in the repair stages of periodontal disease.
4.2 Materials & Methods

The effect of sub-toxic levels of LPS on TLR expression and cytokine profile was investigated at mRNA and protein levels. Gene expression of TLR2, TLR4, CD14 receptors and cytokines IL-1β, IL-1RA, IL-6, TGF-β, RANKL, OPG, TNF-α, TNFR were examined by RT-PCR. The extracellular expression of TLR2, TLR4 and CD14 in response to sub-toxic levels of LPS was investigated by immunocytochemistry and cytokine profiles were evaluated by means of ELISA.

4.2.1 RT PCR

Periodontal ligament (PDL)-like cells were obtained as explants from alveolar bone chips as described in section 3.2.3. Cells from passage 2 were used for the experiments. PDL cells were reseeded at a concentration of 1x10⁴ cells/cm² in 6 well plates. Cells were allowed to adhere to the plate overnight and then stimulated with LPS at a concentration of 0ng/ml, 50ng/ml and 100ng/ml. Existing media was replaced with fresh media every 48 hrs. On days 1 and 2 post seeding, RT-PCR was carried out as described in section 3.2.9.1. Primer sequences used in the PCR reactions are listed in the table 4.2 below. PCR products were run on 2% agarose gels (Sigma, Aldrich) as described in section 3.2.2.4 and visualised on Gel Doc™ scanner (Bio-Rad, Hemel Hempstead, UK) using UV light and digital images recorded.
<table>
<thead>
<tr>
<th>Target gene (receptors)</th>
<th>Primer sequence</th>
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| TLR 2                  | F: 5' GGGAGCGGCGGCTGGAGGACTCCTAGGC-3'  
| R: 5'TCGCACCAGCTCGGAAGTACCATGGCCCA-3' |
| TLR 4                  | F: 5'AAGGAATGCCCCGCTTTTACCTTGCGCT-3'  
| R:5'GGGCTCTCGGTCCATAGCACAGAGCCCAAGG-3' |
| CD14                   | F: 5'CCCCAACCCTCTGGAAGCGCCAGGACC-3'  
| R: 5'GGGCGTCTCCCATCCCCCGTTACGCAGC-3' |

<table>
<thead>
<tr>
<th>Target gene (cytokines)</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| IL-1β                   | F:5'TTGTGGCTGGAGTGGAGTGGGTGGCCGTT-3'  
| R:5'TCCAGCTGCAGGGGTGCTCCGA-3' |
| IL-1RA                  | F:5'TTGGGCATCGCCGGAGGAGCTTGCGCCGC-3'  
| R:5'TGGCAGGGGTAGGGGTGGGCTAGAGA-3' |
| IL-6                    | F:5'CACGCGCTTCTTACTCTACAAGTGCCCG-3'  
| R:5'TCCTTAAGCCACTTCTGGAATCCG-3' |
| TGFβ                    | F:5'ACGTCGGGGCCAGCCTGGGACCACCACAT-3'  
| R:5'TGGGCGAGTGCTCCAAAGGCCGCAC-3' |
| TNFα                    | F:5'CTCACCCACACGGTTAGCCTGCAGAATTC-3'  
| R:5'TCTTGAAAGGTCTGAAGCTT-3' |
| TNFR                    | F:5'ACGGTGCGCGGTCTGGGACAGAGCAGGCA-3'  
| R:5'AGGGTCCCTGGGCTGCTGAGT-3' |
| RANKL                   | F:5'ATCGCGCCAGCCAGGAGCTACGGAAGTAC-3'  
| R:5'TCAGTTGTCTGCAATGTTTTGGAAGCCCA-3' |
| OPG                     | F:5'CCGCTCTCTGAGATGTGCTGGGCTGCT-3'  
| R:5'GCGCTCAGGCTTGGCTGGCACA-3' |
| β-Actin (House keeping gene) | F:5'CAGGTCCCGCGCCAGCCAGGTCCAGACGC-3'  
| R:5'CTGTCGAGTCCGTCAGCCACGCA-3' |

**Table 4.2:** A list of primer sequences used in the PCR reactions. Primers were designed using Primer Blast to ensure specificity for the intended amplification targets.
4.2.2 Immunolocalisation

Immunodetection of TLR2, TLR4 and CD14 was carried out using goat polyclonal antibodies against mouse TLR2, TLR4, and CD14 followed by exposure to FITC labelled antibodies and fluorescence microscopy as described below. Round glass coverslips (Thermo scientific, UK) were autoclaved and placed at the base of wells of 6-well plates. Cells obtained from passage 2 were reseeded in the 6 well plates at a density of 1x10^4 cells/cm^2 in culture conditions as described previously in section 3.2.3. After 24 hrs, the media was replaced with fresh media containing LPS at 0ng/ml, 50ng/ml and 100ng/ml. At specific time points (Day 1, 2) media was decanted out from the wells and the cells washed with PBS. The cells were then fixed in freshly prepared paraformaldehyde (2%) for 30 mins and then washed (x2) with PBS. In order to avoid non-specific staining, the cells were incubated in blocking buffer (1% BSA in PBS) at room temperature for 1 hr. Primary antibody (anti TLR2, TLR4 and CD14 goat polyclonal antibodies, Santa Cruz Biotechnology Inc, Santa Cruz, USA) diluted in 1% BSA in PBS was prepared at a dilution of 1:50. Blocking buffer was discarded and the cells were incubated in primary antibody at room temperature for 1 hr. Replacement of primary antibody with goat IgG was used for isotype control and negative control was obtained by the omission of primary antibody. The cells were then washed (x3) in PBS for 5 mins and then exposed to mouse anti-goat IgG FITC conjugated secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, USA) diluted in 1% BSA/PBS solution at a dilution of 1:1000 and incubated for 1 hour at room temperature in the dark. The cells were then washed (x3) in PBS for 5 mins each time. Using fine tweezers, the glass coverslips were then removed from the bottom of the wells and the edges carefully blotted to remove excess solution. A droplet of mounting medium for fluorescence microscopy viewing containing DAPI (4', 6-diamidino-2-phenylindole, VECTASHIELD, Vector Laboratories Inc, Burlingame, USA) was added onto the coverslip. The coverslip was mounted with the cells facing the microscope slide (Polysine slides, Thermos scientific, UK). The cells were then viewed under an Olympus AX70 fluorescent microscope and images were captured using a Nikon digital camera DXM 1200.
4.2.3 ELISA

ELISA was performed to analyse cytokine levels in PDL cells stimulated with *P.gingivalis* LPS at concentrations of 0ng, 50ng and 100ng/ml. 96 well plates (Microplate Immuno MaxiSorp, Fischer) were coated with 50μl/well capture antibody (1:500 dilution of anti-mouse IL-1β in coating buffer (0.1M NaHCO\(_3\), pH - 8.5) and incubated overnight at 4°C. The wells were then washed (x4) with wash buffer (0.05% v/v Tween-20 in TBS) and then blocked with blocking buffer (10% FBS in PBS) for 2 hrs at 37°C to minimise non-specific binding. Plates were once again washed (x4) using wash buffer.

Standards were prepared by diluting the supplied protein standards with a starting concentration of 5ng/ml. 50μl of double diluted standards in triplicates at concentrations in the range of 5ng/ml to 0.078ng/ml were added to the coated ELISA plate. Diluent buffer was used as control. 50μl of supernatant collected from each condition were added in triplicates to the coated plates and incubated at 4°C overnight. Plates were then washed with wash buffer (x4) and 50μl of 1/500 diluted biotinylated detection antibody (eBioscience, San Diego, CA) was added to each well and incubated at 37°C for 2 hrs. The plates were washed (x5) and 50μl of 1/500 diluted HRP Strep Avidin (eBioscience, San Diego, CA) was added to each well and incubated at 37°C for 1.5 hrs.

The plates were washed (x6) and 50μl of Super Aqua blue ELISA substrate (eBioscience, San Diego, CA) was added and the plate incubated for about 30 minutes. The substrate contains ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) which is oxidised by HRP resulting in a soluble blue green colored end product. The absorbance was measured using a microplate reader (BioTek instruments Ltd) at 405nm.
4.2.4 Slot Blot

Slot Blot was carried out as an alternative technique for cytokine detection. A nitrocellulose membrane pre-soaked in TBS for 5 mins was assembled onto the slot blot apparatus and vacuum applied to the apparatus. Unused wells were blocked by a plate sticker. Slots were washed (x3) with TBS and then 150μl of supernatant collected from each condition were loaded onto the wells of the slot blot apparatus taking care not to puncture the membrane or introduce bubbles. Vacuum was applied to the wells until all the samples were absorbed. The slots were then washed (x3) with TBS and the wells were allowed to dry. The vacuum was turned off, the apparatus unclamped and the membrane was removed carefully. Immunodetection was carried out as detailed in 3.2.9.2.4.
4.3 Results

4.3.1 RT PCR

mRNA expression of TLR2, TLR4, CD14, IL-1β, IL-1RA, IL-6, TGFβ, RANKL, OPG, TNF-α and TNFR were investigated by RT-PCR. Fig 4.1A shows the expression of TLR2 and CD14 was strong compared to TLR4. On densitometric analysis of the bands following standardisation against β-actin, the most notable difference (as demonstrated by a twofold difference) in mRNA expression was observed for TLR2 and CD14, 24 hrs post stimulation (Fig 4.1B). However this effect was not observed for TLR4.
Fig 4.1 PDL cells were stimulated with LPS (0.50ng/ml and 100ng/ml) and expression of CD14, TLR2 and TLR4 were investigated by RT-PCR. Total RNA was extracted and mRNA expression of CD14, TLR2 and TLR4 was analysed by RT-PCR (Fig A). The mRNA levels of CD14, TLR2 and TLR4 were normalised to β-actin levels in individual samples. Fig B shows the semi-quantification of mRNA expression by densitometry showing a general trend of increased expression of TLR2 and CD14 when compared to TLR4 (not possible to compare absolute levels as technique used is semi-quantitative).
Gene expression for RANKL, OPG, TNF-α, TNFR, IL-1β, IL-1RA, IL-6 and TGFβ were observed as shown below (Fig 4.2 A and B). On densitometric analysis following standardisation against β-actin, a strong expression of TNFR was observed at all time points which was not dependent on LPS stimulation. However, on day 4, an increased expression (>2 fold) was observed for TGF-β and IL-1β in response to stimulation with 100ng LPS.

On comparing pro-inflammatory vs anti-inflammatory cytokines, RANKL expression was stronger when compared to OPG and IL-1β stronger than IL-1RA. However, the reverse was true for IL-6 and TNF-α with stronger expression observed for TNFβ and TGFβ when compared to TNF-α and IL-6 (Fig 4.2 A and B). The techniques used in this study (RT-PCR) are semi-quantitative and therefore the absolute levels of mRNA expression between different conditions and time points were not possible.
Fig 4.2: PDL cells were stimulated with LPS (50ng/ml and 100ng/ml) and expression of RANKL, OPG, TNFα, TNFr, IL-1β, IL-6 and TGFβ were investigated on days 4, 8 and 12 by RT-PCR. Total RNA was extracted and mRNA expression of RANKL, OPG, TNFα, TNFr, IL-1β, IL-1ra, IL-6 and TGFβ was analysed by RT-PCR (A). The mRNA levels were normalised to β-actin levels in individual samples. Fig B shows the semi-quantification of mRNA expression by densitometric analysis. Increased expression of TNFr was observed at all time points (not dependent on LPS stimulation) and increased expression of TGFβ and IL1β was observed in response to stimulation with 100ng LPS. It was not possible to compare absolute levels as technique used is semi-quantitative.
4.3.2 Immunolocalisation

FITC labelled antibodies and fluorescent microscopy was used in this study to label and identify the cell surface proteins. Images resulting from the immunolocalisation of receptors in response to stimulation with various concentrations of LPS are shown in Figures 4.3 to 4.6. Nuclei are seen stained blue with DAPI and receptors stained green with FITC conjugated antibody.

TLR2 expression was observed at all time points and at all concentrations (Fig 4.3). An increase in expression was observed between stimulated and unstimulated cells. On day 2 an increase in TLR2 expression was observed (Fig 4.3). TLR4 expression was observed on cells stimulated with LPS at concentrations of 0ng, 50ng and 100ng on Day 1(Fig 4.4). A dose dependent difference was not observed.

CD14 receptors were expressed at all concentrations on day 1(Fig 4.5). An increase in expression was observed between stimulated and unstimulated cells as observed with TLR2 expression.

Negative controls:

Typical examples of isotype control and negative control are shown in Fig 4.6. Both of these negative controls were performed for all receptors investigated and at all time points. No positive staining for receptors were observed as shown in Fig 4.6.
**Fig 4.3:** Expression of TLR2 on immunostaining. PDL cells were stimulated with LPS (0, 50ng/ml and 100ng/ml) on days 1 and 2. The cells were fixed with 4% paraformaldehyde and treated with anti-TLR2 antibody and then visualised with FITC conjugated antibody (receptors stained green) and DAPI (nuclei stained blue).
**Fig 4.4:** Expression of TLR4 on immunostaining. PDL cells were stimulated with LPS (0, 50ng/ml and 100ng/ml) on days 1 and 2. The cells were fixed with 4% paraformaldehyde and treated with anti-TLR4 antibody and then visualised with FITC conjugated antibody (stained green) and DAPI (nuclei stained blue).
Fig 4.5: Expression of CD14 receptors on immunostaining. PDL cells were stimulated with LPS (0, 50ng/ml and 100ng/ml) on days 1 and 2. The cells were fixed with 4% paraformaldehyde and treated with anti-CD14 antibody and then visualised with FITC conjugated antibody (stained green) and DAPI (nuclei stained blue).
Fig 4.6: Typical examples of negative controls demonstrating no positive staining of receptors.
4.3.3 ELISA

The effect of sub-toxic levels of LPS on cytokine profile was investigated by means of ELISA. A standard curve was generated for IL-1β by plotting the absorbance against the corresponding concentrations of standards provided in the kit. With an increase in concentration, an increase in absorbance was observed thereby indicating immunoreactivity in the ELISA's. The line of best fit had a correlation coefficient of $R^2=0.6$. The concentration of cytokines in the samples was determined from the standard curve. However, detectable levels of protein (IL-1β and TNF-α) were not present in the culture supernatants.

4.3.4 Dot blot

On dot blot analysis, results were similar to ELISA in that, detectable levels of proteins (IL-1β and TNF-α) were not present in the supernatant samples.
This study has investigated the effects of sub-toxic levels of LPS on the expression of receptors TLR2, TLR4 and CD14 and cytokine profile by PDL cells at both mRNA and protein levels with a view to further our understanding of the response of PDL cells and the mechanistic pathways by which cytokines are released into the GCF. The results from this study report for the first time that on exposure to sub-toxic levels of LPS, an increased expression of TLR2 and CD14 at both protein and mRNA levels were observed when compared to TLR4. Further, the expression of IL1-β, IL-1RA, TNF-α, TNFR, IL-6, TGF-β, RANKL and OPG were observed at mRNA levels, but significantly these cytokines were not detected to be secreted by the PDL cells. These results suggest that firstly, although *P. gingivalis* LPS activates both TLR2 and TLR4 receptors, there is a preferential utilisation of TLR2 receptors (Fig 4.1A). The absence of cytokines in the supernatant may indicate that the resident PDL cells may not contribute to the inflammatory status of the periodontal tissues, leading to connective tissue destruction or towards the cytokine pool detected in the GCF.

As the binding of LPS to TLRs activates signal transduction leading to transcription of pro-inflammatory cytokines, it is important to identify TLR signalling pathways to further our understanding of how TLR stimulation determines the outcome of immune responses. It has been reported that TLR2 is mostly involved in the recognition of a variety of different bacterial components such as peptidoglycan and lipoproteins (Lien et al. 1999) whereas TLR4 has been shown to specifically recognize LPS of Gram-negative bacteria and acts in cooperation with several protein components such as lipopolysaccharide-binding protein and CD14 (Yoshimura et al. 2002). However, a large body of evidence suggests that *P. gingivalis* LPS stimulates TLR2 and not TLR4 (Pulendran et al. 2001; Yoshimura et al. 2002; Kikkert et al. 2007). In contrast, Gram-negative enterobacteria can stimulate both TLR2 as well as TLR4 (Mandell et al. 2004). In addition, Kikkert et al, demonstrated that Gram-negative periodontal
bacteria primarily interact with TLR2 (Kikkert et al. 2007). However, only *Aggregatibacter actinomycetemcomitans* and *Veillonella parvula* were capable of stimulating both TLR2 and TLR4. This study has demonstrated further that *P. gingivalis* LPS is capable of stimulating both TLR2 and TLR4 receptors. In healthy tissues, mRNA and protein expression of both TLR2 and TLR4 has been demonstrated but the expression of both receptors were markedly up-regulated with inflammation (Sugawara et al. 2006). In chronic periodontitis tissues, both TLR2 and TLR4 were detected at mRNA and protein levels, whereas in healthy tissues, there was only a weak expression of TLR2 and no expression of TLR4 (Ren et al. 2005). Our results demonstrate that in the presence of low levels of virulence factors, there is an increased expression of TLR2 receptors when compared to TLR4 which may suggest that as the bacterial load increases, an up-regulation of these receptors occurs. Therefore, the expression of TLR2 and TLR4 receptors is dependent on the severity of periodontal inflammation.

Although the overall influence of these receptors in health and disease is not clearly understood, it is evident that these receptors play an important role in mediating the LPS response. The cytokine expression response of PDL cells to exposure of sub-toxic levels of LPS was investigated at both mRNA and protein level. Interestingly, the mRNA expression of most cytokines were similar in that a decreased expression was observed over time whereas IL-1β, TGF-β and TNFr were consistently expressed over time. The techniques used in this study (RT-PCR) are semi-quantitative and therefore the absolute levels of mRNA expression between different conditions and time points was not possible. The results presented herein, provide us with a profile of the general trend observed. Further information could be obtained by using more sensitive techniques such as qPCR that will help us to quantify specifically the differences in the mRNA levels between cells cultured in various conditions and time points. Interestingly, detectable levels of cytokines were not present in the culture supernatants, despite its expression at mRNA levels. This may be attributed to the possibility that although these cytokines were expressed at gene level, protein translation may
not have taken place or these cytokines may not have been secreted extracellularly. Alternatively, these cytokines may be secreted at levels too low to be detected by techniques such as ELISA.

In considering the mRNA profile of the cytokines investigated, a gradual decrease in mRNA levels over time were observed from day 4 to 12 for all cytokines except TNFR and TGF-β. The mRNA expression of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were observed although the expression of IL-1β and IL-6 was stronger than that for TNF-α. Detectable levels of cytokines were not present in the supernatant on analysis by ELISA. As an alternate technique, Slot Blot was used to investigate the presence of cytokines. The results obtained by both techniques confirmed that PDL cells do not secrete high levels of cytokines in response to stimulation with sub-toxic levels of LPS. However, we are aware that these cells are capable of secreting cytokines as demonstrated by studies using orthodontic models investigating the effect of tensile stress on PDL cells where these cells respond to mechanical stress by the production of cytokines such as IL-1 and IL-6 (Meikle 2006). Recent studies using human PDL cells have reported that these cells produce IL-1β, IL-6 and IL-8 in response to stimulation with *P. gingivalis* LPS (Tang et al. 2011; Kato et al. 2014). However the concentration of LPS used was much higher than that used in this present study. Therefore, although studies have reported that PDL cells are capable of secreting cytokines in response to stimuli (mechanical as well as due to infection), the results from this study suggests that the PDL cells may not necessarily contribute to the pro-inflammatory cytokine profile in GCF collected from patients with periodontal disease.

Previous studies investigating effects of *P. gingivalis* LPS on other cell types in the periodontium have reported that *P. gingivalis* LPS stimulates host cells including macrophages and fibroblasts to produce cytokines (Takada et al. 1991). Gingival fibroblasts, the most abundant cells in periodontal tissue which is responsible for the synthesis and
degradation of connective tissues, were reported to secrete a variety of immunoregulatory cytokines and chemical mediators (Okada and Murakami 1998). On stimulation with microbial factors such as LPS and cytokines such as IL-1 and TNF-α, gingival fibroblasts were reported to induce IL-6 mRNA expression (Agarwal et al. 1995). Cultured gingival fibroblasts stimulated with LPS from *Porphyromonas* species secreted increased levels of IL-1, IL-6 and IL-8 (Takada et al. 1991; Tamura et al. 1992; Sakuta et al. 1998). On stimulation with *P. gingivalis* LPS, gingival fibroblasts produce cytokines such as IL-6 which in turn activated osteoclasts but this process could be inhibited by IL-10. (Wang et al. 1999a). In vitro, *P. gingivalis* LPS is shown to stimulate secretion of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-18 and TNF-α in monocytes (Zhou et al. 2005; Bostanci et al. 2007a; Bostanci et al. 2007b; Hamedi et al. 2009). Monocytes, a source of precursor cells for osteoclasts on stimulation with *P. gingivalis* LPS have been reported to up regulate RANKL and down regulate OPG expression at both mRNA and protein levels, thereby promoting osteoclastogenesis (Reddi et al. 2008). Additionally, *P. gingivalis* LPS promotes the secretion of pro-inflammatory cytokines in dendritic cells (Pulendran et al. 2001). PDL cells however, host a heterogeneous population, are involved in multiple regulatory roles such as formation and degradation of tissues, repair and remodelling, very high turnover rate, maintaining flexibility during function as detailed in Chapter 3. The progenitor population in the PDL contribute to the repair of both mineralised and ligamentous tissue, maintain insertion points of Sharpey’s fibres but most importantly, they are unique in the way high turnover of the extracellular matrix is carried out by endocytosis of the collagen fibres. The key findings in this study that that at low levels LPS appears to have more of a significant effect on cell behaviour resulting in altered matrix synthesis (as demonstrated in Chapter 3) as compared to its contribution to the pro-inflammatory cytokine profile further indicates that the altered behaviour results in formation of tissue less resistant infection. Further the high turnover rate may be further accelerated leading to further tissue destruction.
Recent research has highlighted the important role played by the PDL cells in the repair and remodelling process. Although the response of PDL cells to the continual presence of factors such as LPS may affect the repair and remodelling stages in periodontal disease, these responses are difficult to elucidate, as within the periodontal environment, in addition to LPS and other virulence factors, there exists cytokine networks that play key roles in the host’s response to bacterial challenge. The key finding that cytokines were not detected on exposure of the PDL cells to sub-toxic levels of *P. gingivalis* LPS, despite the presence of receptors, suggests that these resident cells may have a minimal contribution towards the cytokine pool detected in the GCF. Therefore, a more prominent role may be played by the cells recruited during the inflammatory-immune response. Due to the complexities involved in the wound healing process, in addition to the various factors that may influence this process, further investigations are necessary to gain a clearer understanding of the processes involved in the repair stages of the disease.
Chapter 5

Altered cytokine profile in GCF in patients with periodontal diseases: a cross-sectional study

5.1 Introduction

The response of the resident PDL cells in the presence of virulence factors such as LPS was investigated in the previous chapters to assess the contribution of these cells to the inflammatory response. The presence of microbial factors stimulates a local inflammatory response resulting in activation of the innate immune system. The innate response is primarily involved in the recognition of microbial components such as LPS, bacterial DNA, peptidoglycan (Mahanonda and Pichyangkul 2007) with the aid of pathogen recognition receptors such as TLRs expressed by resident cells, which subsequently lead to the release of pro-inflammatory cytokines. It is now recognised that the progression of the disease is determined by the nature of the host immune response to specific microorganisms within the biofilm (Graves 2008; Preshaw 2008). An unregulated inflammatory response leads to the activation of the adaptive immune response by antigen presenting cells to the T and B cells (Cutler and Jotwani 2004; Xu and Banchereau 2014), resulting in a Th1, Th2, Th17, Treg response and antibody production respectively. Within this scenario, cytokines produced by these subsets of cells will determine phenotypically distinguished immune responses with the Th1 and Th2 cells, respectively associated with cellular and humoral immunity (Murphy and Reiner 2002) and the more recently described Th17, Treg cells presenting antagonistic roles as effector and suppressive cells (Appay et al. 2008; Sallusto and Lanzavecchia 2009; Weaver and Hatton 2009). Besides T-cells, B-cells are also activated and transform into plasma cells which produce antibodies in response to bacterial antigens. Consequently, tissues affected by
periodontitis become populated with both subtypes, of which B cells predominate (Berthelot and Le Goff 2010).

Several early studies have demonstrated that the development of periodontitis involves a switch from a gingivitis lesion, mainly mediated by T cells to one involving large numbers of B cells and plasma cells (Ohlrich et al. 2009) and that this shift is mediated by a balance between the Th1 and Th2 subsets, with gingivitis being mediated by Th1 cells and periodontitis by Th2 cells (Ohlrich et al. 2009). This concept proposed that a strong innate immune response leads to the production of high levels of IL-12 by both PMN’s and macrophages which in turn stimulates a Th1 response, cell mediated immunity, protective antibody and a stable periodontal lesion. In contrast, a poor innate immune response with polyclonal B cell activation leads to a Th2 response, non-protective antibody formation and a progressive periodontal lesion (Kinane and Bartold 2007). The Th1 phenotype are characteristically cellular and pro-inflammatory in nature and secrete IL2, IFN-γ, in addition to suppression of B cells and plasma cells. In contrast, Th2 cells induce predominantly B cell humoral immune response, secrete IL-4, IL-5 and IL-10, while their secondary function is the suppression of T cell mediated response (Ohlrich et al. 2009). Thus, the immunoregulatory control was considered to be dependent on the balance between these two T cell subsets. However, certain additional cytokines, which did not fit into either the Th1/Th2 category, seem to play an important role in the pathogenesis of periodontitis, of which were cytokines belonging to the recently identified Th17 and Treg subsets. Recent studies have characterised the Th17 cell lineage as an IL-17 producing CD4 T-cell subset, which has been implicated in numerous auto-immune and inflammatory conditions (Dong 2008; Sallusto and Lanzavecchia 2009; Maddur et al. 2012). Th17 cells develop through cytokine signals distinct from and antagonised by products from the Th1 and Th2 lineages (Appay et al. 2008; Dong 2008; Sallusto and Lanzavecchia 2009). These cells are characterised by the production of pro-inflammatory cytokines such as IL-17, IL-21, IL-22 of which IL-17 is the most important effector cytokine. IL-17 promotes inflammation by inducing various pro-inflammatory cytokines, chemokines, recruiting
neutrophils, enhancing antibody production, and activating T cells (Iwakura et al. 2008). It has been suggested that the unregulated proliferation and dysfunction of the Th17 cells could lead to the amplification of local inflammation, thus intensifying the tissue damage. Tregs, in addition to these Th cells, are engaged in maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases by suppressing and regulating the effector functions of Th cells (de Rezende et al. 2010). Thereby, they play a significant role in the maintenance of immunological self-tolerance and the modulation of immune response (Mougiakakos et al. 2010). These cells predominantly mediate suppression by the release of suppressor cytokines such as TGFβ and IL-10 (Yuan et al. 2010). In light of the opposing functions between these subsets of T cells, it has been suggested that an imbalance between them may be involved in the occurrence and development of many chronic diseases.

Accumulated data have demonstrated quantitative or functional imbalance between Th1/Th2 and Th17/ Treg in chronic diseases such as osteoporosis (Pacifici 2012; Tyagi et al. 2012), rheumatoid arthritis (Andersson et al. 2008; Oh et al. 2010), bone marrow malignancies (Hideshima et al. 2007; Noonan et al. 2010). Subsequent research in periodontal disease conditions to investigate the link between Th1/Th2/Th17/Treg (Kinane and Bartold 2007; Kinane et al. 2011) have highlighted the important role played by these cells in maintaining homeostasis. As mentioned above, an imbalance between Th1/Th2 was originally thought to bring about progression of periodontal disease. However, recent research has highlighted the additional important roles of the Th17/Treg subsets. For example, studies have reported that pathogenic as well as commensal organisms present within the oral cavity, are capable of stimulating the production of mediators characteristic of T-cell responses and markers characteristic of the T cell subsets have been demonstrated within diseased periodontal tissues (Gemmell et al. 2002a; Garlet et al. 2003b; Kopitar et al. 2006; Cardoso et al. 2008; Gaffnen and Hajishengallis 2008; Cardoso et al. 2009). Specifically, *P. gingivalis* has been reported to induce CD4+ T cells to differentiate into Th17 cells, and stimulate CD4+ T cells to
produce IL-17 (Oda et al. 2003). Furthermore, it has been reported that human periodontal ligament cells on stimulation with pro-inflammatory cytokines such as IFN-γ, IL-17, produce significant increase of immunomodulatory cytokines and induced recruitment of leucocytes (Konermann et al. 2012a).

In attempting to establish a link between abnormal T cell response and bone loss, studies have identified that Th1 and Th2 cells inhibit osteoclastogenesis by producing inhibitory cytokines, IFN-γ and IL-4, respectively whereas, Th17 cells and related cytokines have the capacity to induce osteoclastogenesis (Takayanagi 2012). Additionally, IL-17 induces RANKL on osteoclastogenesis-supporting mesenchymal cells, such as osteoblasts and synovial fibroblasts, enhances local inflammation by increasing IL-6 and IL-1, which further promote RANKL expression and activity. Moreover, Th17 cells may also contribute directly to bone loss by producing RANKL. Therefore, the infiltration of Th17 cells into the inflammatory lesion may be the link between the abnormal T-cell response and bone damage (Okamoto and Takayanagi 2011). Recent clinical studies have reported the presence of Th17 cells within chronic periodontal lesions (Cardoso et al. 2009; Adibrad et al. 2012) and the cytokines associated with these cells have been detected in tissues obtained from periodontal lesions (Takahashi et al. 2005; Vernal et al. 2005; Ohyama et al. 2009). Similarly, immunohistological and gene expression study has shown increased Tregs and Foxp3 in periodontitis (Nakajima et al. 2005b). Intracellular IL-10 analysis showed a higher frequency of IL-10 producing CD4+ T cells in inflamed gingiva compared to normal tissue thereby suggesting potential role for Treg cells in the downregulation of inflammatory responses through the production of IL-10 (Kobayashi et al. 2011). Furthermore, Tregs can inhibit osteoclast formation via IL-10 and TFGβ signalling pathways (Luo et al. 2011). Therefore, in light of their anti-inflammatory and antiresorptive properties, the presence of Tregs within the periodontal tissue suggests that this subset has a potential protective role and that the balance between Treg and Th17 is particularly essential in maintaining homeostasis. However, very few clinical studies have investigated this relationship.
The understanding that GCF contains a vast array of biochemical factors with the potential to provide information crucial in the assessment of the status of the underlying tissues has led to its popular use in the evaluation of cytokines. Analysis of cytokine profiles in periodontal tissues affected by periodontal disease has been done previously by various techniques such as *in situ* hybridization and immunohistochemistry (Lappin et al. 2001), ELISA (Gorska et al. 2003; Havemose-Poulsen et al. 2005; Duarte et al. 2010), RT-PCR (Garlet et al. 2003a; Suarez et al. 2004) and more recently with cytomeric bead array (CBA) (de Queiroz et al. 2008; Andrukhov et al. 2011). Among the various techniques, conventional ELISA is the most widely used technique for cytokine analysis but is limited by its ability to measure only a single cytokine in each sample. Recent developments in serum cytokine quantification technology include multiplex arrays such as CBA, which has the additional advantage of analysing multiple cytokines simultaneously using small sample volume and thereby allow for comparisons between cytokines. However, limited studies have used CBA to analyse GCF in comparing cytokine profiles in health and periodontal diseases.

Against this background, the aim of this study was to further evaluate the profile of cytokines associated with the Th1/Th2/Th17/Treg cells using bead array technology. GCF levels of cytokines were compared using a CBA kit, in periodontal health and disease conditions (gingivitis, chronic periodontitis and aggressive periodontitis) in a cross-sectional study. Further, the potential of utilising abnormal cytokine ratios in assessing health of periodontal sites and identify individuals susceptible to rapid periodontal disease destruction was investigated.
5.2 Materials and Methods

R & D approval was obtained from the Joint Trust/ University Peer & Risk Review Committee. Ethical approval was provided by South East Wales Local Research Ethics Committee. The study was conducted as per Good Clinical Practice regulations and informed consent was obtained from each patient (Data provided in enclosed CD).

5.2.1 Recruitment of patients

A total of 40 patients representing 10 patients with chronic periodontitis, 10 patients with generalised aggressive periodontitis, 10 patients with gingivitis and 10 healthy subjects were recruited for this study from patients referred to the Restorative Clinic at Cardiff University Dental School. None of the patients recruited, had a history of any systemic disease that may impair the immune response or had received any antibiotic or periodontal treatment in the previous 6 months. Exclusion criteria included pregnancy, any medical conditions or medications that may have an effect on the periodontal health, intake of any antibiotic or anti-inflammatory drugs in the previous six months or a history of periodontal therapy in the previous six months. Patients were recruited between October 2008 and December 2010. The recruitment of selected patients were based on the clinical and radiographic criteria proposed at the 1999 International World workshop for classification of periodontal disease conditions (Armitage 1999). The Generalised Aggressive group consisted of 3 male and 7 females between the age group 28-40 years. These patients demonstrated a generalised pattern of severe destruction in the absence of local factors with probing depths ranging from 6-10mm in the selected sites. The Chronic Periodontitis group consisted of 4 females and 6 males between the age group 33-52 yrs with generalised moderate to severe bone loss associated
with the presence of local factors and probing depths ranging from 6-10mm. The gingivitis group consisted of 4 male and 6 female between the age group of 28 to 52 yrs with varying degrees of gingival inflammation but no loss of attachment. The healthy group consisted of 5 males and 5 females between the age group 28-42 yrs with no clinical signs of periodontal disease. Two deep sites were selected from each patient for GCF collection from the aggressive periodontitis and chronic periodontitis group. In the gingivitis group, two sites from each patient were selected that demonstrated signs of clinical inflammation and in the healthy group two sites were selected that showed no signs of inflammation.

5.2.2 Sample collection

Following isolation of the selected teeth with cotton rolls and high volume suction to prevent contamination with saliva, supragingival plaque was gently removed, the tooth air dried and GCF was collected using small diameter 2μl capillary tubes (Drummond Microcaps, Drummond Scientific Co, Pennsylvania, USA) placed at the gingival margin for a period of 10 minutes. Fluid collected into the tube by capillary action. When the tube became full or blocked due to plaque debris it was refreshed with a new tube. GCF was collected prior to clinical measurements to ensure collection of GCF reflecting disease activity rather than the exudate released as a response to inflammation caused by probing. Samples visibly contaminated with excessive blood were discarded. GCF volume was determined using the formula:

\[
\text{Volume of GCF} = \frac{\text{Linear distance the fluid collected in the tube}}{32} \times 2 \, \mu\text{l} \, (\text{total volume of tube})
\]

The samples were immediately placed in 1.5ml eppendorf tubes and stored at −80°C, under HTA regulations, until further analysis.
5.2.3 Measurement of clinical parameters

Clinical parameters were recorded using the Florida Probe (Florida Probe with PASHA probe-pressure controlled, automated, standardised handpiece from Florida Probe Corporation, Gainesville, FL, USA). Probing depths and recession were recorded at six points per tooth. Bleeding on probing and suppuration were recorded as present or absent for each site after probing. The probe was calibrated for each individual patient prior to recording indices. All measurements and sample collection were carried out by a single operator.

5.2.4 Multiple cytokine analysis of GCF fluid

Analysis of GCF samples for the detection of cytokine levels of IL-2, IL-4, IL-6, IL-10, TNFα, IFN-γ and IL-17 was carried out using BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA.). This kit contains seven bead populations with distinct fluorescence intensities that have been coated with capture antibodies specific for IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17 proteins.

GCF samples were prepared by adding 15 µl of diluents to each sample and allowed to stand for 10 minutes. The mix was then centrifuged at 1000g for 5 minutes. The resulting supernatant was collected in a sterile eppendorph and assay diluent added to make up the total volume to 35 µl. Each capture bead suspension was vortexed vigorously to obtain a uniform mix as the antibody-conjugated beads tend to settle out of suspension over time. A master mix was prepared by mixing 193 µl of each capture bead into a bijou and vortexing it further to make up the final mix. This mix was prepared immediately before using them in the assay. The assay was carried out by adding 35 µl of bead master mix and 35 µl of PE detection reagent to the prepared samples or standards and mixed by pipetting gently. The eppendorphs were incubated at 4°C overnight in dark, followed by equilibration to room temperature for 1 hour. 700 µl of wash buffer was added to each eppendorph and then
centrifuged at 200g for 5 minutes. The supernatant was collected carefully without disturbing the pellet and discarded. 200 µl of wash buffer was added to each tube and vortexed to resuspend the beads. Sample data was acquired using a BD FACSCalibur flow cytometer (BD Biosciences). FCAP Array™ software was used to generate results in a tabular and graphical form.

5.2.5 Statistical analysis

Total cytokine levels (pg/ml) were analysed and reported for each cytokine. Normality tests were carried out to assess distribution of data. As the data was non-uniformly distributed, non-parametric tests were used for analysis. Concentration of cytokines was expressed in pg/ml and presented as means and standard deviation. The statistical significance of the difference in cytokine levels between the controls, aggressive periodontitis, chronic periodontitis and gingivitis groups was determined using a Kruskal-Wallis Test. In each case the level of significance was set at p<0.05. Multiple comparisons were adjusted using Dunn’s Multiple Comparison test. The Pearson correlation (R) was applied to ascertain association between cytokine ratios within the four studied groups. In addition, correlation between cytokine levels and disease severity was also evaluated. SPSS Statistics 20.0 software was used to analyse the data. Statistical advice was provided by Professor R. Newcombe and Professor Syed Herawi (Cardiff University).
5.3 Results

5.3.1 Cytokine levels in health and disease

Levels of individual cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL17) were investigated in GCF samples from the four different groups. The observed trend as shown in Fig 5.1 and 5.2, was for higher cytokine levels in gingivitis samples compared to control, aggressive and chronic periodontitis; higher cytokine levels in aggressive and chronic periodontitis samples compared to control and similar cytokine levels in chronic and aggressive periodontitis groups. The levels of all cytokines examined were significantly higher in inflammatory gingivitis than in healthy sites (p<0.01) and significant for IL-2, IL-4, IL-17 and IFN-γ on comparing aggressive vs gingivitis groups (p<0.05). Additionally, IL-10 levels were higher in aggressive periodontitis and gingivitis compared to chronic periodontitis.
Fig 5.1: Cytokine levels (IL-2, IL-4, IL-6, IL-10) in GCF samples from patients with Aggressive Periodontitis (AP), Chronic Periodontitis (CP), Gingivitis (G) and healthy controls (C) presented as mean and SD (n=20 in each group). All groups investigated demonstrated variations in cytokine levels but significant differences were observed only on comparing (G) with (C) (p<0.01) for all cytokines (IL-2, IL-4, IL-6 and IL-10) and between AP and G for IL-2 and IL-4 (p<0.05). Large SD's in the AP and G groups may suggest variations in disease activity (*p<0.05, ** p<0.01 and ***p<0.001).
Fig 3.2: CBA analysis of cytokines (IFNγ, TNFα and IL17) in GCF samples from patients with Aggressive Periodontitis (AP), Chronic Periodontitis (CP), Gingivitis (G) and healthy controls (C). n=20 in each group. All groups investigated demonstrated variations in cytokine levels but significant differences were observed only on comparing (G) with (C) (p<0.01) for all cytokines (IL-2, IL-4, IL-6 and IL-10) and between AP and G for IL-2 and IL-4 (p<0.05). Large SD’s in the AP and G groups may suggest variations in disease activity (*p<0.05, ** p<0.01 and ***p<0.001).
5.3.2 Cytokine correlations in health and disease

In order to evaluate whether the increase in cytokine levels were correlated to one another, a correlation analysis was carried out. On plotting cytokines one against another, a linear relationship was observed between IL-2/IL-4, IL-2/IFN-γ, IL-2/IL-17, IL-4/IFN-γ, IL-4/IL-17 and IFN-γ/IL-17 in healthy controls, chronic and aggressive periodontitis as shown in Fig 5.3. Whereas in aggressive periodontitis group, a linear relationship was observed only between IL-2 and IL4 (Fig 5.3, Table 5.1).

Statistical analysis revealed strong correlations between IL2/IFNγ in chronic periodontitis group (R=0.979, p<0.01), gingivitis group (R=0.933, p<0.01) and control (R=0.994, p<0.01) suggesting a strong Th1 response, as IL2 is produced by all T cells and IFNγ by Th1 cells. However, a low correlation was observed for the aggressive periodontitis group (R=0.296). As shown in Table 5.1 and Fig 5.3, positive correlations were observed between IL2/IL4 for gingivitis (R=0.997, p<0.01), aggressive periodontitis (R=0.967, p<0.01), chronic periodontitis (R=0.993, p<0.01) and control (R=0.999, p<0.01). These results indicate a strong Th2 response as IL4 is produced by Th2 cells. Strong correlations were also observed for IL2/IL17 for gingivitis (R= 0.982, p<0.01), chronic periodontitis (R=0.969, p<0.01), control (R=0.988, p<0.01) and moderate correlation for aggressive periodontitis (R=0.623, p<0.05). This suggests a strong Th17 response for all groups except aggressive periodontitis. Similar responses were noted for IL4/IL17. Other cytokine combinations showed no correlation as shown in Fig 5.4 to 5.6.
Fig 5.3: On comparing cytokines representing Th1/Th2/Th17 subsets, a linear relation is observed between (IL2/IL4, IL2/IFNγ, IL2/IL17, IL4/IFNγ, IL4/IL17, IL17/IFNγ) suggesting a strong Th1/Th2/Th17 response in all groups except aggressive periodontitis. Clustering of samples observed for AP suggesting very low levels of cytokines whereas gingivitis samples demonstrate a linear spread indicating higher levels of cytokines.

- Aggressive Periodontitis, – Chronic Periodontitis, X – gingivitis and O-healthy control.
Fig 5.4: On comparing cytokines representing Th1/Th2/Treg subsets a non-linear relation between (IL6/IL2, IL10/IL2, TNFα/IL2, IL6/IL4, IL10/IL4, TNFα/IL4) was observed in all groups. Clustering of samples was observed indicating low levels of cytokines in both AP and CP.

- Aggressive Periodontitis, ▲ – Chronic Periodontitis, ▼ gingivitis and O-healthy control.
Fig 5.5: On comparing cytokines representing Th1/Th2/Th17 subsets, a non-linear relation between (IL17/IL6, IL17/IL10, IL10/IL6, IL10/TNFα, TNFα/IL6 and IL6/IFNγ) was observed in all groups. Clustering of samples was observed indicating low levels of cytokines in both AP and CP.

- Aggressive Periodontitis, △—Chronic Periodontitis, ×—gingivitis and O—healthy control.
Fig 5.6: On comparing cytokines representing Th1/Th2/Th17 subsets, a non-linear relation between (IL10/IFNγ, TNFα/IFNγ and IL17/TNFα) was observed in all groups. Clustering of samples was observed indicating low levels of cytokines in both AP and CP.

- Aggressive Periodontitis, – Chronic Periodontitis, X – gingivitis and O-healthy control.
5.3.3 Cytokine ratios in health and disease

For ratios of IFNγ /IL-2, IL-17/IL-4, IFNγ /IL-4, IFNγ /IL-17 a variable spread was observed for samples from aggressive periodontitis compared with IL-4/IL-2, suggesting variation in the production of these cytokines within the aggressive periodontitis group. On the other hand, a narrower spread of ratio values was observed for samples from “healthy” control, chronic periodontitis and gingivitis sites (Fig 5.7)
Fig 5.7a: Analysis of cytokine ratios: For ratios of IFNγ/IL-2, larger spread of ratio values was observed compared to IL-4/IL-2 for samples from Aggressive periodontitis. A narrower spread of ratio values was observed for samples from “healthy” control, chronic periodontitis and gingivitis sites indicating variable responses within the Aggressive periodontitis groups.
Fig 5.7b: Analysis of cytokine ratios: For ratios of IFNγ/IL-2, larger spread of ratio values was observed compared to IL-4/IL-2 for samples from Aggressive periodontitis. A narrower spread of ratio values was observed for samples from “healthy” control, chronic periodontitis and gingivitis sites indicating variable responses within the Aggressive periodontitis groups.
<table>
<thead>
<tr>
<th>Cytokine Ratios</th>
<th>Control</th>
<th>Aggressive</th>
<th>Chronic</th>
<th>Gingivitis</th>
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<td>Pearson r</td>
<td>Pearson r</td>
<td>Pearson r</td>
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<td>0.982**</td>
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<tr>
<td>IL6/IL-10</td>
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<td>0.877**</td>
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<td>0.382</td>
<td>0.939**</td>
<td>0.915**</td>
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</table>

**Correlation significant at the .01 level, correlation significant at the .05 level

*Table 5.1:* Evaluation of cytokine ratios in GCF samples of patients with Aggressive periodontitis (n=20), Chronic periodontitis (n=20), Gingivitis (n=20) and in healthy individuals.
5.3.4 Cytokine correlation with attachment loss

For all cytokines analyzed, there was no correlation between cytokine levels and attachment loss. Data for IFN-γ and IL17 presented (Fig 5.8).
Fig 5.8: Plots representing loss of attachment and cytokine levels (IFNγ and IL-17) show no relationship in health or disease thereby indicating that cytokine levels do not relate to pocket depths rather relate to disease activity.

- Aggressive Periodontitis, □ – Chronic Periodontitis, X – gingivitis and ◊ – healthy control
5.4 Discussion

The progression of periodontal disease occurs as a consequence of an altered immune response brought about by a dysregulation of molecules released by specific cell populations, many of which are involved in bone regulation and maintenance, an imbalance of which leads to altered remodelling and repair. Therefore, this present study examined the expression of cytokines that defined the Th1/Th2/Th17/Treg response types (IL-2, IL-4, IL-6, IL-10, TNFα, IFN-γ and IL-17) and supports preceding hypothesis that, periodontal disease is brought about by an imbalance in the Th1/Th2/Th17/Treg response. Although these cytokines have been categorised as belonging to the Th1/Th2/Th17/Treg cells, it is imperative to emphasise that sources other than Th1/Th2/Th17 cells may contribute to the presence of these cytokines in the GCF. This is the first study evaluating cytokine ratios in GCF using cytokine bead array (CBA), which has distinct advantages to other techniques in that, it allows for simultaneous measurement of multiple cytokines from a single sample thereby minimising methodological errors. Our results showed that levels of IL-2, IL-4, IL-6, TNFα, IFN-γ and IL-17 in gingivitis were significantly higher, when compared to aggressive periodontitis and chronic periodontitis which may be suggestive of an important role played by the T-cells in inflammatory conditions as in gingivitis. Furthermore, chronic periodontitis and gingivitis indicated a strong and balanced Th1/Th2/Th17 response which was matched by minor responses seen in the “healthy” control group as demonstrated by the linear response between cytokines IL2/IL4, IL2/IFNγ, IL2/IL17, IL4/IFNγ, IL4/IL17 and IL17/IFNγ. Whereas, in aggressive periodontitis group, a consistent Th2 response dominated alongside a mixed Th1/Th17 response as shown by a linear response only to IL2/IL4. However, clustering of samples were observed in the aggressive periodontitis groups, on comparing cytokines belonging to Th1/Th2/Th17/Treg against each other, reflecting low levels of cytokines whereas a more linear relation was observed in the gingivitis group. Additionally, large standard deviations were also observed
within the aggressive periodontitis and gingivitis groups reflecting possible wide variations in T cell responses. The immunosuppressive cytokine, IL-10 was higher in aggressive periodontitis and gingivitis compared to chronic periodontitis. Collectively, these results suggest that measuring cytokine ratios may provide important information for identifying patients susceptible to rapid periodontal destruction. Correlation between cytokines and attachment levels were also investigated. No correlation was observed, thereby confirming that cytokine levels relate to disease activity and not severity. Indeed, an absence of correlation reflects on the unique process of disease progression in that, there are periods of exacerbation and quiescence. A longitudinal study design would provide more information linking cytokine levels with disease activity.

In health, the presence of low levels of all cytokines investigated, may indicate that they are essential and contribute towards the maintenance of homeostasis within the periodontal environment. Additionally, the detection and identification of these cytokines gives us an indication of the pattern of cytokine profile in health.

**Th1 cytokines** (IFN-γ, TNF-α) were significantly higher in the gingivitis group when compared to healthy controls. Aggressive and chronic groups expressed similar levels although higher than healthy controls. IFN-γ and TNF-α, are primarily involved in the activation of T cytotoxic cells and macrophages consequently stimulating cellular immunity and inflammation. On the other hand, IL-2 is necessary for the growth, proliferation, and differentiation of T cells to become 'effector' T cells. Additionally, it augments cytokine production, cytolytic activity, enhances antibody secretion and induces apoptosis of activated T-cells (Gaffen and Liu 2004). Correlation regression analysis identified strong correlations between IL2/IFN-γ in chronic periodontitis group (R=0.979), gingivitis group (R=0.933) and control (R=0.994) suggesting a strong Th1 response, as IL2 is produced by all T cells and IFN-γ by Th1 cells. These results indicate that all patients produced Th1 cells in proportion to the overall T cell response.
However, a low correlation was observed for the aggressive periodontitis group (R=0.240) which was suggestive of a variable response within this group (Table 5.1).

**Th2 cytokines** (IL4, IL-6) were higher in the gingivitis group when compared to the other groups. However, IL-6 levels were higher in the chronic groups compared to aggressive. On examining cytokine ratios, a stronger correlation was observed for IL6/IL2 (R=0.613), IL-6/IL-4(R=0.635), IL-6/TNF-α(R=0.833), IL6/IFN-γ(R=0.636) and IL6/IL-17(R=0.647) in chronic periodontitis samples compared to other groups. IL-6 has a direct growth stimulatory effect on cells and regulates cell proliferation, differentiation and cell survival through cross talk with growth factor and several signalling pathways (Ara and Declerck 2010; Poncet et al. 2011).

Additionally, IL-6 can promote MMP-2, MMP-7 and MMP-9 activity which play a role in extracellular matrix degradation, promote angiogenesis, T-cell differentiation and activation, and more recently IL-6 has been identified as a major regulator of the balance between Treg and Th17 cells (Taniguchi and Karin 2014). Specifically, IL-6 along with TGFβ induces the generation of Th17 cells, while it inhibits differentiation of Treg cells thereby promoting a pro-inflammatory milieu. Therefore strong correlations between IL-6/TNFα(R=0.833), IL6/IFN-γ(R=0.636), IL6/IL-17(R=0.647), IL6/IL2 (R=0.613) and IL-6/IL-4(R=0.635) in chronic periodontitis compared to aggressive periodontitis, gingivitis and healthy control is suggestive of a balanced Th2 response in the chronic periodontitis group.

Higher levels of IL-4 and IL-10 were observed in aggressive periodontitis compared to chronic periodontitis and healthy controls. IL-10 (Treg cytokine) along with IL-4 (Th2 cytokine) have been considered as potent anti-inflammatory agents (Dinarello 2000) and it has been suggested that their lack may cause increased production of inflammatory mediators such as TNFα and IL1β in periodontal tissue (Shapira et al. 1992). Furthermore, IL-10 has been regarded as a key immunoregulatory cytokine capable of controlling inflammation in various pathophysiological settings by inhibiting activity of Th1 cells, Th2 cells, NK cells, monocytes, macrophages and dendritic cells. Within the control group, the levels of IL-4 and IL-10 were
lower when compared to chronic periodontitis and aggressive periodontitis which was contradictory to previous clinical studies which have reported lower levels of IL-4 and IL-10 in GCF samples from chronic periodontitis compared to healthy controls (Bozkurt et al. 2006; Cetinkaya et al. 2012). However, it has also been reported that an increased number of IL-10 producing CD4+ T cells were present in inflamed gingival tissue compared to normal tissue in experimental periodontitis model, suggesting a potential central role for Treg cells in down-regulation of inflammatory response by IL-10 production in periodontal inflammation and alveolar bone loss (Kobayashi et al. 2011). Therefore, the increased expression of IL-10 in the aggressive periodontitis group may indicate a potential role in inhibiting cell mediated immunity and B-cell response. Although previous studies have documented the anti-inflammatory properties of IL-10, recent research has reported IL-10 to possess pro-inflammatory effects by promoting proliferation, differentiation and antibody production by B cells, with subsequent detrimental immune-complex deposition thereby suggesting a dual role (O’Garra et al. 2008; Rutz and Ouyang 2011; Hofmann et al. 2012; Kubo and Motomura 2012). Clinical studies have disclosed perplexing pro-inflammatory functions of IL-10, contradictory to data obtained in defined rodent models of disease which propose IL-10 as potent and reliable anti-inflammatory cytokine (Muhl 2013). Recent reports have suggested that on priming by type 1 interferon, IL-10 family cytokines (IL-10, IL-22) promote Th-1 like inflammation, promote apoptosis and control of tumor growth (Kubo and Motomura 2012; Bachmann et al. 2013), thereby suggesting plasticity of IL10 production by various cell types or the presence of certain cytokine environment. Similarly, IL4 causes a Th2 response with B-cell activation and production of antibodies which help in containing the infection. However, nonprotective antibodies may also be produced leading to persistence of infection and high levels of IL-1 resulting in tissue destruction (Gemmell et al. 2002b). Therefore, it is less clear whether the dominant role of the Th2/Tregs are host protective or destructive.

**Th17 cytokine** (IL-17) levels were significantly higher in the gingivitis and aggressive periodontitis groups compared to healthy controls (Fig 5.1). Early studies reported on the
crucial function of the Th1/Th2 paradigm in the host immune responses in chronic periodontitis (Gemmell and Seymour 2004) with Th1 cells being associated with a stable lesion and Th2 cells associated with disease progression. However, with the discovery of Th17 and Treg subsets, with equally important roles in host immune inflammatory response, it is clear that the molecular responses underlying periodontal tissue destruction involves additional factors. In recent years research has identified Th17 and Tregs to play important roles in the host immune-inflammatory response. Studies have demonstrated that Th17 cells are involved in periodontal inflammation and tissue destruction in periodontitis (Zhao et al. 2011; Adibrad et al. 2012) whereas Tregs play a protective role in the development of periodontitis (Ernst et al. 2007; Garlet et al. 2010). An imbalance in the Th17/Treg ratios has been reported recently in several chronic and autoimmune diseases such as Rheumatoid arthritis (Wang et al. 2012), pemphigus (Xu et al. 2013), sarcoidosis (Huang et al. 2013), systemic lupus erythematosis (Mengya et al. 2013), in addition to cancer(Zhao et al. 2013) and as a natural consequence of aging (Schmitt et al. 2013). For example, an enhanced Th17 cell response and a weakened Treg response has been associated with rheumatoid arthritis(Wang et al. 2012). In contrast, a weakened Th17 cell response and an enhanced Treg response has been demonstrated in non-small cell lung cancer (Zhao et al. 2013). With regards to periodontal disease which is also a chronic condition, data obtained from murine experimental periodontitis model, have reported that levels of both Th17 and Treg related cytokines were elevated and that inhibiting IL-17 alleviated periodontal destruction, whereas inhibiting Treg cell function exacerbated periodontal lesions (Garlet et al. 2010; Eskin et al. 2012). Clinical studies have reported that Th17 cells were found infiltrated in periodontal tissues in chronic periodontitis, and IL-17 level in GCF was significantly increased (Cardoso et al. 2009). Gingival concentrations of IL-23, IL-17, 1L-6, IL1β and TNFα were significantly higher at sites with severe attachment loss compared to healthy sites (Lester et al. 2007). Additionally, it has been reported that Th17 cell related cytokines correlated positively with periodontal destruction whereas Treg cell related cytokines correlated negatively with periodontal destruction in patients with periodontitis (Dutzan et al. 2012) indicating that Th17/Treg imbalance may be associated with the
pathogenesis of periodontitis which lends support to the results presented from this present study showing a significant correlation between IL-17 expression and severe disease condition. Since Th17 responses are indicated to drive periodontal disease progression, abnormal Th1/Th2/Th17 response in the aggressive periodontitis group strongly suggests a contribution to the development of an exacerbated manifestation of the disease. It may be that, patients with aggressive periodontitis may be predisposed to a potential cellular hyperactivity which may contribute to the exacerbated tissue destruction seen in aggressive periodontitis.

In conclusion, this pilot study has further contributed to the characterization of the cytokine profile presented in health and disease with distinct variations observed within the four groups investigated. It is possible that this variability or lower frequency of detection may be useful in determining the presence or severity of the disease. Additionally, this study demonstrated for the first time, that an abnormal immune response exists in the severe disease group. In the absence of signs and symptoms as in chronic conditions like periodontitis, early stages of the disease are usually undetected until clear evidence of bone destruction is present. Appropriate early intervention may prevent progression of the disease if diagnosis were possible in the early stages of the disease. Within this scenario, it is possible that susceptible patients may be identified on the basis of differential immune response. To this end, cytokine profiling may be of prognostic value in assisting identification of high risk patients as well as monitoring response to treatment. However, further longitudinal studies to investigate the intricate balance between the cytokines belonging to the various T subsets would be necessary to validate its use as a prognostic tool in the management of patients with periodontal disease.
Chapter 6

DISCUSSION

The inability of current clinical methods to accurately diagnose disease activity, identify high risk patients and predict disease progression have been major predicaments in the management of patients presenting with periodontal diseases, as detailed in Chapter 1. Therefore, through a series of in-vitro and in-vivo studies, this study set out to further our understanding of the molecular mechanisms involved in the pathogenesis of periodontal disease, that may help implement the use of markers detected in GCF to aid clinicians in the management of patients with periodontal diseases. Previous studies have identified CS to be a matrix metabolite component released as a consequence of inflammatory degradation during periodontitis. In this study, the diagnostic potential of CS as a marker of disease activity was evaluated longitudinally in patients with chronic periodontitis and results identified CS as a product released continuously at the point of sampling, increased CS levels were associated with sites demonstrating progressive attachment loss, identified periods of activity and inactivity, with few sites demonstrating disease activity over a 21 month period. Furthermore, in-vitro studies investigated the cellular synthesis of this proteoglycan in a pathological condition by examining the biological effects of P. gingivalis LPS on PDL cells. In the presence of P. gingivalis LPS, an alteration in cell behaviour was observed with an increase in cell proliferation and a decrease in matrix formation, further suggesting that the degradation products detected in GCF (CS), were as a consequence of tissue destruction and not as a result of repair or remodelling. Collectively, these results highlight the potential of CS present in GCF as a marker of disease activity.
Cytokines detected in GCF are released as a consequence of the inflammatory-immune process activated in response to a bacterial challenge, an imbalance of which lead to periodontal tissue destruction. A cross-sectional study investigating cytokine profiles in GCF of patients with periodontal disease to investigate the prognostic value of cytokine profiling in identifying the high risk patient, demonstrated a variable response in the severe type, which was suggestive of an imbalance in Th1/Th2/Th17/Treg responses, thereby affirming the predominant role of an altered host response in disease progression. Cytokine ratios were examined between different groups and the results demonstrated an inconsistent Th1/Th17 response in the aggressive periodontitis group compared to a consistent Th1/Th2/Th17 in the other groups further suggesting that cytokine profiling may be of prognostic value in the identification of high risk patients. Further, non-immune sources were also considered as potential contributors to the variation in response. Consequently, in-vitro studies demonstrated that although TLR receptors were present on PDL cells, no cytokines were released on exposure to P. gingivalis LPS further suggesting that the cytokines detected in GCF were released by the recruited immune cells, an imbalance of which causes periodontal tissue destruction and alveolar bone loss.

CS has previously been detected in GCF and proposed as a marker of active tissue destruction (Last et al. 1985; Last et al. 1988; Samuels et al. 1993; Waddington et al. 1994; Okazaki et al. 1995; Smith et al. 1995; Waddington et al. 1996; Waddington et al. 1998; Baldwin et al. 1999). Based on the above pilot data, this study investigated further, the validity of CS as an ideal biomarker of periodontal destruction. This study also addressed the potential of CS as a marker of disease activity at the time of sampling. Longitudinal analysis revealed a statistically significant increase in sGAGs associated with sites that demonstrated continuing signs of disease activity and progressive attachment loss, thereby confirming its potential as a good marker of disease activity. However, within the active and inactive sites, large variations were present which possibly suggests, the sites being in different stages of an active/inactive period. It is possible that a burst of activity without 2 mm loss of attachment
may have occurred but not been included in the study or a burst of activity may have occurred in the 3 month period between visits and therefore not be detected. Therefore, future work would involve determination of the cut-off point so as to increase the specificity/sensitivity of the marker, as it is possible that we may otherwise misdiagnose disease activity due to the considerable overlap in GAG levels. Sites demonstrating high levels of GAGs may need to be monitored closely over a shorter period of time to detect periods of activity. Furthermore, cytokine profiling may be of additional value in monitoring these sites, as a concurrent increase/decrease in specific cytokines may be useful in identifying sites that need further monitoring. Significantly, only 30% of the sites demonstrated disease activity over a period of 21 months. These results have serious clinical implications in terms of managing patients diagnosed with periodontal disease, as these results are suggestive that current treatment modalities provide treatment that may not be necessary for the patient and therefore, further highlight the need for a biomarker that can accurately detect disease activity. Deeper sites (≥ 9mm) demonstrated increased disease activity when compared to less deeper pockets. These findings are supported from alternative clinical studies which have reported that further attachment loss mostly occurred at deeper sites (Grbic et al. 1991; Grbic and Lamster 1992). The techniques used for the detection of GAGs are however time consuming and technique sensitive. For its use as a diagnostic test, the assay must be relatively easy to perform, quick and reproducible. Ideally, an immunoassay would best serve the purpose, however, specific antibodies to specific components to the GAG chains would need to be developed.

This study further addressed the source of these proteoglycans in the GCF, in that CS may be released into the GCF as a consequence of either turnover or an increase in tissue destruction. As periodontal disease destruction is largely attributed to local inflammation resulting in interaction between host and microbial factors such as bacterial lipopolysaccharides (LPS), which alter the host immune response, the effect of LPS on periodontal ligament cell behaviour was investigated with a view to further understand the release of these markers in GCF. The effect of LPS on the various cell types present within
the periodontium such as fibroblasts (Takada et al. 1991; Tamura et al. 1992; Sakuta et al. 1998), osteoblasts (Bostanci et al. 2007a; Hamedi et al. 2009), epithelial cells, dendritic cells (Pulendran et al. 2001) have been extensively investigated and reported in the literature as detailed in Chapter 2. Several of these reports contradict each other and this may partly be due to the variations in cell types examined in that, some studies have used primary cells whereas others used cell lines. The source and method of LPS extraction and purification may also have contributed to the conflicting results as structural variations between strains, contamination with protein or DNA, have been reported to exhibit an effect on cellular activity (Wang and Ohura 2002). However, the effect of LPS on the cells contributing to the repair process is less understood. In order to model the complex nature of a wound healing process, an in-vitro culture system utilising a possible high proportion of progenitor cells derived from the periodontal ligament was used, as it is these cells which are possibly recruited during attempts at periodontal tissue repair. At toxic levels, viability was reduced to less than 50% suggesting that the presence of an increased microbial load within a periodontal pocket, would have a deleterious effect on the cells of the periodontal ligament and therefore lead to tissue destruction. However, in the presence of sub-toxic levels of LPS, an increase in cell expansion was observed, with a consequent decrease in matrix production. Within a clinical scenario, this may represent the post treatment stage where the microbial load has been reduced and the tissues are in a state of attempting repair. As it is almost impossible to totally eradicate microorganisms from a pocket, its presence in low levels within a healing pocket may be responsible for delayed/impaired healing and therefore the non-responding sites within responding patients, as well as the limited regeneration observed post treatment. Thus, sub-toxic levels of LPS may alter the biological events associated with the synthesis of a matrix and consequently influence the potential of the tissues in attempting repair. The results from our study along with other studies examining effects of LPS on various cell types are suggestive of a stimulatory effect by *P. gingivalis* LPS on the cell growth phase, thereby prolonging the initial cell growth phase and delaying the formation of matrix, with a further concomitant effect on maturation and mineralisation as demonstrated by an alteration in
decorin and biglycan synthesis. It is possible that within the heterogenous cell population, LPS may increase the cell growth of one or more subsets of the cell population which may further have a detrimental effect on the other subsets. Therefore, the decrease in ALP activity observed, may possibly be due to the decrease in the number of osteogenic precursor cells brought about by an increase in the other progenitor cell population in response to LPS stimulation. Alternatively, LPS may also affect the ability of the cells to induce and sustain matrix production. Previous work reporting the absence of DS decorin and biglycan in GCF (Last et al. 1988) along with the results from this study that indicates a decrease in expression of CS decorin and biglycan, is suggestive of an increase in processing and removal from the extracellular compartment. As a consequence of this, the remodelling and repair process may be affected and this implies that the degradation products such as proteoglycans or CS decorin and biglycan, detected in the GCF are as a consequence of tissue destruction. The effect of sub-toxic levels of LPS on other cells of the periodontium have not been investigated, therefore further studies to address this effect on other cell types, with a view to understand their behaviour during the repair process in the presence of virulence factors such as LPS, may provide us with more understanding of impaired/delayed wound healing processes. The isolation of the various populations of progenitor cells may also be useful to further study their individual behaviour during healing with a view to consider alternate techniques to promote repair and regeneration.

The presence of matrix degradation product (CS) in GCF as reported above, is attributed to an inappropriate host response resulting in an imbalance between the pro-inflammatory and anti-inflammatory cytokines released by specific cell populations such as the Th1/Th2/Th17/Treg cells. On cross-sectional examination of cytokine levels in gingivitis, chronic periodontitis, aggressive periodontitis, and healthy controls, significantly increased levels of IL-2, IL-4, IL-6, IL-17, IFN-γ and TNF-α were observed in gingivitis. Further examination of cytokine ratios in gingivitis, chronic periodontitis, aggressive periodontitis and healthy controls revealed an inconsistent Th1/Th17 response in patients with aggressive
periodontitis compared to a consistent Th1/Th2/Th17 in the other groups further confirming the potential of cytokine profiling in identifying high risk patients. Non-immune sources were also considered as potential contributors to the variation in response by means of in-vitro studies and results demonstrate that in spite of the presence of TLRs, which are necessary for downstream signalling and activation of NFkB resulting in cytokine secretion, the PDL cells did not release detectable levels of cytokines on stimulation with sub-toxic levels of LPS. These results confirmed that the cytokines detected in GCF may be released by the recruited immune cells, an imbalance of which causes periodontal tissue destruction and alveolar bone loss. Future work may involve the investigation of the role of other recruited cells such as the neutrophils and monocytes to the inflammatory pool. Additionally, the use of an ex-vivo murine mandible model for the study of inflammatory bone destruction (Sloan et al. 2013) to which T cells are introduced, may allow for the observation of co-expression of cytokines by specific cell types.

Although this study was cross-sectional in nature, it helped us to identify general trends and answer the question as to whether cytokine profiling may be of any value in identifying high risk patients. Further longitudinal studies investigating the relationship of cytokines to clinical loss of attachment would perhaps provide us with more information on its ability to specifically identify high risk patients and also its ability to assist in diagnosing disease activity. The longitudinal monitoring of these ratios to assess changes in values in response to treatment, will provide us with further information on its ability to monitor or alter host response in periodontal disease. However, care must be taken in the interpretation of these results, as the level of cytokines may be altered non-specifically in both gingivitis as well as periodontitis.

In conclusion, this study demonstrated that, CS is a good marker of diagnostic value as it has the ability to detect disease activity at the time of sampling. Results from in-vitro studies are suggestive of the degradation products in GCF being a product of tissue destruction rather than as a consequence of remodelling or repair further confirming the potential of CS as a good marker. However, on its own, CS/cytokines may not be ideal biomarkers. The specificity
of these biomarkers may be enhanced when used in combinations. Therefore, a chair-side test composing of a panel of markers such as CS and specific cytokine ratios, may be of significant value to clinicians in identifying high risk patients as well as sites, that require further monitoring. With the development of new diagnostic technologies such as microarray and microfluidics along with the use of oral fluids such as GCF, the development of rapid, non-invasive, site based risk assessment and comprehensive screening for biomarkers may be possible in the near future. Biomarker screening will allow for enhanced patient assessment which may enable provision of customized therapies that target treatment at individual level.
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