PERIODONTITIS IN COPD AND ALPHA-1 ANTITRYPSIN DEFICIENCY

Stephanie Ann Hobbins

ABSTRACT

This cross-sectional study reviews the relationship between periodontitis and chronic obstructive pulmonary disease (COPD) by exploring epidemiological and pathological data from patients with both diseases and a cohort with alpha-one antitrypsin deficiency (AATD).

Diagnostic criteria for periodontitis is not well standardised in the literature and thus leads to diversity in reported prevalence. In this study, the prevalence in non-deficient COPD patients was found to be increased (1.2 – 97.6%) in comparison to AATD patients (0 – 88.2%) regardless of the definition of periodontitis used (p<0.001), and both groups had a significantly higher prevalence than the general UK population. Common risk factors to both diseases were associated with periodontitis but did not fully account for the increased prevalence. Periodontitis prevalence did not relate to respiratory physiology in non-deficient COPD, but correlations were seen in the AATD group.

As periodontitis, COPD and AATD are all destructive, neutrophilic inflammatory diseases; they likely share a common pathophysiology and the role of peripheral and salivary CRP and IL-8 was explored. Peripheral IL-8 and salivary and peripheral CRP levels were significantly higher in non-deficient COPD compared to AATD patients, but neither accounted for the increased prevalence of periodontitis in non-deficient COPD patients.

To conclude, the criterion used to diagnose periodontitis in epidemiological studies has a significant impact on the published prevalence and associations with other chronic lung disease. This data finds increased prevalence in non-deficient COPD compared to AATD but the association with the severity of pulmonary impairment is stronger in AATD suggesting

the deficiency enhances periodontal damage irrespective of other demographic

confounders. The pathophysiology underlying this requires further exploration.

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LIST OF ABBREVIATIONS

AAP	American Academy of Periodontology
AAT	Alpha-1 Antitrypsin
AATD	Alpha-1 Antitrypsin Deficiency
ADAPT	Antitrypsin Deficiency Assessment and Programme for Treatment
ADHS	Adult Dental Health Survey
ATS	American Thoracic Society
BAL	Bronchoalveolar Lavage
BMI	Body Mass Index
BOP	Bleeding on Probing (of the gingiva)
BPE	Basic Periodontal Examination
CAL	Clinical Attachment Loss
CDC	Centers for Disease Control and Prevention
CDRC	Chronic Disease Resource Centre
CEJ	Cement-enamel Junction
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
CRP	C- Reactive Protein
CT	Computed Tomography
ELISA	Enzyme-Linked Immunosorbent Assay
ERS	European Respiratory Society
FEV_1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity

GCF	Gingival Crevicular Fluid
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HR	Hazards Ratio
hs-CRP	High Sensitivity C-Reactive Protein
ICS	Inhaled Corticosteroid
IL-8	Interleukin 8
IMD	Index of Multiple Deprivation
K_{CO}	Transfer factor per alveolar volume
LABA	Long Acting Beta-2 Agonist
LAMA	Long Acting Muscarinic Antagonist
mMRC	Modified Medical Research Council
NHANES	National Health and Nutrition Examination Survey
NS-SEC	National Statistics Socio-economic Classification
OR	Odds Ratio
PPD	Pocket Probing Depth
PYH	Pack Year History
ROS	Reactive Oxygen Species
SABA	Short Acting Beta-2 Agonist
SPSS	Statistical Package for the Social Sciences
TL_{CO}	Transfer Factor of the Lung for Carbon Monoxide
TMB	3,3',5,5'-Tetramethylbenzidine

STATEMENT OF ROLES

All patients were recruited between 2014 and 2016.

- Dr Adam Usher recruited 88 non-deficient COPD patients and 51 AATD patients.

- Dr Stephanie Hobbins recruited 17 AATD patients.

All respiratory physiology measurements were undertaken by members of the respiratory physiology department at Queen Elizabeth Hospital, Birmingham.

Dental assessments were performed by dentists Miss Sabrina Parmar and Mr Simon Crichard.

Clinical reviews were performed by Dr Adam Usher and Dr Stephanie Hobbins.

All laboratory work was undertaken by Dr Stephanie Hobbins

Statistical tests were performed by Dr Stephanie Hobbins following advice from Statistician Dr Peter Nightingale based at Queen Elizabeth Hospital, Birmingham.

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1   CHRONIC OBSTRUCTIVE PULMONARY DISEASE

1.1.1   Definition

Chronic obstructive pulmonary disease (COPD) is a chronic, progressive disease of airflow obstruction due to an enhanced chronic inflammatory response [1, 2] resulting in airway remodelling and parenchymal destruction [3]. The most common symptoms include cough, breathlessness and sputum [4]. COPD is heterogeneous and includes different phenotypes including emphysema predominant and those with chronic bronchitis [5] but the extent to which phenotype is predominant is individual to the patient.

Emphysema is the pathological enlargement of airspaces distal to terminal bronchioles with non-fibrotic destruction of alveolar walls. Centrilobular emphysema is characterised by discrete enlargement of airspaces and is upper lobe predominant and is the type most associated with smoking related disease. Panlobular emphysema affects the whole acinus more uniformly and is often seen in non-smokers [6, 7].

Chronic bronchitis describes an inflammatory condition involving the bronchi, but also describes bronchial mucous gland enlargement and excessive mucus secretion. This is usually defined as chronic or recurrent productive cough most days ≥3months per year for ≥2 years [4].

1.1.2 Diagnosis and Severity Classification

The most frequently employed measure to diagnose COPD is the presence of post-bronchodilator FEV1:FVC ratio <70% [4]. Severity of disease is then classified according to the percentage of predicted FEV1. GOLD stage 1 (mild) is characterised by FEV1 ≥80%; GOLD stage 2 (moderate) by FEV1≥50% but <80%; GOLD stage 3 (severe) by FEV1 ≥30% but <50% and GOLD stage 4 (very severe) by FEV1 <30% [4].

1.1.3 Prevalence

Prevalence data is widely variable due to methods of measurement, diagnosis and reporting but has been reported to be 9-10% in people ≥40years of age [8] with a global prevalence of 11.7% [4]. There is evidence to suggest that COPD prevalence is increasing in the UK; by 27% in the last decade[9].

1.1.4 Mortality and Economic Burden

COPD accounts for 5.3% of total UK deaths and 26.1% of all deaths from lung disease [9].

In 2010, the cost of COPD in the USA was approximately US$50 billion, including $20 billion in indirect costs and $30 billion in direct health care expenditures [10]. In the UK the NHS annual cost is approximately £800 million, or £1.3 million per 100000 people [11]. COPD is associated with other chronic inflammatory conditions such as cardiovascular disease and diabetes which occur at a higher prevalence than seen in age-matched patients without COPD [12] and treating these co-morbidities approximately doubles health care expenditure [13].

1.1.5 Risk factors

Cigarette smoking remains the main risk factor for the development of COPD in the developed world. However, not all smokers develop disease and COPD is seen with other environmental exposures [14] and in non-smokers [15]. Familial and genome wide association studies suggest there is a genetic susceptibility to COPD with the most documented being alpha-1 antitrypsin deficiency (AATD) [16](see section 1.2).

1.1.6 Pathogenesis

COPD results from an abnormal and excessive inflammatory response to noxious stimuli in the lungs leading to destruction of tissues [17]. The ongoing activation of innate and acquired host inflammatory responses eventually results in lung damage [18]. Contributing to excessive inflammation, there are two processes hypothesised to be involved in the pathogenesis of COPD; the proteinase-anti-proteinase theory [19] and the oxidative stress theory [18].

The proteinase-anti-proteinase hypothesis states that tissue destruction occurs when the action of proteinases is no longer controlled by anti-proteinases. This can occur due to genetic factors e.g. AATD, functional loss of anti-proteinases through proteolytic or oxidative damage, or due to excessive recruitment or activation of proteinases [19].

The oxidative burden is increased in COPD. The main source of excess oxidant is cigarette smoke, but additional oxidants can also be released by inflammatory cells such as neutrophils [20, 21]. Oxidative stress inactivates anti-proteases, amplifying inflammation [22].

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1.1.7 Inflammation in COPD

COPD is also associated with enhanced inflammation systemically, with studies describing the activation of inflammatory mediators and the secretion of cytokines [23]. Cigarette smoke and other atmospheric noxious substances can directly activate many cells including those of the respiratory epithelium to release cytokines and chemokines leading to inflammatory responses and tissue destruction [3, 24]. In susceptible individuals this inflammation is sustained even after smoking cessation [25]. The mechanism of systemic inflammation resulting from pulmonary inflammation is not clear but may result from an overspill of cytokines from the airways into the systemic circulation; such as seen with Surfactant Protein D- a glycoprotein only produced by type 2 pneumocytes, yet found in peripheral circulation of patients with COPD [26], cellular cross talk between respiratory and systemic compartments (e.g. release of microvesicles into the circulation as seen by increased numbers of circulating endothelial microparticles of pulmonary origin in patients with COPD exacerbations [27]) or the presence of local inflammatory mediators causing peripheral release of other mediators and cytokines [28]. Increased inflammatory mediators and cytokines have been seen in the peripheral circulation of COPD patients, and are more pronounced during exacerbations [29-31].

1.1.8 Neutrophils in COPD

Neutrophils are the most abundant circulating leucocyte, derived from pluripotent stem cells in bone marrow and characterised by multilobed nuclei and cytoplasmic granules.

Neutrophils are the predominant cell seen in airway secretions in COPD and have been implicated in causing tissue damage by extracellular release of destructive mediators such as the proteinases neutrophil elastase and proteinase 3 [3].

Many studies have shown an increase in neutrophils in both systemic circulation and airways of smokers and patients with COPD. Bronchoalveolar lavage (BAL) fluid from chronic smokers shows dose-dependent increased level of neutrophils when compared to non-smokers [32], and numbers were highest in induced sputum of smokers and COPD patients than non-smokers, with the highest levels being seen in severe COPD patients, correlating with FEV1 percent predicted [21]. Peripheral and airway neutrophil counts have been shown to be positively related to accelerated decline in FEV1 in COPD patients [33-35].

The neutrophils from COPD patients also appear dysfunctional. Circulating neutrophils produce more reactive oxygen species (ROS) than those from healthy non-smokers or smokers with normal lung function [20], and have enhanced, rapid and tortuous chemotactic responses to inflammation and increased degranulation and tissue destruction both in vitro and systemically [36, 37].

The level of neutrophilic inflammation in airways correlates with disease severity and progression in COPD whether measured by lung physiology, radiologically or self-reported health status [21, 33-35, 38]. This supports the concept that activated neutrophils play an important role in lung injury including development of emphysema through neutrophilic release of inflammatory cytokines and proteases [39-41]. Cigarette smoking enhances recruitment of activated neutrophils with increased release of tissue destructive proteinases [42], supporting the faster physiological decline seen in COPD patients who continue to

smoke. Importantly, neutrophilic inflammation is sustained in ex-smokers with COPD although it abates in ex-smokers without COPD [43] again suggesting susceptibility to self-perpetuating inflammation.

1.1.9   Co-morbidities in COPD

COPD is recognised as having a series of co-morbidities hypothesised to be related to a systemic or common organ specific inflammatory process. This presence of co-morbidity is reflected in the updated GOLD classification systems for COPD [4]. An observational study found more than 97% of a COPD cohort had at least one other co-morbidity, with more than 50% having at least 4 [44] when systematically sought. Respiratory co-morbidities associated with COPD include bronchiectasis [45], asthma, pulmonary fibrosis [46] and lung cancer [46], but extra thoracic chronic inflammatory disease also co-occurs [47]. Cardiovascular diseases are the most common [48] including hypertension [46], venous thromboembolism, ischaemic heart disease, arrhythmias [46, 49] and heart failure [46, 50]. Non-cardiovascular co-morbidities include cognitive impairment [51], diabetes [48, 52], skeletal muscle dysfunction [53], low bone mineral density and osteoporosis [50, 54]. This high prevalence may reflect the presence of common risk factors including age, smoking and sedentary lifestyle, but even when such factors are accounted for, there remains increased risk, supporting linked pathogenic drivers of disease.

1.2   ALPHA-1 ANTITRYPSIN DEFICIENCY

1.2.1   Definition

Alpha-1 antitrypsin (AAT) is predominantly synthesised in the liver [55] and its primary function is the inhibition of neutrophil elastase (which it inhibits on a one-to-one molar basis). However AAT also has an anti-inflammatory role regulating the expression of many pro-inflammatory cytokines [56].   Alpha-1 antitrypsin deficiency (AATD) was first described in 1963 where it was noted that serum from patients with early, severe emphysema lacked α1 bands [57].

Although there are more than 100 detectable alleles for AAT, the most common variants are M, S and Z alleles. The M allele is the wild type, and is the most prevalent variant, providing adequate levels of AAT, whilst the Z allele is the most common deficiency variant in northern Europeans [58]. Null variants are characterised by a complete absence of circulating AAT due to transcriptional or translational errors interrupting their synthesis [59].

Patients with AATD, in particular homozygotes for Z allele are at increased risk for the development of early onset, severe emphysema and airflow obstruction [60]. This risk is increased in patients who smoke cigarettes but can occur in non-smokers. Patients with AATD can also suffer with bronchiectasis, liver cirrhosis, panniculitis and vasculitis [59].

1.2.2   Diagnosis and Severity Classification

Individuals with normal alleles for AAT generally have serum concentrations of AAT of 20–53µmol/L. Deficient alleles generate concentrations < 20µmol/L, but in severe cases of AATD such as homozygotes for Z allele, the concentration is <11µmol/L with only 15-20% of normal circulating AAT present [59].

Aside from measuring concentration of circulating AAT in serum, genotyping is also recommended as it has the advantage of identifying null genes, rare deficiency alleles and forms of AATD where the level of the protein is normal, but has abnormal function [61].

1.2.3   Prevalence

AATD is an autosomal co-dominant disorder affecting 1 in 3000 live births [62]. Accurate prevalence data is lacking but it is suggested that the prevalence in England is 8.1 per 1000 population for the more severe, PiZZ genotype [63].

1.2.4   Morbidity & Mortality

A UK longitudinal study of patients with PiZZ AATD revealed that FEV_1 GOLD stage, Kco and GOLD group were estimated to be the most significant factors related to mortality. For all-cause mortality, patients in GOLD group D had a survival of less than 40% at 15years compared to more than 70% in groups A, B and C [64].

A review of the American National Heart, Lung, and Blood Institute Registry of Individuals with Severe AAT Deficiency found an overall 5-year mortality rate of approximately 3% per

year and those with initial $FEV_1 < 15\%$ predicted had a significantly higher mortality rate than those with FEV_1 percentage of predicted > 50%. The most common causes of death were emphysema and cirrhosis [65].

A significant relationship exists between smoking and lung function decline in adults with PIZZ. Research has shown current smokers have more decline in FEV1, whereas ex and never smokers show a slower decline [66]. Long-term loss of lung function also correlates with the incidence of respiratory infections and exacerbations [67, 68].

1.2.5   Pathogenesis

Emphysema in AATD is thought to develop due to excess levels of unopposed neutrophil elastase in the lung [69] resulting in a wider area of tissue destruction [70]. In individuals with PiZZ, the Z allele can also act as an additional chemoattractant for neutrophils, perpetuating their recruitment [71]. As with usual COPD, there is significant heterogeneity in patient presentation and cigarette smoking can cause oxidative and proteolytic inactivation of AAT [72] function, contributing to the noted accelerated clinical decline.

1.2.6   Co-morbidities in AATD

Less data is available on diseases commonly seen as co-morbid with AATD but AATD patients with PiZZ or PiZnull genotype have a higher prevalence of inflammatory bowel disease and hypothyroidism than that predicted for the general UK population; (1.5 vs 0.4%), and (8.5 vs 1.2% of women and 0.4% of men) respectively. In the same study, 0.8% of patients had a

diagnosis of Wegener's granulomatosis and 0.9% had panniculitis [73]. Conflicting evidence

is available for the association of AATD and bronchiectasis, pancreatitis, cardiovascular

disease and renal disease [74].

1.2.7   AATD as a mechanistic model

Investigating relationships with COPD and other diseases can be hampered by confounders

such as smoking, age and severity of airflow obstruction which can be difficult to correct for.

For this reason, patients with AATD are often used as a comparative group to usual COPD

patients as AATD is characterised by an exaggerated inflammatory response, similar to usual

COPD but usually identified at an earlier age [74] earlier stage of disease [75] and many of

these patients have not usually been exposed to the significant environmental risk factors

such as smoking [67, 76, 77] that usual COPD patients have.

1.3 PERIODONTITIS

1.3.1   Definition

Periodontitis is a common, chronic, bacterial-initiated inflammatory disease. The accumulation of a biofilm above and below the gingival margin leads to a dysbiosis, which drives a dysfunctional host-immune-inflammatory response, leading to connective tissue destruction and the formation of a periodontal pocket [78].   Without treatment, the persistent inflammation leads to destruction of alveolar bone and subsequent tooth loss [78].

1.3.2   Diagnosis

Periodontitis is usually diagnosed in the presence of a combination of periodontal inflammation, (bleeding on probing (BOP) of the gingiva), number and depth of periodontal pockets, level of clinical attachment loss (CAL) and presence of radiologically evaluated alveolar bone loss [79-81]. Probing pocket depth (PPD) and CAL are measured using purposefully designed periodontal probes. The PPD is the distance from the gingival margin to the base of the gingival sulcus (or periodontal pocket). CAL is the distance from the cemento-enamel junction to the base of the sulcus or periodontal pocket [79], see Figure 1.
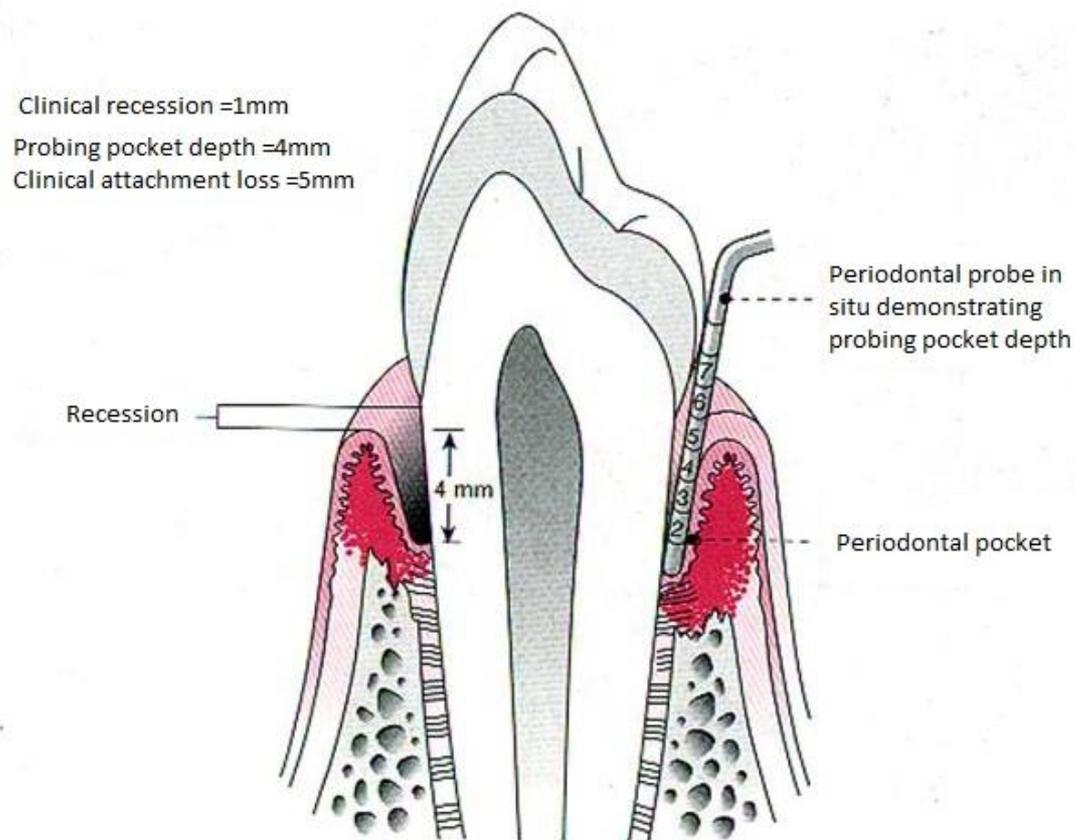
Figure 1: *Measurement of clinical attachment loss and probing pocket depth in the assessment of periodontitis.* *Note.* Adapted (with permission) from "Understanding Periodontal Diseases: Assessment and Diagnostic Procedures in Practice: 1" by Iain L.C. Chapple, Angela D. Gilbert, Nairn H.F. Wilson, 2002, *Published by* Quintessentials: Endontics

However, there is no consistent use of one definition of periodontitis in the literature. Despite the clear criteria defined by the American Association or Periodontology [97], a systematic review found only 15/104 studies providing any proposed definition of periodontitis [81] and published studies use a range of periodontal measurement parameters for analysis [82-86] reflecting the lack of a uniformly applied definition of periodontitis [82-88, 90-94] in epidemiological studies. Classifying periodontitis has been the subject of many changes and reviews [79-81, 95, 96]. National Health Examination

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Surveys have been conducted in the United States since 1960, and indices of measurement in these surveys have evolved from visual inspection of inflammation to a combination of PPD, CAL and furcation involvement [79].

PPD is generally accepted as a reflection of current disease status, whilst CAL represents cumulative disease experience [94]. In younger populations, PPD and CAL correlate well, but as people age, gingival recession and therefore measures of attachment loss become a normal part of the ageing process. Increased PPD can occur as a false positive clinical finding when gingival tissues become swollen due to inflammation hence moving the gingival margin upwards (false pocketing). The use of CAL alone may over estimate periodontitis because attachment loss can occur with non-inflammatory gingival recession, e.g. trauma from over tooth brushing [79].

There have been attempts to provide a unifying definition for periodontitis. The Division of Oral Health at the Centers for Disease Control and Prevention (CDC) alongside the American Academy of Periodontology (AAP) defined severe periodontitis as involvement of two or more interproximal sites with CAL ≥6mm and one or more interproximal sites with PPD ≥5mm. Moderate periodontitis required involvement of two or more interproximal sites with CAL ≥4mm or ≥2 interproximal sites with PPD ≥5mm [79]. Since then, Eke et al additionally defined mild periodontitis as ≥2 interproximal sites with attachment loss ≥3mm and ≥2 interproximal sites with PPD ≥4mm, or one with PPD ≥5mm [97].   During the 2005 5th European Workshop in Periodontology a two-threshold level criterion was proposed to diagnose periodontitis. This was the presence of attachment loss of ≥3mm in ≥2 non-adjacent teeth to diagnose incipient cases and the presence of proximal attachment loss of

≥5mm in ≥30% of teeth to determine severe disease. However, the authors stated that a single index measurement does not reflect the disease and that these criteria are designed for use in studies determining risk factors for, and not the prevalence of, periodontitis [96].

The current American classification system is based on the 2014 task force meeting who developed a clinical interpretation of the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions proposal [98], recognising the importance of attachment levels but also difficulties in accurate measurement and interpretation especially in those with non-inflammatory recession [99].

The Basic Periodontal Examination (BPE) recommended by The British Society of Periodontology in clinical practice [100] is used routinely in the UK which defines evidence of periodontitis being present when the PPD is ≥3.5mm but does not utilise measurements of CAL in its definitions.

1.3.3   Prevalence

Accurate prevalence data is limited given the lack of a uniformly accepted definition for the diagnosis of periodontitis. However, the most recent adult dental health survey reports that periodontitis affects approximately 45% of adults [101], with 8-11% of adults suffering from severe periodontitis [101, 102]. In this survey, periodontal health was recorded in the absence of bleeding, calculus, periodontal pocketing of 4mm or more and in the case of adults aged 55years or more, loss of periodontal attachment of 4mm or more anywhere in the mouth

1.3.4   Morbidity

Periodontitis is an important public health concern given the consequences of tooth loss, including maintaining nutrition, effects on speech production and the financial burden associated with its treatment estimated to be £2.8 billion in the UK in 2008 [103]. Periodontitis has also been linked to other chronic systemic diseases including diabetes mellitus [104, 105], rheumatoid arthritis [105, 106], and cardiovascular diseases.

1.3.5   Risk factors

There are several well recognised risk factors for the development of periodontitis. The most reported are age [107-109]and smoking [107, 110, 111], with prevalence and severity increasing as patients' age and in those with a significant smoking history. Others include diabetes, poor dental habits [109, 112]and low socio-economic status [101, 109, 111]. Evidence has shown that people taking long term corticosteroids have increased CAL and deeper PPD [113]. Inhaled corticosteroids also affect bone mineral density and increase the risk of tooth loss [114] and inhaled beta-2 agonists reduce salivary production and thus a consequent decreased output of antibacterial components which promotes bacterial colonization, plaque growth and potentially periodontitis [115]. There is no evidence that LAMA use has any relationship with periodontitis but a dry mouth is a commonly reported side effect due to reduction in oral fluid production and reduced salivary flow has been implicated in periodontitis [115].

1.3.6 Pathogenesis

The pathogens implicated in periodontitis adhere to the gingival margins and produce biofilms which induces a local inflammatory response activating host innate and acquired immune pathways. This results in local infiltration of gingival tissues with neutrophils and other inflammatory mediators including cytokines, chemokines and neutrophil derived proteases. The active periodontal lesion is B-cell and neutrophil dominant with repeated bacteraemia causing activation of the acute-phase response and subsequent systemic inflammation [116]. This is thought to prime peripheral blood neutrophils causing an exaggerated release of ROS and pro-inflammatory cytokines [117], which can lead to breakdown of supporting structures and destruction of bone in susceptible individuals.

1.3.7 Neutrophils in Periodontitis

Periodontitis is characterised by a neutrophilic infiltrate [118]. Systemic neutrophil chemotaxis studies in chronic periodontitis showed reduced speed (movement in any direction), velocity (speed of movement towards the chemoattractant) and accuracy (of movement towards the chemoattractant) of neutrophils [119] which improved after treatment of the periodontitis [120]. This observation supports the notion of a causal link between periodontitis and systemic cell function. Impaired neutrophil function could render the host less able to deal with the burden of bacteria in the periodontium, thereby perpetuating the inflammatory response and destructive processes pathognomonic of periodontitis.

Further supporting the link between neutrophils and periodontitis there is an increased

prevalence of periodontitis in conditions characterised by abnormal neutrophil function

[121], including defective neutrophil production (autoimmune or chemotherapy induced

neutropenia) defective neutrophil release from bone marrow as seen in WHIM (warts,

hypogammaglobinaemia, infections, and myelokathexis), inherited leucocyte adhesion

deficiency, excessive neutrophil activation, deficient clearance of neutrophils as in

autoimmune inflammatory diseases including systemic lupus erythematosus and the

Papillon Lefevre syndrome(neutrophil cathepsin C deficiency).

1.4 PERIODONTITIS AND SYSTEMIC DISEASE

1.4.1   Periodontitis and Systemic Co-Morbidity

Periodontitis has also been linked to a number of other chronic systemic diseases including

diabetes mellitus [104, 105], rheumatoid arthritis [105, 106], cardiovascular diseases [122-

124] and stroke [125, 126].

1.4.2   Periodontitis and Respiratory Disease

A meta-analysis identified 11 observational studies of intubated patients where periodontal

disease was associated with respiratory infections [127]. Half of these studies link the

patient's dental status to the development of a ventilator-associated pneumonia, and one

study reported the implicated pathogen had first colonized the dental plaque biofilm [128]. Other studies have demonstrated an association between poor dental status, the presence of oral bacteria and occurrence of pneumonia [129-132], although these studies were predominantly undertaken in patients admitted to intensive care or residing in care homes rather than the general population.

Another meta-analysis [127] included several intervention trials (improved oral hygiene and use of antibiotics for oral disease) [133-137] to reduce the bacterial load in the mouth. It found these treatments significantly reduced the risk of developing pneumonia, supporting a causal link between oral pathogens and lower respiratory tract infections. Furthermore, the use of mouthwashes in intubated patients can reduce the nosocomial respiratory infection rate [138, 139].

The association between poor dental health and respiratory disease is believed to exist as a result of dental plaque becoming colonized with respiratory pathogens and subsequently acting as a periodontal reservoir for infectious micro-organisms. This is more problematic in patients who are already compromised due to frailty or ill health as they are more likely to aspirate oral secretions containing these pathogens and struggle to clear them from the respiratory tract by normal protective mechanisms [86, 129].

1.4.3   Periodontitis and COPD

There is evidence that COPD and periodontitis occur more commonly in the presence of the other [83, 84, 86-88, 92, 94, 140-145], but published studies are variable in approach. Some authors assessed the prevalence of periodontitis patients having COPD as co-morbidity,

whilst others have assessed the prevalence of patients with COPD having periodontitis as co-morbidity.

Studies assessing COPD as a co-morbidity of periodontitis have all described an association, but of varying strength. A meta-analysis representing 3988 COPD patients and 22871 controls reported a pooled odds ratio (OR) for having COPD in populations diagnosed with periodontitis as 2.08, (95% CI 1.48-2.91; p<0.001) [143]. Hayes et al found for each 20% increase in mean whole mouth alveolar bone loss, a person's risk for subsequently developing COPD was increased by 60% [141], whilst a link was found between poor oral health status with bacterial colonisation and the presence of COPD in chronic care facility residents [146]. A large cross sectional study correcting for age, smoking, socio-economic status and dental habits, found moderate periodontitis was associated with COPD in male patients only and the relative risk of severe periodontitis in the male group was 1.23 (95% CI 1.06-1.56) [92]. The lack of a link in women may have been due to insufficient power in the female group.

Studies assessing the co-morbid prevalence of periodontitis amongst patients with COPD have found similar results. Data from a longitudinal COPD cohort study found a significantly increased cumulative incidence of increased periodontitis with adjusted hazard ratio (HR) of 1.20 (95% CI 1.15-1.25) rising to 3.17 (95% CI 2.81-3.57) in a sub group requiring hospitalisation for COPD [144]. A further in-patient study noted increased markers of periodontitis in a hospitalised COPD group compared to an in-patient control group (PPD 2.57 +/-0.85 vs 1.76 +/- 0.7, p<0.0001 and CAL 3.16 +/- 0.53 vs 2.12 +/- 0.92, p<0.0001) [83]. Several studies assessing non-exacerbating COPD and periodontitis report worse periodontal

indices in patients with COPD when compared to non-COPD controls [84, 86-88]. Leuckfeld et al assessed orthopantomograms in a retrospective cross sectional study of patients evaluated for lung transplant and found a significant difference in radiographically determined periodontitis, with 44% of COPD patients affected compared to 7% of non-COPD patients [145].

However, other studies have failed to find such associations [85, 90, 93]. Bergstrom et al found that smoking rather than COPD was the major risk factor for the existence of periodontal pockets [93], and the NHANES III survey were unable to identify any relationship between COPD and periodontitis [90]. Kim et al found a relationship with airflow obstruction in males and loss of natural teeth, but no relationship with periodontitis [91].

These conflicted studies may reflect inconsistency in case definitions, data collection and analysis. Additionally, not all studies describing an association between COPD and periodontitis correct for potentially significant confounding factors. For example, a meta-analysis that reported a pooled odds ratio for having COPD in populations with periodontitis as 2.08, (95% confidence interval 1.48-2.91; p<0.001) reduced this to 1.78 (95% CI 1.23-2.58) following correction for age, gender and smoking [143]. There remains some uncertainty, therefore, whether the association between COPD and periodontitis simply reflects the shared risk factors of age, smoking, dental habits and socio-economic group.

1.4.4   Neutrophils linking periodontitis & COPD

As described already, neutrophil dysfunction occurs in both COPD and periodontitis. Both diseases are characterised by non-resolving inflammation and the subsequent activation and

consequences of the actions of neutrophils as a part of the host response to this persistent inflammation.

1.5 C- REACTIVE PROTEIN

1.5.1   Introduction

CRP is predominantly synthesised in the liver [147] and is a major acute-phase plasma protein, the concentration of which can reach a 1000-fold increase in response to inflammation or tissue injury within 24–48 hours of the insult [148, 149] and fall rapidly after resolution of the precipitating event [149].

Experiments have shown CRP can reduce the chemotactic responses of neutrophils [150, 151] but also enhances phagocytosis [150]. CRP can also interrupt the adhesion of neutrophils to endothelium [152] and thus impede their migratory capacity.

Raised systemic CRP levels have been associated with an increased risk of cardiovascular disease [153-156], diabetes [157], stroke [154, 156, 158] and death from these diseases [157, 159]. However, there is no consensus whether high levels of CRP are part of the mechanism through which inflammation contributes to these disease processes or whether it simply reflects the presence of atherosclerosis or vascular damage.

1.5.2   CRP and COPD

Published evidence supports an association between CRP and the presence of COPD. A meta-analysis of five studies by Gan et al confirmed a significant increase in CRP levels in COPD patients compared with controls [160]. The ECLIPSE study found baseline CRP levels were significantly higher in COPD patients compared to smoking and non-smoking controls [161]. Similar findings of increased CRP have been seen with patients with airflow obstruction confirmed on spirometry and pre-existing diagnoses of COPD [28, 162, 163]. He et al also note a positive correlation between sputum and blood CRP levels in the COPD patients; r = 0.443, P < 0.01 [21], suggesting local inflammation in the respiratory tract promotes systemic inflammation causing a rise in blood CRP alongside sputum CRP.

There are studies which have found significant associations with levels of CRP and lung function in COPD patients [21, 147, 162, 164-167]. Other studies note an association between CRP and exercise capacity [162, 163] and oxygenation [162, 166] and higher CRP levels over time have been associated with a faster FEV1 decline [33, 165]. Further studies have found an association with CRP and exacerbations of COPD [161, 166], and CRP has been found to be a significant predictor of hospitalisation and mortality in patients with chronic respiratory failure [168]. Many COPD patients use inhaled corticosteroids (ICS), and interestingly, the CRP level in COPD ICS users was found to be significantly lower than in those not using ICS [163].

The mechanism of systemic inflammation in COPD is not yet clear, but overspill of ROS and cytokines from airways into systemic circulation or peripheral liberation of pro-inflammatory cytokines by inflammatory and/or structural cells are feasible hypotheses.

1.5.3   CRP and Periodontitis

CRP has been shown to be raised in the peripheral circulation of patients with periodontitis when compared to people without periodontitis [169-172]. Serum CRP has also shown to be correlated with the presence of bleeding on probing of the gingiva and the number of pathological periodontal pockets [173].

There is considerable evidence supporting treatment of periodontitis, (non-invasive and surgical), successfully reduces the level of circulating CRP [174-181], and a reduction in peripheral CRP is associated with an improvement in measured periodontal indices [178, 181].

1.5.4   Salivary CRP

The use of salivary CRP as a diagnostic tool has received increasing attention due to its easy availability and non-invasive collection methods[182]. Several studies have reported the potential use of salivary CRP as a clinical biomarker in thyroid disease [183] , metabolic syndrome [184], myocardial infarction[185, 186] atherosclerosis [187], COPD [83, 188] and periodontitis [189]. In studies comparing salivary and circulating CRP levels, results show a good correlation between the two [83, 186-188, 190, 191].

CRP ELISA kits have been previously validated for use in saliva and found to show reasonable association with peripheral CRP [192, 193]. However there are reports where this association has not been seen [194] and in one validation study, several samples had salivary CRP values that exceeding more than 2 standard deviations from the mean reported by the manufacturer of the ELISA kit and despite dilution, adequate spike and recovery was not reliably achieved [193].

1.5.4.1   Salivary CRP in COPD

Bhavsar et al measured serum and salivary CRP in a review of COPD and oral health status. A significant positive correlation between serum and salivary levels in patients and controls was seen, but CRP levels were significantly higher in the COPD patients [83].

Patel et al also measured serum and salivary CRP in patients with COPD, smokers with normal spirometry and never smokers and found that salivary CRP correlated with serum CRP but was approximately 200 times lower than serum CRP. The authors reported an increased CRP in COPD patients and smokers compared with never-smokers [188], a finding replicated by Azar et al [195]. Patel et al, found acutely exacerbating COPD patients had a significantly increased salivary CRP in comparison to non-exacerbating patients. No association was observed between salivary CRP and FEV1, but it was weakly correlated with mMRC score in the COPD patients (r = 0.16, p < 0.01) [188].

1.5.4.2 *Salivary CRP in Periodontitis*

There is limited published evidence on studies looking at salivary CRP in periodontitis, as much of the literature examines CRP in gingival crevicular fluid [171, 196, 197]. However, Shojaee et al found salivary CRP levels in patients with chronic periodontitis were higher than those for healthy controls, and report positive correlations between CRP and periodontal indices [189].

1.6 Interleukin-8

1.6.1 Introduction

Interleukin-8 (IL-8) is a pro-inflammatory cytokine synthesized in response to an inflammatory insult. It is a member of the neutrophil-specific CXC subfamily of chemokines and is a potent neutrophil chemotactic and activating factor and can be secreted from many host cells.

1.6.2 IL-8 in COPD

Several studies have reported increased levels of IL-8 in the sputum of patients with COPD [3, 38, 43, 198] and IL-8 levels also appear to correlate with the bacterial load in the airways [3], measures of disease severity [199] and airflow limitation [38, 198, 199]. Bhowmik et al found increased sputum IL-8 was associated with increased neutrophil counts in COPD

patients in a stable state and significantly higher IL-8 levels in patients classed as frequent exacerbators [200].

Measurement of peripheral circulating IL-8 is unreliable and produces conflicting results [38, 201, 202]. Circulating IL-8 is not always detectable in COPD patients, and is frequently undetectable in healthy individuals [29, 38]. Peripheral IL-8 does not always correlate with sputum levels, airflow obstruction [38] or smoking status [201].

Kuschner et al found dose related higher levels of IL-8 in the bronchoalveolar lavage (BAL) fluid of smokers compared to non-smokers. They also report a significant association between the numbers of neutrophils in the BAL and the IL-8 in the BAL, consistent with the known established neutrophil chemoattractant effects of IL-8 [32].

1.6.3   IL-8 in Periodontitis

In periodontitis, microbial toxins stimulate connective tissue epithelial cells to release inflammatory mediators including IL-8, which can then can pass through the connective tissue epithelium and enter the gingival crevicular fluid (GCF) [203].

However, there are conflicting results regarding levels of IL-8 in GCF of periodontitis patients with some studies reporting lower levels in disease [204] and others significantly higher levels in periodontitis [203, 205, 206]. There is also evidence to suggest treatment of periodontitis reduces the levels of IL-8 in GCF [205] and levels correlate with clinical periodontal parameters [203, 207]. Similarly conflicting results exist for peripheral IL-8 in the circulation of patients with periodontitis with some evidence for there being no

difference in health and disease [208] and other evidence that plasma IL-8 is raised in periodontitis [209].

Some studies have investigated salivary IL-8, but found it was detectable in only 50 % of participants, and with no significant difference in the levels between those with periodontitis and healthy controls. However, weak but significant correlations were seen with salivary IL-8 and mean pocket depth [210].

1.7 Summary

There is evidence suggesting that periodontitis and COPD could be co-morbidities of each other but variable definitions of disease, lack of uniformly applied diagnostic criteria and the presence of significant confounders weakens the current evidence. The mechanisms underlying the potential association are yet to be fully explored but likely centre around the shared inflammatory hypothesis with the neutrophil as the key effector cell in both disease processes.

1.8 Study Hypotheses

1.8.1   The prevalence of periodontitis is increased in patients with COPD and AATD

compared to the prevalence in the UK general population as quoted in published

literature.

1.8.2   This increased prevalence reflects shared pathogenic mechanisms of

neutrophilic inflammation, and so will relate to the severity of the lung disease.

1.9 Study Aims

1.9.1   To calculate the prevalence of periodontitis in COPD and AATD patients and

importantly, determine this using the different definitions of periodontitis

previously described and correct for common confounders.

1.9.2   To validate commercially available ELISA kits for the measurement of salivary

CRP and salivary IL-8.

1.9.3   To measure CRP and IL-8 in peripheral blood and saliva and correlate with

indices of lung function and disease severity.

1.9.4   To measure CRP and IL-8 in peripheral blood and saliva and correlate with

indices of periodontitis.

CHAPTER 2

METHODS

2 METHODS

2.1 Ethics

All patient recruitment, collection of data and biological samples and experiments conducted were done so with ethical approval from the National Research Ethics Service (REC13/WM/0044) and all patients provided informed written consent to take part.

2.2 Patient cohort

Patient characteristics are described in Chapter 3. Non-AATD COPD patients were recruited from the Chronic Disease Resource Centre (CDRC) COPD cohort. AATD patients were recruited from the Antitrypsin Deficiency Assessment and Programme for Treatment (ADAPT) cohort at Queen Elizabeth Hospital Birmingham, between 2014 and 2016.

COPD was diagnosed by post bronchodilator spirometry and staged by GOLD criteria [17]. The AATD patients included only those with the PiZZ and PiZ0 null genotypes (confirmed by isoelectric focusing of finger prick blood spots at Heredilab Salt Lake City USA).

Patients were not recruited if they had any medical condition precluding a dental examination, were unable to perform lung function tests to ERS/ATS standards or had significant alternative lung diseases. All patients were in the stable state and exacerbation free for at least 6 weeks prior to review.

All validatory work was conducted on patient samples.

2.3 Pulmonary Function Testing

All patients had pulmonary function testing performed by appropriately qualified Respiratory Physiologists at Queen Elizabeth Hospital Birmingham. All testing was done in concordance with International Guidelines [211]. Percent of predicted lung function values [212] were chosen for all analysis of lung function parameters as these correct for age, sex and height.

2.4 Blood Samples

30ml of peripheral venous blood was taken from each patient using the BD Vacutainer system® (Becton, Dickinson and Company, Plymouth, UK). The first aliquot was allowed to clot and then serum was removed for routine testing in the hospital clinical laboratories. The second aliquot was collected into lithium heparin tubes to obtain plasma. This was prepared by centrifugation at 3000rpm for 10minutes at 4°C and then collection of the supernatant which was subsequently stored at -80°C.

2.5 Saliva Samples

Patients were asked to not eat, drink, smoke or brush teeth prior to sampling appointment. Sterile water was used by the patient to rinse their mouth, then after 10 minutes, a sterile marble was placed in the mouth to stimulate saliva production which was then expectorated into a sterile tube.

The saliva was stored on ice before centrifugation at 2,500 rpm for 10 minutes at 4°C to remove debris. The supernatant was snap frozen in liquid nitrogen and subsequently stored at -80°C until analysis.

2.6 Clinical Review

All patients attended the department on one occasion and underwent a clinical review by a trained respiratory physician to ascertain the following: Age, BMI, past medical history, medications and smoking history. Respiratory symptom burden was assessed using the modified Medical Research Council scale (mMRC scale) [213] which is the most commonly used validated scale to assess dyspnoea in daily living in chronic respiratory diseases [213]. Bronchitis was defined as self-reported cough and sputum production for at least 3 months of the year on two consecutive years and annual exacerbation rate (increase in symptoms usually requiring an increase in medication) was self-reported. Note was also made of medication use and a review of previous thoracic CT scans was undertaken to assess for radiological evidence of emphysema.

2.7 Dental Examination

A fully qualified dentist conducted a general oral examination and recorded the number of teeth present. Periodontal measurements were carried out on all teeth present using the UB-WHO-CF15 periodontal probe (Implantium.co.uk). For each tooth, 4 sets of periodontal measurements were taken; the pocket probing depth and clinical recession on the mesial

and distal aspects of the buccal and palatal/lingual surfaces. In order to standardise all of the measurements taken the same criteria were used in each examination. Measurements were taken as per standard dental examination procedures. The probing depth was measured to the nearest millimetre from the base of the periodontal pocket to the gingival margin. The recession was measured to the nearest millimetre from the cement-enamel junction (CEJ) to the gingival level [79]. f the gingival level was at the CEJ, the recession was recorded as 0mm. The total attachment loss was recorded as the sum of the probing depth and recession. A dichotomous record of bleeding on probing (BoP) was recorded as either present or absent for each site probed. These periodontal measurements allowed for determination of the presence of periodontitis as per the definitions outlined in Section 3.2.

2.8 Demographic Questionnaire & Assessment of Socio-economic Status

All patients were asked to complete a questionnaire at the time of dental assessment. This included questions on current residential postcode, age at leaving full time education and qualifications gained, current and where applicable, previous employment status, category of occupation and household income, see Appendix 1.

Socio-economic data was determined by calculating the English Index of Multiple Deprivation (IMD) score and the National Statistics Socio-economic Classification (NS-SEC) score. IMD score was determined by the self-reported residential postcode of each individual and comprises 7 elements of deprivation: Income Deprivation; Employment Deprivation; Health and Disability Deprivation; Education, Skills and Training Deprivation;

Barriers to Housing and Services; Crime Deprivation and Living Environment Deprivation [214]. NS-SEC defines socio-economic position by employment category [215] and was determined by the self-reported occupation of the patients.

2.9 Dental Questionnaire

All patients were asked to complete a questionnaire on dental habits including tooth brushing frequency, use of mouthwash and floss, date of last routine dental examination, history of previous dental disease and tooth extraction and changes in the appearance of teeth and gums. This is because a major risk factor for the development of periodontitis is having poor dental habits [109, 112], and thus it was important to be able to control for this as a potential confounder. See Appendix 1 for questionnaire.

2.10 Plasma hs-CRP ELISA

Plasma hs-CRP was measured by ELISA using a commercially available kit (Cambridge BioScience, Cambridge, UK) as per manufacturer's instructions. Patient plasma samples were diluted 1:1000 with the provided sample diluent. The prediluted standards were diluted further with sample diluent to achieve a 1:100 dilution. $100\mu L$ of each standard and patient sample were added to each well of the plate in duplicate. The wells were covered and incubated for 30minutes at room temperature. One litre of washing solution was prepared by diluting 50ml of concentrated washing solution in 1000ml of distilled water.

Each well was washed 3 times with 300μl of washing solution. 100μl of chromagen solution was added to each well before a further incubation of 10minutes in a light protected environment. The reaction was stopped by adding 50μl of stopping solution to each well and the absorbance was then read at 450nm using a microplate reader (Biotech Plate Reader, Synergy HT, Winooski, USA).

The hs-CRP concentration in the samples was calculated by interpolation from the standard curve.

The minimal detectable concentration was 0.02μg/ml as per the manufacturers product information leaflet.

2.11   Validation of CRP ELISA for saliva – Salimetrics and Abcam

A spike and recovery experiment to measure CRP in saliva was conducted on a commercially available ELISA kit (Abcam, Cambridge, UK). The manufacturers state the kit has been validated for measuring CRP in saliva. Two saliva samples from different individuals were used for the experiment. 60mls of saliva was added to 3 separate Eppendorfs and to each Eppendorf, 60mls of a chosen standard was added. The standards were prepared as per the manufacturers' protocol. 50ul of standards, neat saliva and spiked saliva samples were added to the wells in duplicate. The plate was covered and incubated for two hours. Washing buffer was produced by diluting 30mls of the supplied wash buffer with 570mls of

distilled water. Each well was washed five times with 300ul of washing buffer. 50ul of

prepared CRP antibody was added to each well and the plate incubated for a further

30minutes. Each well was again washed five times with 300ul of washing buffer. 50ul of

prepared SP conjugate was added to each well and the plate incubated for a further

30minutes. Again, each well was washed five times with 300ul of washing buffer. 50ul of

chromogen substrate was added to each well and incubated for 10minutes and the reaction

then stopped by adding 50ul of stop solution to each well. Absorbance was read at 450nm

and 570nm using a microplate reader (Biotech Plate Reader, Synergy HT, Winooski, USA).

The CRP concentration in the samples was calculated by interpolation from the standard

curve.   The percentage recovery of the spiked sampled was determined using:

 % recovery = (assay result for spiked sample/ (assay result for neat sample + amount

spiked)) x 100.

A further spike and recovery experiment to measure CRP in saliva was conducted on a

different commercially available ELISA kit (Salimetrics Europe, Newmarket, UK). The

manufacturers state the kit has been validated for measuring CRP in saliva. Two saliva

samples from different individuals were used for the experiment. Saliva samples were

spiked with different amounts of the CRP standard and diluted with variable amounts of

sample diluent to ensure the final saliva sample was diluted 1:10 as per manufacturers'

protocol, see Table 1

VOLUME OF SALIVA	VOLUME OF 6000PG/mL CRP STANDARD	VOLUME OF DILUENT
15uL	75uL	60uL
15uL	37.7uL	97.5uL
15uL	18.75uL	116.25uL
15uL	9.38uL	125.62uL
15uL	4.69uL	130.31uL
15uL	2.35uL	132.65uL
15uL	0	135uL.

Table 1: Volumes of saliva, standard and diluent used to create spiked samples for Salimetrics ELISA

The standard curve was prepared as per manufacturers' protocol. 50uL of standards and saliva samples were pipetted into wells in duplicate. 150uL of 1:250 diluted enzyme conjugate was added to each well. The plate was covered and incubated for 2hours on a rotatory shaker at 500rpm. Washing buffer was created by diluting 50mL of washing buffer to 450mL distilled water. Each well was washed four times with 300ul of washing buffer. 200ul of TMB substrate was added to each well and the plate incubated on the rotary shaker at 500rpm in a light protected environment for a further 30minutes. The reaction was stopped by adding 50ul of stop solution to each well and mixing on the rotary shaker for 3minutes.

Absorbance was read at 450nm and 630nm using a microplate reader (Biotech Plate Reader, Synergy HT, Winooski, USA).

The CRP concentration in the samples was calculated by interpolation from the standard curve. The percentage recovery of the spiked sampled was determined using:

% recovery = (assay result for spiked sample/ (assay result for neat sample + amount spiked)) x 100.

The Salimetrics kit was subsequently validated for linearity of dilution. The same technique was used as described above to prepare the standards and run the assay. The saliva samples were diluted to the concentrations in Table 2 and the same dilutions also spiked with a known quantity of the top CRP standard of 3000pg/ml also as shown in Table 2. The recovery was determined in the same manner as previously described.

OVERALL SALIVA DILUTION	VOLUME OF SALIVA (ul)	VOLUME OF 3000pg/ml CRP (ul)	VOLUME OF DILUENT (ul)
1:2	75	0	75
1:5	30	0	120
1:10	15	0	135
1:20	7.5	0	142.5
1:50	3	0	147
1:100	1.5	0	148.5
1:2 plus spike	75	75	0
1:5 plus spike	30	75	45
1:10 plus spike	15	75	60
1:20 plus spike	7.5	75	67.5
1:50 plus spike	3	75	72
1:100 plus spike	1.5	75	73.5

Table 2: Volumes of saliva, standard and diluent used in the Salimetrics linearity of dilution validation.

2.12   Plasma IL-8 ELISA

Plasma IL-8 was measured by ELISA using a commercially available kit (R&D Systems Ltd, Abingdon, UK) as per manufacturer's instructions. 100ul of assay diluent was added to each well. The standards were prepared as per manufacturer's instructions. 50ul of standards and plasma samples were added to the well and the plate was incubated for 2 hours. Wash buffer was produced by adding 20ml of concentrated wash buffer to 480ml of distilled

water. Each well was washed 4 times with 300ul of washing buffer. 100ul of IL-8 conjugate was added to each well and the plate incubated for a further 1 hour. Each well was again washed 4 times with 300ul of washing buffer. Colour reagents A and B were mixed together and 200ul of the mixed solution was added to each well and the plate incubated for 30moiniutes in a light protected environment. The reaction was stopped by adding 50ul of stop solution to each well.

The absorbance was then read at 450nm and 630nm using a microplate reader (Biotech Plate Reader, Synergy HT, Winooski, USA).

The IL-8 level in the samples was calculated by interpolation from the standard curve.

The mean minimal detectable concentration was 3.5pg/ml as per the manufacturers product information leaflet.

2.13   Validation of IL-8 ELISA in saliva

A spike and recovery experiment to measure IL-8 in saliva was conducted on a commercially available ELISA kit (R&D Systems Ltd, Abingdon, UK). This kit had not been validated for use in saliva previously.

Two saliva samples from different individuals were used for the experiment. After thawing the samples, they were ultra-centrifuged at 29000rpm for 20minutes at 4°C and supernatant removed. 75mls of saliva supernatant was then added to 7 separate Eppendorfs and to this 75mls of each standard was added and the mixture vortexed. This was replicated for the

second saliva sample. The assay was then run in the same manner as for plasma IL-8 per manufacturer's instructions.

The IL-8 in the samples was calculated by interpolation from the standard curve.   The percentage recovery of the spiked sampled was determined using:

 % recovery = (assay result for spiked sample/ (assay result for neat sample + amount spiked)) x 100.

The kit was subsequently validated for linearity of dilution. The same technique was used as described above to prepare the standards and run the assay. The saliva samples were diluted to 1:2, 1:10 and 1:50 and the same dilutions also spiked with a known quantity of IL-8 standard of 2000pg/ml as shown in Table 3. The recovery was determined in the same manner as previously described.

OVERALL SALIVA DILUTION	VOLUME OF SALIVA (ul)	VOLUME OF 3000pg/ml CRP (ul)	VOLUME OF DILUENT (ul)
1:2	75	0	75
1:10	15	0	135
1:50	3	0	147
1:2 plus spike	75	75	0
1:10 plus spike	15	75	60
1:50 plus spike	3	75	72

Table 3: Volumes of saliva, standard and diluent used in the IL-8 linearity of dilution validation in saliva.

2.14   Statistical Analysis

Statistical analyses were performed using the SPSS statistical program (version 24.0 IBM, Chicago, USA). Data were tested for normality using the Shapiro-Wilk test and then the appropriate statistical test was used to compare groups.

Parametric data was analysed using student's t-test and non-parametric data by Mann Whitney U Test. Chi square, Cochran's Q and Kendall's tau-b tests were used to assess for differences across groups and binary logistic regression models were used to calculate odds ratios (see results). Spearman's rank testing was used to assess correlations of non-parametric data. 2-tailed p values are quoted throughout the results and a p value <0.05 was used to indicate a statistically significant result.

CHAPTER 3

RESULTS

3 RESULTS: Patient Demographics & Prevalence of Periodontitis

3.1 Epidemiology

3.1.1 Introduction

Studies have suggested mechanistic associations between COPD and periodontitis (that of increased neutrophilic inflammation), but many COPD patients have known confounding factors for the development of periodontitis such as advancing age, smoking, poor dental habits and lower socioeconomic status. Here AATD provides a useful model of study since patients have often smoked less and are younger. Data regarding dental habits or socio-economic grouping are not usually available in many studies.

To test the hypothesis in section 1.81 and the aim in section 1.91, we describe the prevalence and severity of periodontitis in a cohort of patients with AATD and non-AATD COPD using several recognised definitions of periodontitis. We then compare the prevalence and severity of periodontitis to parameters of lung disease severity, both with and without correcting for potentially confounding risk factors for periodontitis.

3.1.2 Patients, Clinical Review and Questionnaires

This study included 88 dentate (presence of ≥1 natural tooth) non-deficient COPD patients recruited from the Chronic Disease Resource Centre (CDRC) and 68 dentate AATD patients recruited from the Antitrypsin Deficiency Assessment and Programme for Treatment (ADAPT) cohort at Queen Elizabeth Hospital Birmingham, between 2014 and 2016.

Clinical data gathering, questionnaire responses and dental assessments occurred as described in Chapter 2.

3.1.3 Statistical analysis

Data analysis was performed using SPSS computer package version 24.

All continuous data is presented as mean and standard deviation. Parametric data was analysed by student's paired t-test and non -parametric data by Mann Whitney U-test. Binary data is presented as numbers and percentage of the total. Categorical data is presented as median with range where appropriate. Categorical data is analysed by Chi square and Kendall's tau-b tests.

3.1.4 Results

The baseline demographics and socio-economic data for the non-deficient COPD and AATD groups are shown in Table 4.

Variable	Non-deficient COPD		AATD		p-value
	Total n included.		Total n included.		
Male n. (%)	88	62 (70.5%)	68	39 (57.4%)	0.09
Age years (SD)	88	68.6 (7.6)	68	54.6 (12.5)	**<0.001**
BMI (SD)	88	27.8 (6.2)	68	26.0 (5.1)	0.06
Current smoker n. (%)	88	21 (23.9%)	68	1 (1.5%)	**<0.001**
Ex-smoker n. (%)	88	67 (76.1%)	68	42 (61.8%)	0.052
Never smoker n. (%)	88	0 (0%)	68	25 (36.7%)	**<0.001**
Average smoking PYH of current and ex-smokers (SD)	88	56.0 (42.4)	43	19.9 (21.1)	**<0.001**
Diabetes n. (%)	88	9 (10.2%)	68	1 (1.5%)	**0.044**
Hypertension n. (%)	88	22 (25%)	68	9 (13.2%)	0.068
Vascular disease n. (%)	88	26 (29.5%)	68	1 (1.5%)	**<0.001**
Median IMD Quintile (range)	88	5 (3-5)	65	2 (1-4)	**<0.001**
Average age leaving education – years (SD)	72	15.7 (2.5)	54	18.6 (3.6)	**<0.001**
Median Highest Qualification Obtained (range)	70	1 (1)	55	2 (2-4)	**<0.001**
Median NS-SEC score (range)	69	5 (4-5)	52	2 (2-4)	**<0.001**
Median household income	54	2 (1-2)	48	3 (2-4)	**<0.001**

Table 4 4: Demographic and socio-economic data for non-deficient COPD and AATD groups.

Legend: Data are presented as mean with corresponding standard deviation or median and range

depending on the type of data. Significant results are in bold*. PYH= smoking pack year history,*

vascular disease = any history of peripheral vascular disease, ischaemic heart disease,

cerebrovascular disease, IMD=index of multiple deprivation; a score of deprivation based on postcode

[214]*, Highest Qualification Codes; 0=none, 1= GCSE, 3= A-level, 4=degree, 5=post-graduate*

qualification, NS-SEC = National Statistics Socio-economic Classification; the primary social

classification system used in UK based on occupation; 1=Higher management, 2= Lower managerial,

administrative and professional occupations, 3= Intermediate occupations, 4= Small employers and own account workers, 5= Lower supervisory and technical occupations, 6= Semi-routine occupations, 7= Routine occupations [215]. *Household Income Codes; 1=0-10k, 2=10-20k, 3=20-40k, 4=40-80k, 5=>80k.* Statistical tests used; Mann-Whitney U test for non-parametric data, Chi Square test for nominal categorical data and Kendall's tau b test for ordinal data.

The lung function and symptom burden results for the non-deficient COPD and AATD groups are shown in Table 5.

Variable	Non-deficient COPD		AATD		p-value
	Total n included.		Total n included.		
Median mMRC (range)	88	3 (2-4)	68	2 (2-4)	**<0.001**
Exacerbation Frequency (SD)	87	2.8 (3.0)	68	1.2 (1.2)	**<0.001**
Bronchitis n. (%)	87	37 (42.5)	68	24 (35.3)	0.360
Use of ICS n. (%)	87	74 (85.1)	68	36 (52.9)	**<0.001**
Use of LAMA n. (%)	88	73 (83.0)	68	40 (58.8)	**0.001**
Use of long acting β-2 agonist n. (%)	88	74 (84.1)	68	37 (54.4)	**<0.001**
Use of short acting β-2 agonist n. (%)	88	79 (89.8)	68	34 (50%)	**<0.001**
FEV_1: FVC ratio (SD)	86	46.7(15.9)	68	50.9(16.5)	0.096
% Predicted FEV_1 (SD)	86	54.1 (22.4)	68	70.8 (28.1)	**<0.001**
Median GOLD stage of patients with COPD (range)	76	3 (2-3)	57	2 (2-3)	0.113
% Predicted TLco (SD)	76	39.2 (26.2)	68	64.4 (19.8)	**<0.001**
% Predicted Kco (SD)	76	51.6 (34.4)	68	67.7 (18.4)	**0.014**
Radiological emphysema n. (%)	76	58 (76.3)	63	49 (77.8)	0.838

Table 5: Lung function and symptom burden for non-deficient COPD and AATD groups.

Legend: Data are presented as mean with corresponding standard deviation unless otherwise stated. Significant results are in bold. *mMRC = modified Medical Research Council scale ICS = inhaled corticosteroid, LAMA= long acting muscarinic antagonist.* Statistical tests used; Mann-Whitney U test for non-parametric data, Chi Square test for nominal categorical data and Kendall's tau b test for ordinal data.

The dental habits and symptoms results for the non-deficient COPD and AATD groups are shown in Table 6.

Variable	Non-deficient COPD		AATD		p-value
	Total n included.		Total n included.		
No. of teeth (SD)	85	17 (7.5)	68	24 (5.4)	**<0.001**
Twice daily tooth brushing n. (%)	75	35 (46.7)	65	47 (72.3)	**0.001**
Regular use of mouthwash n. (%)	75	38 (50.7)	65	20 (30.8)	**0.017**
Regular use of floss n. (%)	75	13 (17.3)	65	38.2 (40.0)	**0.003**
Last dental examination ≤6months n. (%)	75	48 (64.0)	65	41 (63.1)	0.172
Previous dental disease n. (%)	75	17 (22.7)	64	13 (20.3)	0.737
Appearance of loss of bone n. (%)	75	9 (12.2)	64	8 (12.5)	0.952
Wider spaces between teeth n (%)	75	30 (40.0)	65	13 (20)	**0.011**
Teeth appear longer n. (%)	75	29 (38.7)	63	34 (54.0)	0.072
Painful teeth/ gums n. (%)	75	11 (14.7)	64	18 (28.1)	0.052
Bleeding gums n. (%)	75	19 (25.3)	65	37 (56.9)	**<0.001**
Previous tooth extraction n. (%)	74	13 (17.6)	65	7 (10.8)	0.255

Table 6: Dental symptoms and habits for non-deficient COPD and AATD groups

Legend: Data are presented as mean with corresponding standard deviation unless otherwise stated. Significant differences are in bold. Statistical tests used; Mann-Whitney U test for non-parametric data, Chi Square test for nominal categorical data and Kendall's tau b test for ordinal data.

3.1.5 Summary of patient demographics

Non-deficient COPD patients were older, more likely to be current or ex-smokers, had a greater smoking pack year history, used more inhaled therapy, had more severe COPD as defined by FEV_1 and measures of gas transfer and a greater incidence of diabetes and vascular disease. The non-deficient group were also of a poorer socio-economic status, had lower income and were less well educated than the AATD patients in keeping with reported differences to a healthy population [86, 91, 92].

Non-deficient COPD patients had fewer teeth, reduced brush frequency and use of dental floss, although they reported use of mouthwash more than AATD patients. In keeping with this, several other studies have also shown COPD patients have significantly reduced brush frequency [83, 85, 87] compared to the general population. There was no significant difference in attendance at dental visits or history of previous dental disease or tooth extraction. However, non-deficient COPD patient were more likely to report that the space between their teeth appeared wider, whilst the AATD group reported more bleeding of gums on brushing. There were no significant differences in any of the other dental symptoms or behaviours on the questionnaire.

This in detail characterisation is essential to establish and account for any potential confounding factors for subsequent comparisons.

3.2 Prevalence of Periodontitis

Definitions of periodontitis were chosen to reflect periodontal measurements reported frequently in the current literature which can vary from ≥2mm for CAL and ≥3mm for PPD [81]. However low values may over represent disease [216] and therefore we chose thresholds beyond the normal ranges to exclude the risk of including healthy periodontium.

The following definitions were used;

1) Average whole mouth PPD ≥ 4mm,

2) Average whole mouth CAL ≥4mm

3) PD ≥4mm at any one tooth

4) CAL ≥ 4mm at any one tooth

5) The Centers for Disease Control and Prevention - American Association of Periodontology (CDC-AAP) criteria [97] as described in section 1.3.2.

6) The Basic Periodontal Examination criteria (BPE) [100] as described in section 1.3.2.

3.2.1   Results

The prevalence of periodontitis varied depending on the definition used. It ranged from 0.7% to 93.5% in the combined cohorts, 1.2 – 97.6% in non-deficient COPD and 0 – 88.2% in patients with AATD; see Table 7.

DEFINITION	WHOLE GROUP	COPD	AATD
Av PPD ≥4mm n. (%)	1 (0.7)	1 (1.2)	0
Av CAL ≥4mm n. (%)	32 (20.9)	29 (34.1) ***	3 (4.4)
Max PPD ≥4mm n. (%)	124(81.0)	72 (84.7)	52 (76.5)
Max CAL ≥4mm n. (%)	143 (93.5)	83 (97.6) *	60 (88.2)
CDC-AAP n. (%)	127 (83)	78 (91.8) **	49 (72.1)
BPE n. (%)	124 (81)	72 (84.7)	52 (76.5)

Table 7: Prevalence of periodontitis according to definition used.

Legend: Data is presented as number of patients affected and corresponding percentage of the group. Statistical tests used; Chi Square Test and Fishers Exact Test. Statistically significant differences between COPD and AATD are denoted by *** = p<0.001, ** = p<0.01 and * = p<0.05.

Cochran's Q test confirmed a statistically significant difference in the prevalence of periodontitis depending on the definition used. This was true for the whole group and the subgroups of non-deficient COPD and AATD patients, p<0.001 for all tests.

With the exception of average and maximum PPD ≥4mm and periodontitis by BPE criteria; non-deficient COPD patients had a significantly higher prevalence of periodontitis compared to those with AATD. For maximum CAL ≥4mm at any tooth, χ^2 = 5.5, p<0.05; average CAL ≥4mm, χ^2 = 20.2; p<0.001, and for CDC-AAP criteria, χ^2 = 10.4; p<0.01.

In comparison to the prevalence of pathological periodontal pockets in the 2009 ADHS (45%), both non-deficient COPD group and AATD group had increased prevalence (84.7 and 76.5% respectively). This was statistically significant for non-deficient COPD; χ^2 = 53.3, p<0.001 and for AATD χ^2 = 26.9, p<0.001.

A statistically significant difference existed between the groups when assessing periodontitis severity according to CDC-AAP criteria. 7% of non-deficient COPD patients had no periodontitis and 2.4%, 47% and 43% had mild, moderate and severe disease respectively. In AATD, 28% of patients had no periodontitis, 50% and 22% had moderate and severe disease respectively and they were more likely to have no or less evidence of severe periodontitis, (τ_b = 0.27, p<0.001) than patients with non-deficient COPD. A similar pattern was seen when periodontitis severity was scored using BPE criteria with AATD patients less likely to have a score of 4 on the BPE, (τ_b = -0.17, p<0.05).

To measure the association between periodontitis and COPD, binary logistic regression was used to determine the odds ratio (OR) for the non-deficient COPD group compared to AATD and this is summarised in Table 8. A result for average PD ≥4mm was not statistically possible as only 1 patient fulfilled this criterion. The results show three of the definitions had an increased OR for the non-deficient COPD group.

PERIODONTITIS DEFINITION	ODDS RATIO FOR NON-DEFICIENT COPD PATIENTS	95% CI	P-VALUE
Average CAL ≥4mm	11.2	3.2-38.3	**<0.001**
Maximum CAL ≥4m	5.5	1.1-27.0	**<0.05**
Maximum PPD ≥4mm	1.7	0.8-3.8-	NS
CDC-AAP criteria	4.3	1.6-11.0	**<0.01**
BPE criteria	1.7	0.8-3.9	NS

Table 8: OR for periodontitis in non-deficient COPD patients compared to AATD.

Legend: Statistical tests used; Binary logistic regression was used to assess the OR for each binary outcome measure (periodontitis definition) individually. Significant results are shown in bold.

This univariate analysis supports an increased association between non-deficient COPD and periodontitis when compared to AATD patients for some of the periodontitis definitions. However, as there was a significant difference in age and smoking history between the COPD and AATD groups, and as these variables are also associated with periodontitis, they were included in a further regression model. After adjusting for these variables, only average CAL ≥4mm remained significantly associated with the non-deficient COPD group but with reduced OR of 7.9, 95% CI 1.8-35.5; p<0.01 (Table 9). For this reason, average CAL ≥4mm was chosen for the later comparisons as this was the only periodontal measurement that remained a significant predictor for non-deficient COPD and AATD *after* correcting for risk factors common to both periodontitis and chronic lung disease. The associations with the other periodontal measurements such as PPD could be attributed to confounding variables such as age and smoking, whereas the regression models show these confounders did not fully explain the association with Average CAL ≥4mm and lung disease. Therefore, it was felt this was more likely to be representative of a genuine predictor of periodontitis in the COPD and AATD rather than a reflection of shared risk factors.

PERIODONTITIS DEFINITION	ODDS RATIO FOR NON-DEFICIENT COPD PATIENTS	95% CI	P-VALUE
Average CAL >4mm Adjusting for age & PYH	**7.9**	**1.8-35.4**	**0.007**
Maximum CAL >4m Adjusting for age & PYH	0.9	0.1-8.7	NS
CDC-AAP criteria Adjusting for PYH	1.3	0.5-3.4	NS

Table 9: Adjusted OR for periodontitis in the non-deficient COPD patient group in comparison to AATD group.

Legend: Statistical tests used: Binary logistic regression was used to assess the OR for each binary outcome measure (periodontitis definition) individually after adjustment for confounders. Significant results are shown in bold.

Previous univariate analyses confirmed other variables as important risk factors for non-deficient COPD. These included IMD score, age when leaving education, highest educational qualification, NS-SEC score, presence of vascular disease, use of inhaled corticosteroids, SABA, LABA and LAMA and brushing teeth less than twice daily. Limited sample size prevented statistical correction for all potential confounders in a standard regression model so additional binary logistic regression models were used to assess the contribution of these variables to the prevalence of periodontitis in the non-deficient COPD compared to AATD group defined by average CAL ≥4mm. As four of these variables (IMD score, age when leaving education, highest qualification gained, NS-SEC score) are also surrogate markers for socio-economic status, IMD score alone was used for the model.

VARIABLES ADDED TO MODEL	ODDS RATIO FOR NON-DEFICIENT COPD PATIENTS	95% CI	P-VALUE
IMD Quintile Vascular Disease	7.5	1.49-38.1	**0.015**
IMD Quintile ICS Use	8.0	1.70-37.8	**0.009**
IMD Quintile Tooth Brushing	11.2	1.8-70.2	**0.01**
IMD Quintile SABA	9.8	2.1-47.2	**0.004**
IMD Quintile LABA	7.8	1.6-37.4	**0.010**
IMD Quintile LAMA	8.6	1.8-40.8	**0.007**
Vascular Disease ICS Use	7.5	2.0-27.6	**0.003**
Vascular Disease Tooth Brushing	7.4	2.0-28.0	**0.003**
Vascular Disease SABA	10.8	2.6-44.4	**0.001**
Vascular Disease LABA	11.0	2.8-43.0	**0.001**
Vascular Disease LAMA	12.2	3.1-48.2	**<0.001**
ICS Use Tooth Brushing	7.3	2.0-26.6	**0.002**
ICS Use SABA	10.8	2.7-42.9	**0.001**
ICS Use LABA	9.2	2.6-33.1	**0.001**
ICS Use LAMA	9.0	2.5-32.3	**0.001**
SABA LAMA	12.2	3.1-48.2	**<0.001**
SABA LABA	11.0	2.8-43.0	**0.001**
LAMA LABA	9.3	2.9-32.9	**0.001**

Table 10: Adjusted OR for patients with non-deficient COPD having periodontitis compared to AATD

Legend: *IMD=index of multiple deprivation; a score of deprivation based on postcode [214], ICS = inhaled corticosteroid use, SABA= short acting β-2 agonist, LABA= long acting β-2 agonist, LAMA= long acting muscarinic antagonist.* Statistical tests used; Binary logistic regression. Significant results are shown in bold.

Table 10 shows the adjusted OR when these variables are added to the model. This confirmed that poorer socio-economic status, use of inhaled therapies, presence of vascular disease and reduced tooth brushing frequency in non-deficient COPD all contribute to, but do not fully explain the increased prevalence of periodontitis in non-deficient COPD compared to AATD.

3.2.2 Summary of periodontal prevalence and association with AATD and non-AATD COPD

Prevalence ranged from 1.2-97.6% for non-deficient COPD and 0-88.2% for AATD patients. The prevalence for the general population in the UK (adults over the age of 16years) has been reported as 45% using PPD ≥4mm [101], although this increases to more than 70% when the age group is greater than 55years. Thus, both the non-deficient COPD group and the AATD group had a significantly higher prevalence than seen in the general UK population (84.7% and 76.5 % versus 45%) when applying the same threshold for diagnosis.

For most definitions, there was greater prevalence of periodontitis in non-deficient COPD compared to AATD patients (p<0.001). After correction for age and smoking, only average CAL ≥4mm still had a greater prevalence in the non-deficient COPD group. The non-deficient COPD patients were of a poorer socio-economic status, had lower income and were less well educated than AATD patients. Regression analyses confirmed these variables contributed to the association between periodontitis in non-deficient COPD compared to AATD in this analysis. Other common risk factors such as age, smoking history, socioeconomic status, vascular disease, use of inhaled therapy and poor dental habits were

also associated with periodontitis but did not fully account for the increased prevalence seen in the non-deficient COPD group compared to AATD.

3.3 Periodontal Measurements and Respiratory Physiology

No correlation of periodontal indices with parameters of lung function was seen in the non-deficient COPD group, although there were significant negative correlations for AATD patients. The results are shown in Table 11. Results for FEV1: FVC ratio and average CAL (mm) for both groups are shown in Figures 2 and 3 and TLco % predicted and AATD in Figure 4.

	AATD		COPD	
VARIABLE	r_s	p-value	r_s	p-value
Average CAL (mm)				
FEV$_1$: FVC ratio	**-0.331**	**0.006**	**-0.411**	**<0.001**
FEV$_1$ % predicted	**-0.460**	**<0.001**	0.039	0.724
TLco % predicted	**-0.361**	**0.002**	0.021	0.861
Kco % predicted	-0.235	0.056	-0.121	0.304
Maximum CAL (mm)				
FEV$_1$: FVC ratio	**-0.149**	**0.225**	**-0.305**	**0.005**
FEV$_1$ % predicted	**-0.390**	**0.001**	0.062	0.577
TLco % predicted	**-0.414**	**<0.001**	0.078	0.512
Kco % predicted	**-0.329**	**0.007**	-0.043	0.716
Average PPD (mm)				
FEV$_1$: FVC ratio	**-0.171**	**0.162**	-0.181	0.102
FEV$_1$ % predicted	**-0.282**	**0.02**	0.003	0.975
TLco % predicted	-0.166	0.177	-0.029	0.809
Kco % predicted	-0.045	0.72	-0.11	0.35
Maximum PPD (mm)				
FEV$_1$: FVC ratio	**-0.109**	**0.376**	-0.226	0.040
FEV$_1$ % predicted	**-0.326**	**0.007**	0.085	0.444
TLco % predicted	**-0.272**	**0.025**	0.103	0.381
Kco % predicted	-0.236	0.054	0.034	0.774

Table 11: Spearman's rank correlations for periodontal indices and lung function parameters. Significant results are displayed in bold.
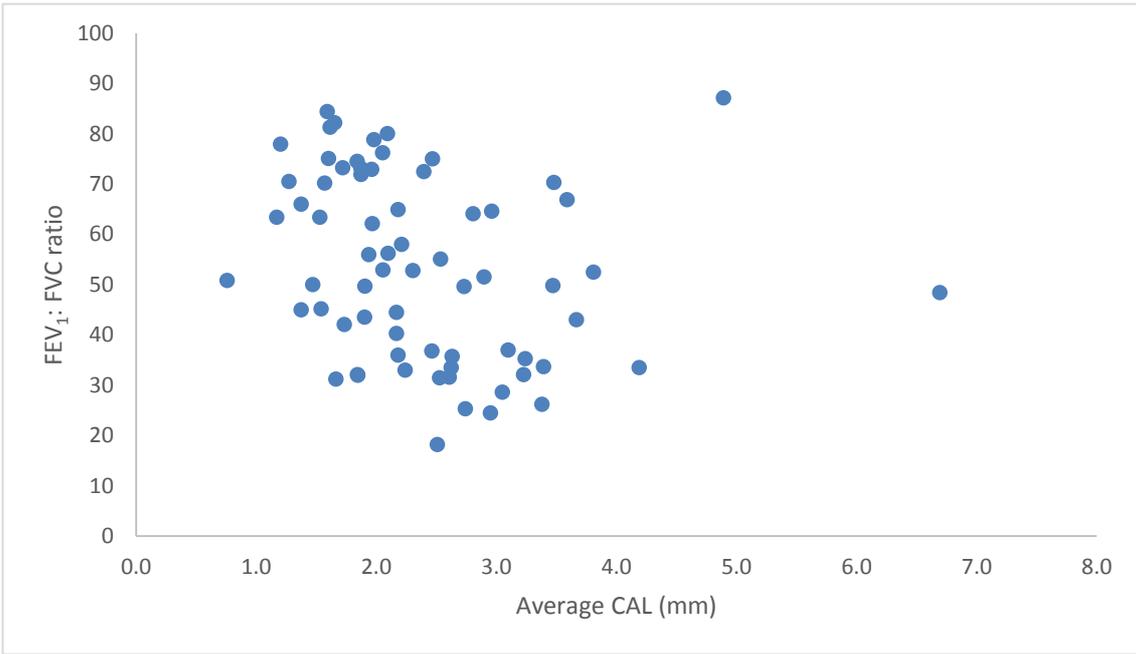
Figure 2: Correlation between Average CAL (mm) and Percent of predicted FEV$_1$ for AATD patients

Legend: 68 patients, each data mark represents one patient. Spearman's Rho shows moderate correlation; r$_s$ -0.331, p<0.01.
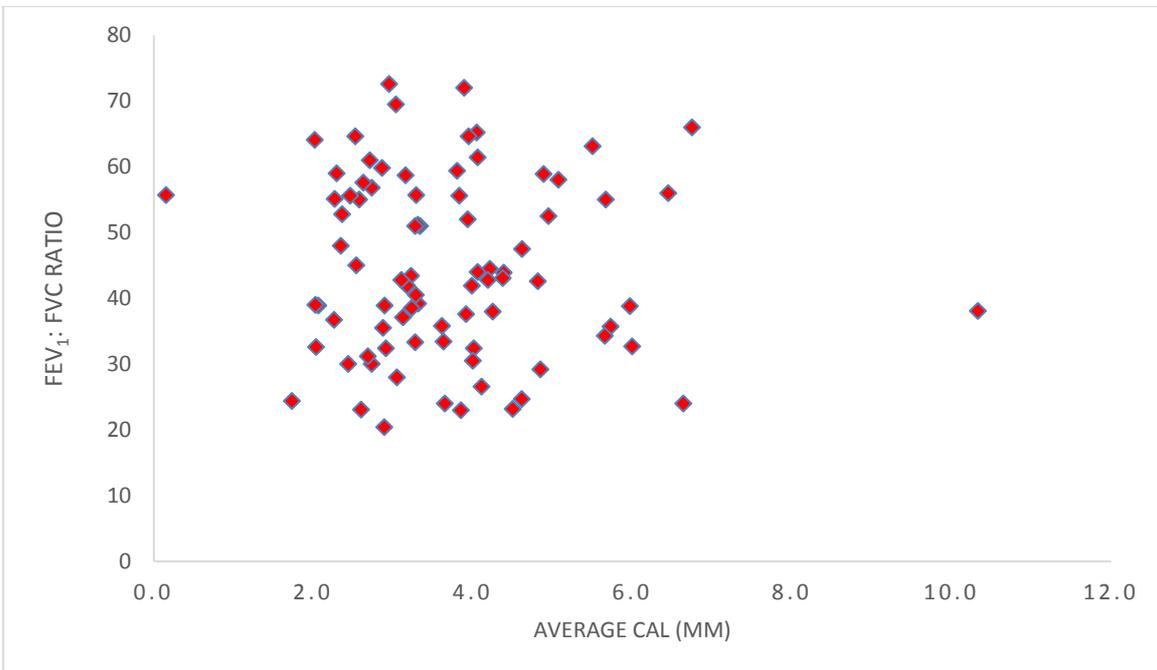
Figure 3: Correlation between Average CAL (mm) and Percent of predicted FEV$_1$ for non-deficient COPD patients

Legend: 83 patients, each data mark represents one patient. Spearman's Rho shows moderate

correlation; r_s -0.411, p<0.001
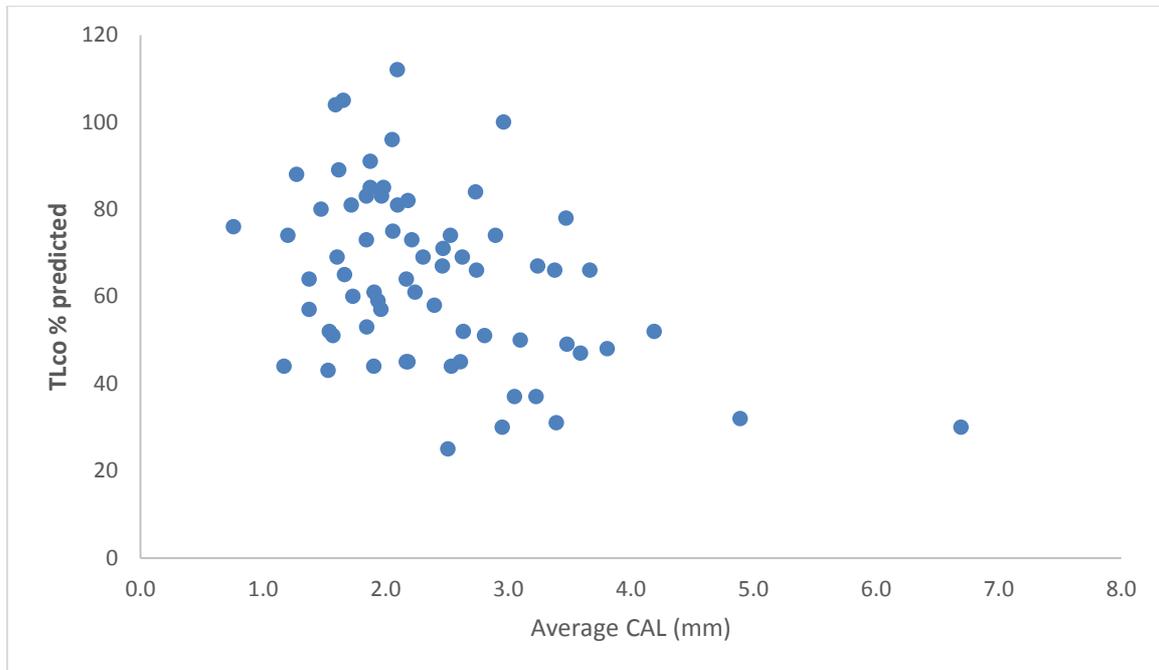
Figure 4: Correlation between Average CAL (mm) and Percent of TL$_{CO}$ for AATD patients.

Legend: 68 patients, each data mark represents one patient. Spearman's Rho shows moderate

correlation; r_s -0.361, p<0.01.

3.3.1   Summary of lung physiology

Moderate correlations were seen for measures of airway obstruction in both non-deficient

COPD and AATD patients and clinical attachment level. Weak to moderate correlations were

seen in the AATD patients for measurements of gas transfer and some periodontal indices.

The reason for seeing this relationship in AATD but not in non-deficient COPD is unclear but

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could relate to the enhanced inflammatory processes seen in AATD. This will be explored in the next chapter.

3.4 VALIDATION OF ELISA KITS FOR USE IN SALIVA

3.4.1   Introduction

It is the policy of this research unit to perform in house validation of any ELISA kits not used previously within this department. Spike and recovery validation was performed for two commercially available ELISA kits which manufacturers claim are valid for the measurement of CRP in saliva, Abcam (Cambridge, UK) and Salimetrics (Europe, Newmarket, UK) and for an ELISA kit designed to measure plasma/ serum levels of IL-8 for use in saliva, R&D Systems, (Abingdon, UK).

3.4.2   Saliva Sampling and Methods

These have been described in section 2.5.

3.4.3   Results

3.4.3.1   Abcam

A standard curve was generated for the Abcam kit (reference number ab108826) as shown in Figure 5.
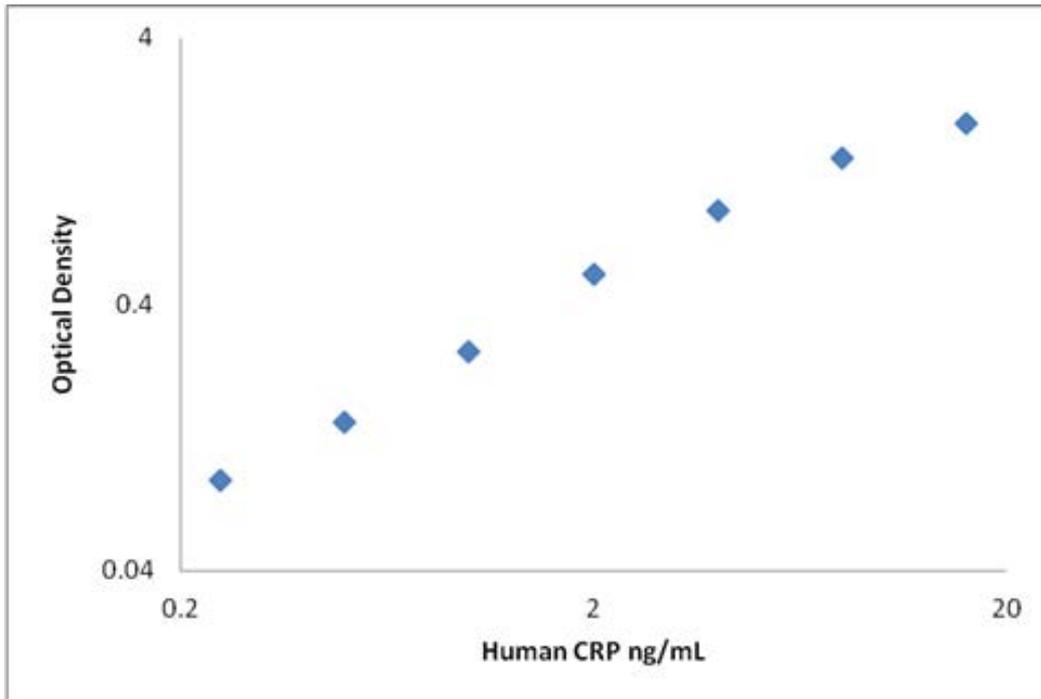
Figure 5: Standard Curve obtained from Abcam CRP ELISA kit ab108826 following manufacturer instructions

Saliva was collected from two non-smoking individuals at Queen Elizabeth Hospital Birmingham, with ethical approval from the National Research Ethics Service (REC13/WM/0044) as part of a larger study investigating inflammation in periodontitis and chronic lung disease. Participant One had psoriasis, which has been associated with higher concentrations of salivary CRP and Participant Two was a sex and age matched healthy control. These subjects were chosen for initial validation as they were thought to be likely to produce samples with higher and lower concentrations of CRP respectively, thus reflecting the ranges described in health and disease.

Three spiking concentrations were chosen, 0.25ng/ml, 2 ng/ml and 16ng/ml, as these represented the lowest, middle and highest standard for the assay.

Participant One's saliva sample had a reported mean concentration of CRP of 6.03ng/ml, which fell within the standard curve, and the CV of duplicate measurements was 4%. Participant Two's saliva sample had a reported mean concentration of CRP of 0.07ng/ml, below the lower limit of detection quoted for the assay and at these low concentrations, the CV of duplicate measurements obtained by interpolation from the standard curve was 30%. For Participant One, recovery of the spike ranged from 59-144%, with the most inaccurate recovery seen at the lower and higher concentrations of spike. For participant two, the spiked recovery was 5-28% and a value was not obtained for the neat plus 0.25ng/ml spike as the optical density reading for this sample was less than the optical density obtained for zero on the standard curve. See Figure 5.

As spike and recovery did not fall within acceptable criteria, no further attempts were made to use this kit in saliva.
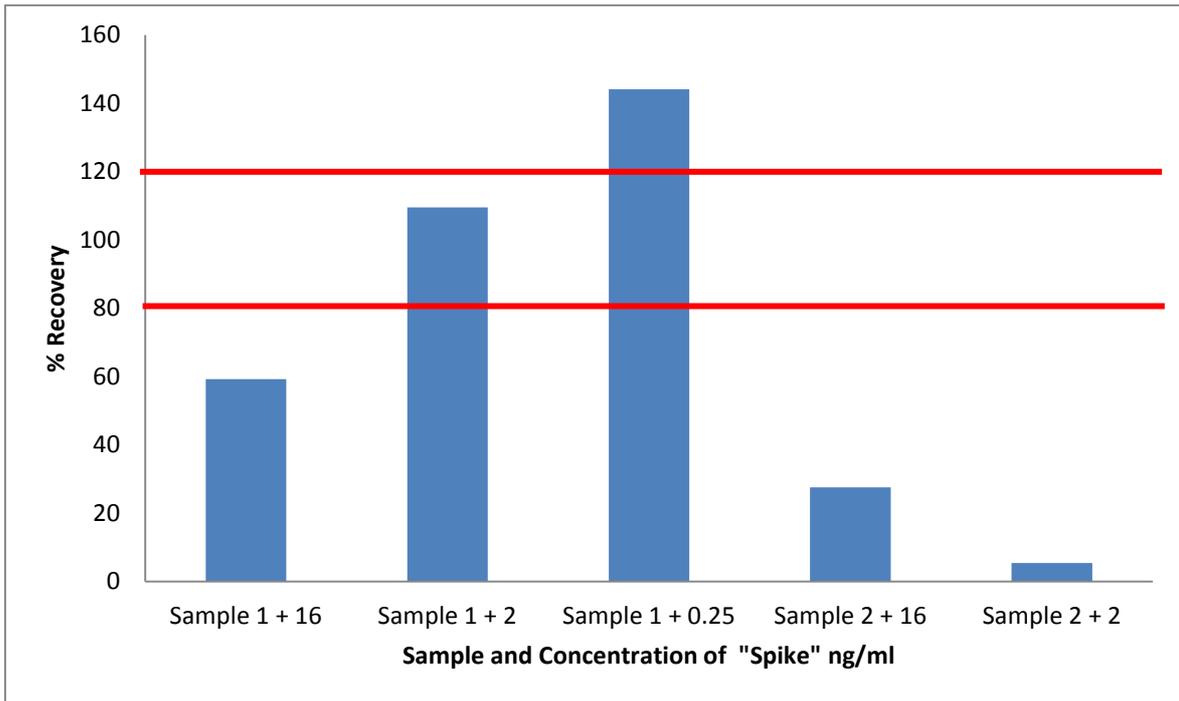
Figure 5: Graphical representation of the results of the spike and recovery experiment for Abcam CRP ELISA kit.

Legend: Horizontal lines indicate percentage of recovery considered acceptable for the kit to be valid (80-120%). Sample 1+16 = saliva sample 1 spiked with a known quantity of the 16ng/ml standard provided in the ELISA kit. Sample 1+2 = saliva sample 1 spiked with a known quantity of the 16ng/ml standard provided in the ELISA kit. Sample 1+0.25 saliva sample 1 spiked with a known quantity of the 16ng/ml standard provided in the ELISA kit. Sample 2 = saliva sample 2, spiked in the same way as sample 1. A result was not possible for Sample 2 +0.25.

Abcam subsequently confirmed that the kit had not been validated for use in saliva by the manufacturers and thus no further validation attempts were made in house.

3.4.3.2 Salimetrics

The same samples were used to validate the Salimetrics kit for use in saliva. Again, a suitable standard curve was generated (see figure 6). Using these samples, Participant 1 had mean CRP concentrations of 2384.6 pg/ml with an intra-measurement CV of 4.35% and Participant 2 had a mean CRP concentration of 295.77 pg/ml with an intra-measurement CV of 3.54%. Spiking was performed for all standards and recovery fell into recommended acceptability criteria, with the percentage of recovery obtained being between 80-111%, shown in Figure 7.
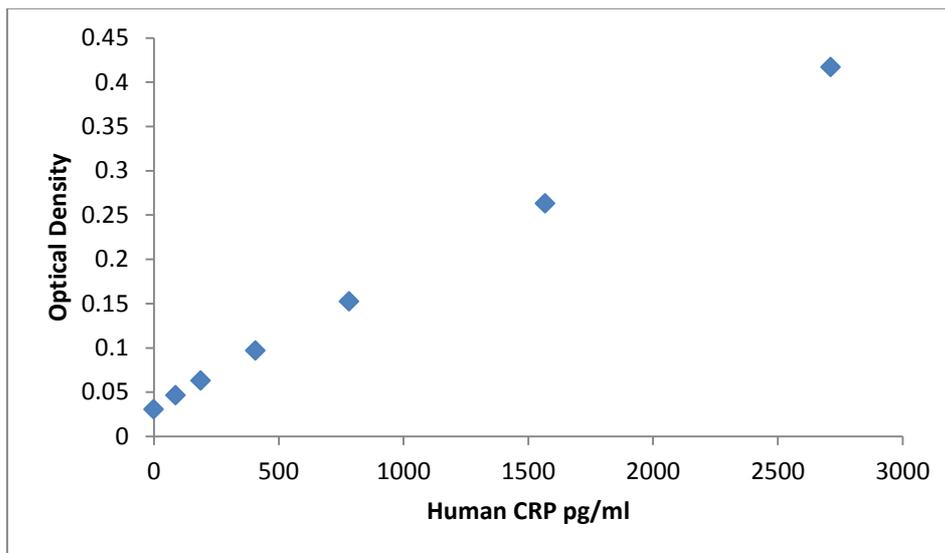
Figure 6: Standard Curve obtained from Salimetric CRP ELISA kit following manufacturer instructions
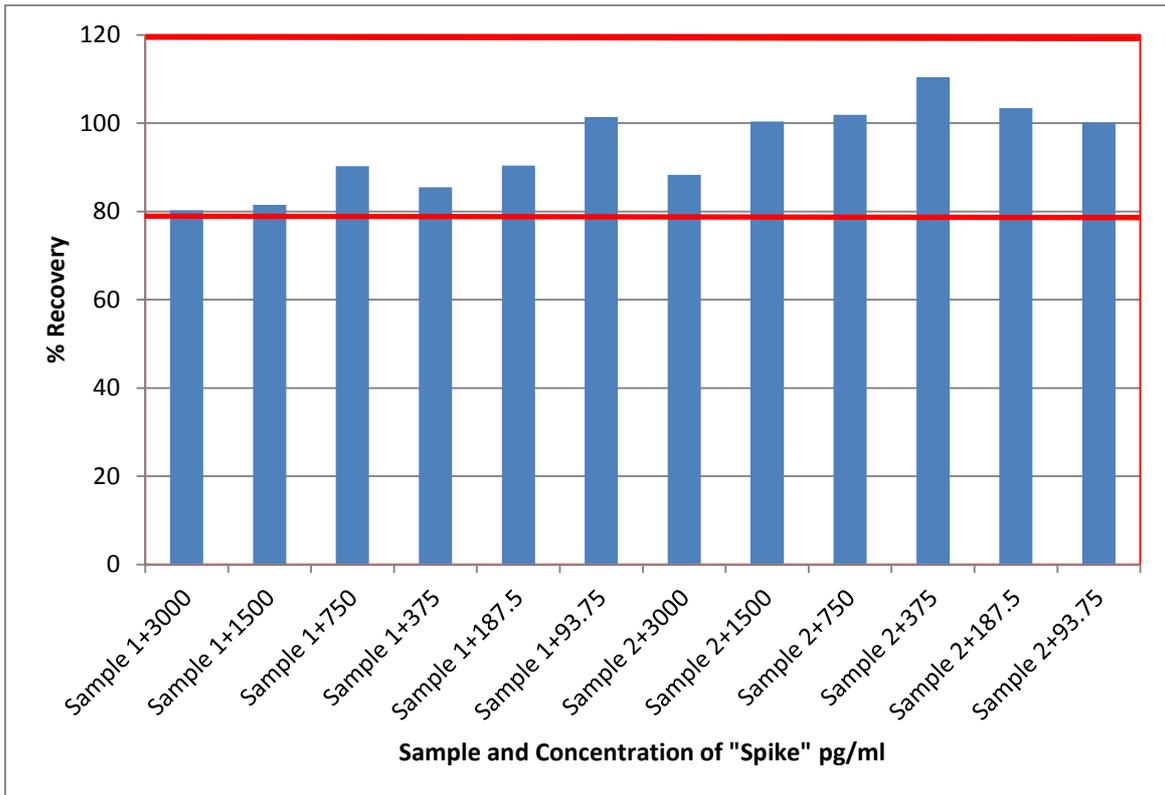
Figure 7: Graphical representation of the results of the Spike and Recovery validation experiment for Salimetrics CRP ELISA

Legend: Horizontal lines indicate percentage of recovery considered acceptable for the kit to be valid (80-120%). Sample 1+3000 = saliva sample 1 spiked with a known quantity of the 3000pg/ml standard provided in the ELISA kit. Sample 1+1500 = saliva sample 1 spiked with a known quantity of the 1500pg/ml standard provided in the ELISA kit and so forth. Sample 2 = saliva sample 2, spiked in the same way as sample 1.

A linearity of dilution experiment was also performed which confirmed the kit produced valid results at dilutions of 1:2, 1:5, 1:10, 1:20, 1:50, 1:100 with recovery of 92-99%, shown in Figure 8.
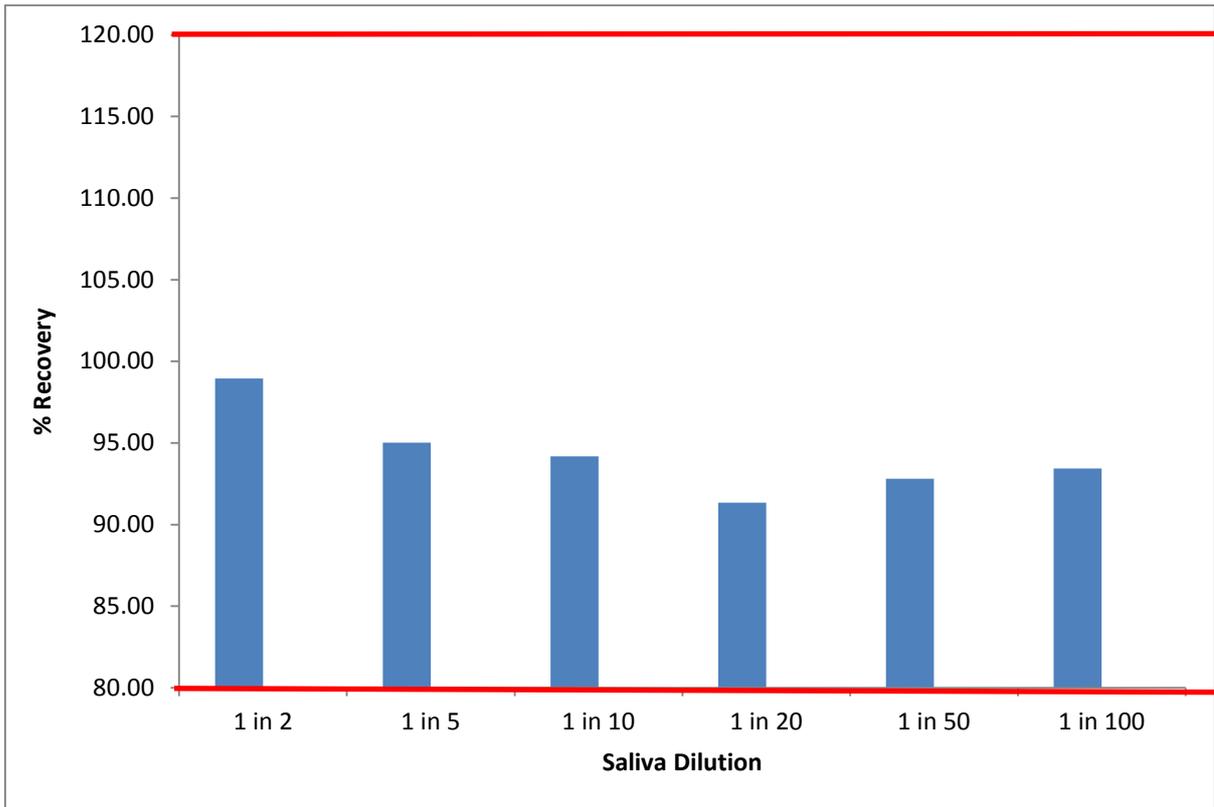
Figure 8: Graphical representation of the results of the linearity of dilution validation experiment for Salimetrics CRP ELISA.

Legend: Horizontal lines indicate percentage of recovery considered acceptable for the kit to be valid (80-120%). Inter-plate assay variation on patient samples was 12.8%.

3.4.3.3   IL-8

We successfully validated the R&D kit for use in measurement of IL-8 in saliva.

A suitable standard curve was generated (see figure 9). The test sample had median IL-8 level of 1801.44 pg/ml with an intra-measurement CV of 1.80%. Spike and recovery was performed for this sample and recovery fell into recommended acceptability criteria between 103-124%, shown in Figure 10.
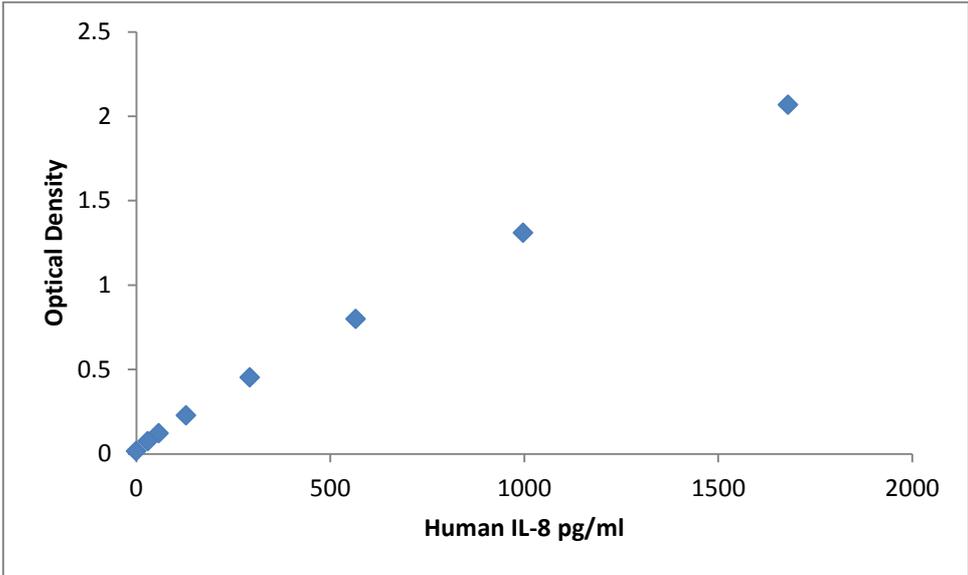
Figure 9: Standard Curve obtained from R&D IL-8 ELISA kit following manufacturer instructions
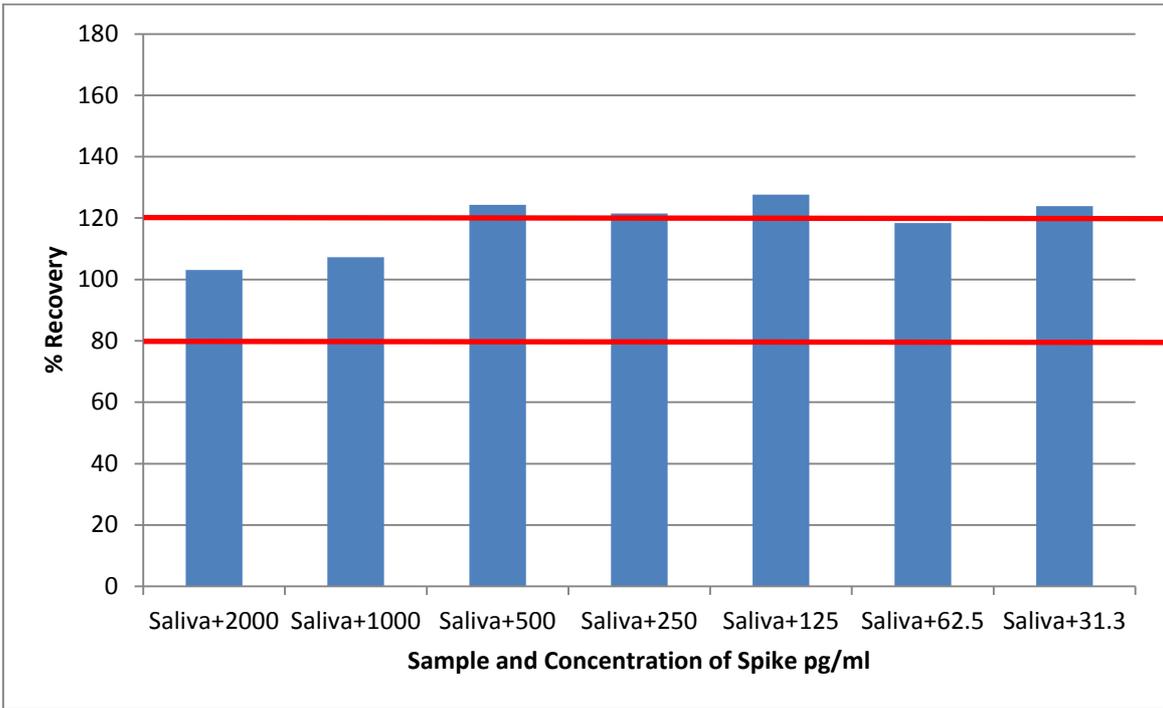

Figure 10: Graphical representation of the results of the Spike and Recovery validation experiment for R&D IL-8 ELISA

Legend: Horizontal lines indicate percentage of recovery considered acceptable for the kit to be valid (80-120%). Sample +2000 = saliva sample spiked with a known quantity of the 2000pg/ml standard

provided in the ELISA kit. Sample +1000 = saliva sample spiked with a known quantity of the

1000pg/ml standard provided in the ELISA kit and so forth.

A linearity of dilution experiment was also performed which confirmed the kit produced

valid results at dilutions of 1:2 and 1:10 with recovery of 85-118%, shown in Figure 11. At

saliva dilution of 1:50 the recovery was less than 80% for one sample and thus concluded not

validated at this dilution factor.
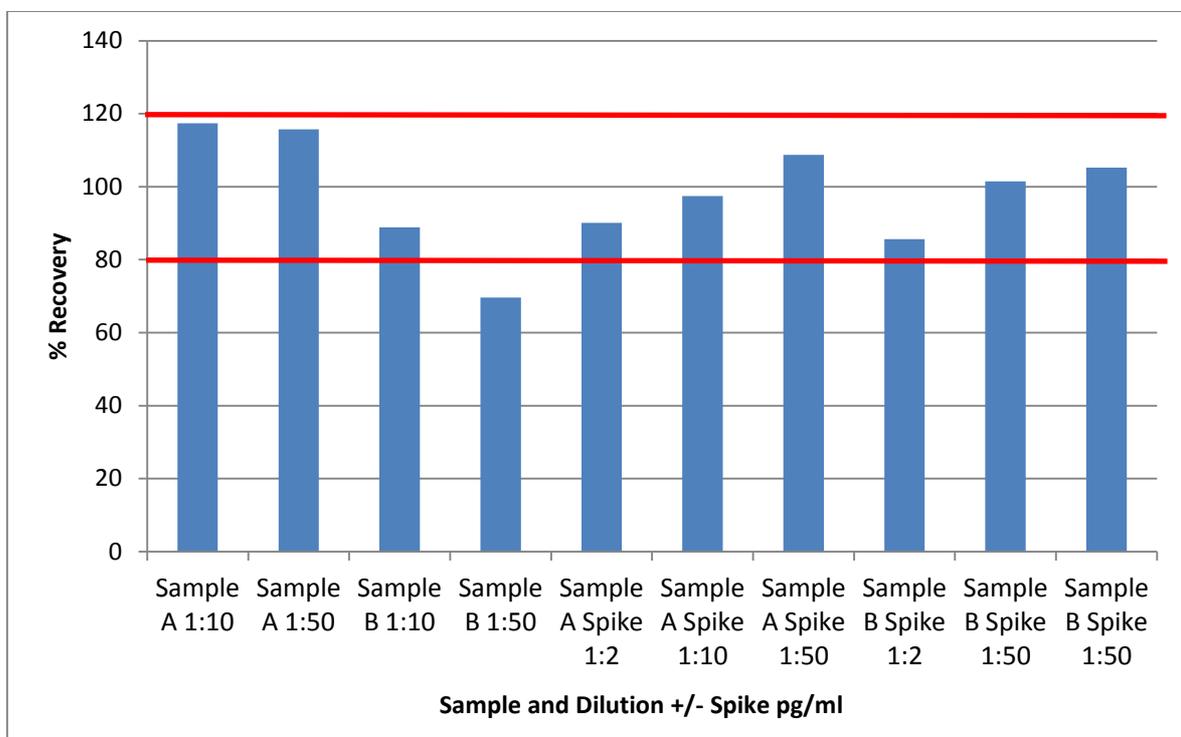
Figure 11: Graphical representation of the results of the linearity of dilution validation experiment for R&D IL-8 ELISA

Legend: Horizontal lines indicate percentage of recovery considered acceptable for the kit to be valid (80-120%). Sample A 1:10 = saliva sample A diluted 1:10 and so forth. Sample A Spike 1:2 = saliva sample A diluted 1:10 and spiked with a known quantity of the 2000pg/ml standard provided in the ELISA kit and so forth.

Inter-plate assay variation on patient samples was 18.2%.

3.4.4   Summary

Following the validation experiments, the Salimetrics CRP kit was used to measure CRP in saliva and the R&D kit for IL-8 in saliva. All saliva samples were initially run "neat" and those that required dilution as the initial assay result was off the top of the curve were re-run at a dilution of 1:10 for both CRP and IL-8.

3.5 MARKERS OF INFLAMMATION

3.5.1   Introduction

To examine inflammatory associations between respiratory disease and periodontitis we analysed the concentrations of CRP and IL-8 both in the peripheral circulation and in saliva.

3.5.2   Patients

The same patients whose data was used in the previous results chapters were used for this analysis and their basic demographics were similar to that of the whole cohort detailed earlier. Details are provided in section 2.2. Plasma hs-CRP results were obtained in 46 dentate COPD patients and 40 dentate AATD patients. Plasma IL-8 results were obtained in

47 dentate COPD patients and 42 dentate AATD patients. Salivary samples were obtained in 84 dentate COPD patients and 66 dentate AATD patients. Data is not available for the whole cohort due to a lack of obtaining blood samples at the same time as dental sampling, and in some cases, dental samples were insufficient for testing. However, this smaller sub-group does not significantly differ in the characteristics already outlined for the main group.

3.5.3 Blood and Saliva Sampling

This is described in sections 2.4 and 2.5

3.5.4 ELISA analysis

This is described in section 2.6

3.5.5 Statistical Analysis

Non-parametric data was analysed by Mann Whitney U tests and Spearman's rank correlation and, parametric data by student's t-test and Pearson's correlation coefficient.

3.5.6 Results

Plasma hs-CRP was above the lower limit of detection for all samples tested. The mean (SD) hs-CRP level in COPD patients was higher (5.81(3.55) ug/ml) than in AATD patients (1.99 (3.02) ug/ml) $p < 0.001$.

Plasma IL-8 was above the lower limit of detection for 76.4% of samples tested, but was more often detectable in COPD patients, n=44 (93.6%) than AATD n=24 (57.1%), p<0.001. The mean IL-8 levels in detectable samples were similar in COPD patients; 5.08 (4.10) pg/ml and in AATD patients; 4.59 (4.46) pg/ml, p=ns, however, the reported lower level of quantification for this assay is 31.3 pg/ml and therefore further analysis of plasma IL-8 results could not be performed as all results fell below this level.

Using the validated kit, salivary CRP was detectable in 100% of samples tested. 18 samples initially produced assay results which were off the top of the standard curve for the ELISA kit. These samples were subsequently diluted 1:100 however 2 samples were still above the top of the curve and were excluded from analysis. 5 diluted samples produced assay results with a CV >20% and were also excluded from analysis. Thus, salivary CRP results were analysed for 81 COPD and 62 AATD patients.

The mean (SD) salivary CRP in COPD patients was higher; 1240.43 (3368.57) pg/ml than in AATD patients; 703.93 (1549.76) pg/ml, p<0.05.

A significant positive relationship between plasma hs-CRP and salivary CRP was seen; Spearman's Rho 0.391, p<0.001, (see Figure 12); but when the groups were analysed separately, the relationship persisted for AATD only; Spearman's Rho = 0.378, p<0.05.
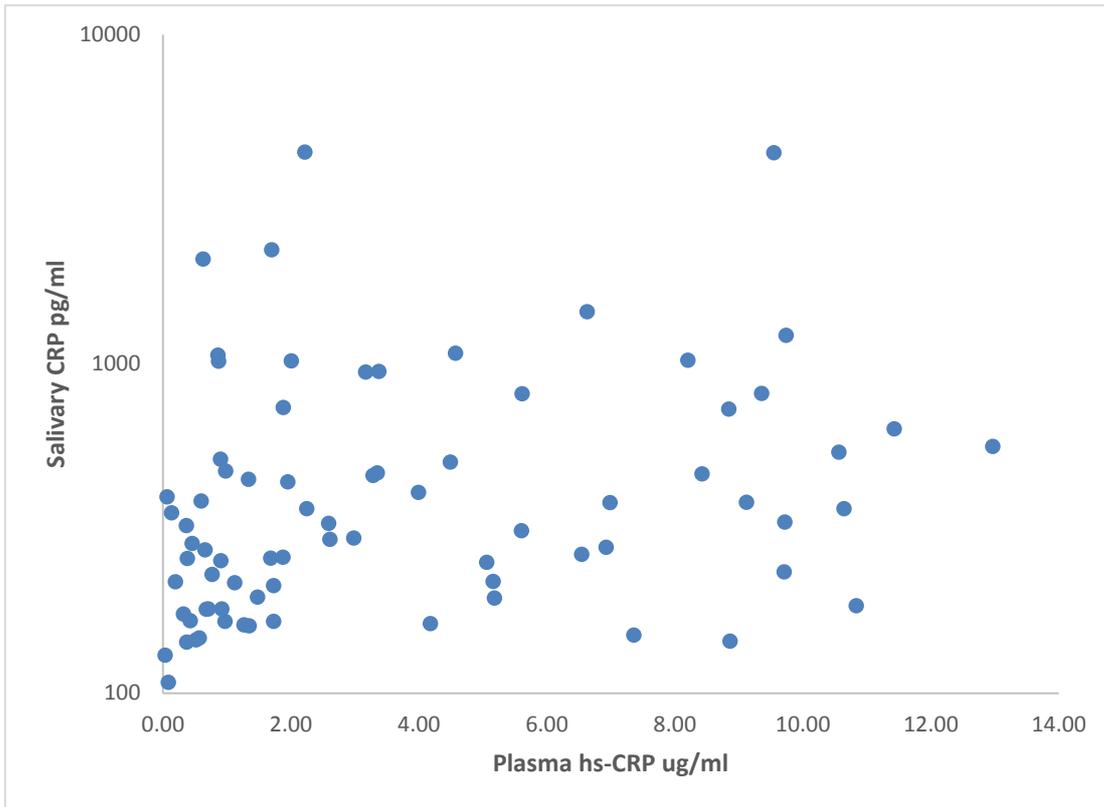
Figure 12: Association between salivary CRP and plasma hs-CRP for all patients

Legend: 78 patients, each data mark represents one patient. Spearman's Rho shows moderate correlation; r_s 0.391, p<0.01.

Using the validated kit, salivary IL-8 was detectable in 100% of samples tested. 2 samples are not included in further analysis as 1 fell below the bottom of the standard curve and the other was off the top of the standard curve despite dilution 1:10. Thus, salivary IL-8 results were analysed for 82 COPD and 66 AATD patients.

The mean (SD) salivary IL-8 in COPD patients and AATD was similar; 1821.55 (3170.48) pg/ml versus 1746.24 (2801.83) pg/ml, respectively, p=ns.

Moderately positive correlations were seen between salivary CRP and salivary IL-8 for COPD

patients, r_s 0.367, p=0.001 and for AATD patients, r_s 0.391, p=0.001, see Figure 13.
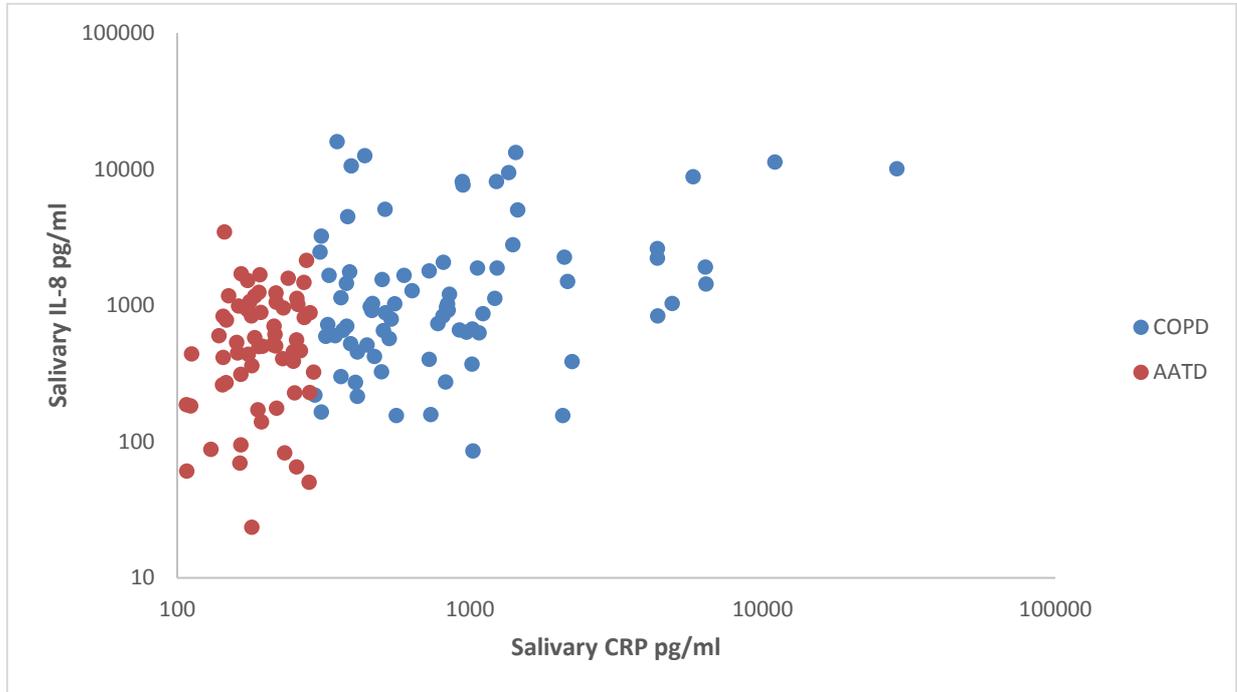
Figure 13: Association between salivary CRP and salivary IL-8

Legend: 79 COPD patients and 62 AATD patients. Each data mark represents one patient.

Spearman's Rho shows moderate correlation; r_s 0.367, p=0.001 for COPD and r_s 0.391, p=0.001 for

AATD patients.

3.5.6.1   Demographics and Socioeconomic Status

No significant associations were seen for plasma hs-CRP, salivary CRP or salivary IL-8 and

gender, age, smoking status or pack year history, co-morbid presence of diabetes mellitus,

hypertension or vascular disease, age at leaving education, NS-SEC score or annual

household income.

Plasma hs-CRP was significantly positively correlated with BMI in AATD patients; r_s 0.331, p=0.037, and IMD quintile in AATD patients, r_s 0.347, p=0.035, but neither was associated with COPD patients.

A weakly positive correlation was seen in the COPD group for salivary IL-8 and highest qualification gained; r_s 0.271, p=0.029 but not for AATD and no relationship was seen for salivary CRP.

3.5.6.2 Respiratory Disease & Lung Function

No significant associations were seen for plasma hs-CRP, salivary CRP or salivary IL-8 and GOLD stage of COPD severity, exacerbation frequency, presence of bronchitis, radiological evidence of emphysema, use of inhaled corticosteroid, SABA, LABA, LAMA or any lung function parameter.

Salivary CRP was positively correlated with mMRC score for COPD patients, r_s 0.328, p<0.01, and AATD patients, r_s 0.308, p<0.05, see Figure 14, but no association was seen for plasma hs-CRP or salivary IL-8.
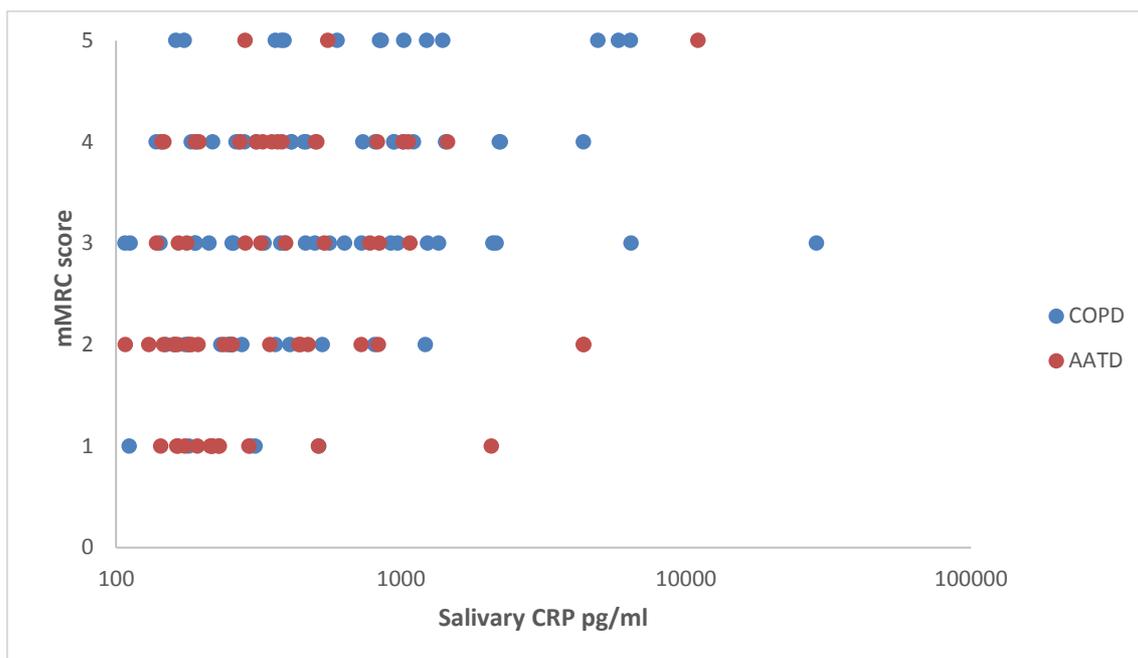
Figure 14: Association between salivary CRP and mMRC score.

Legend: 80 COPD patients and 62 AATD patients. Each data mark represents one patient.

Spearman's Rho shows moderate correlation for COPD patients, r_s 0.328, p<0.01, and AATD patients,

r_s 0.308, p<0.05,

3.5.6.3   *Dental Habits & Symptoms*

No significant associations were seen for plasma hs-CRP, salivary CRP or salivary IL-8 and no.

of teeth, frequency of tooth brushing, use of mouthwash or dental floss, last dental

examination, previous dental disease, appearance of loss of bone, wider spaces between

teeth, bleeding gums, loose teeth or previous dental extractions.

Salivary IL-8 was significantly associated with patients reporting an appearance of longer

teeth in the COPD group only; Mann Whitney U 353.0, p=0.006, and with painful teeth and

gums in the AATD group only; Mann Whitney U 238.0, p=0.023.

3.5.6.4 Periodontitis

Plasma hs-CRP and salivary IL-8 did not correlate with any of the periodontal measurements undertaken.

For COPD patients, there was a significant difference in plasma hs-CRP and diagnosis of periodontitis by maximum probing depth ≥4mm at any tooth and therefore also BPE score; 5.45 +/- 3.56 vs 8.75 +/- 1.87 ug/ml, with patients with periodontitis by these definitions having lower plasma hs-CRP than those who were disease free, p=0.034. No other significant results were found for the other diagnoses of periodontitis applied.

Salivary CRP positively correlated with maximum probing depth (mm) for AATD patients only, Spearman's Rho 0.254, p<0.05 and therefore was also significantly associated with a diagnosis of periodontitis determined by PPD ≥4mm, p0.05 and BPE, p<0.05 for this group of patients.

Salivary IL-8 was not associated with any means of diagnosing periodontitis.

CHAPTER 4
DISCUSSION

4 DISCUSSION

4.1 Prevalence of periodontitis in COPD and AATD

The prevalence of periodontitis in non-deficient COPD and AATD in this study shows variation depending on the diagnostic criteria used. Combined group prevalence ranged from 0.7-93.5% and was 1.2-97.6% for non-deficient COPD and 0-88.2% for AATD patients. The prevalence for the general population in the UK has been calculated from data from The Adult Dental Health Survey (ADHS) 2009 and reported as 45% using PD ≥4mm [101]. Both the non-deficient COPD group and the AATD group in this study had a significantly higher prevalence than this (84.7% and 76.5 % versus 45%) when applying the same threshold for diagnosis.

For most definitions, there was greater prevalence of periodontitis in non-deficient COPD compared to AATD patients (p<0.001). This is in keeping with previous studies reporting relationships between COPD and periodontitis [86, 92, 93, 145] but the current study is the first to compare prevalence in non-deficient COPD and AATD.

Although the American Association of Periodontology has clear criteria [97], these are not always utilised in all epidemiological studies and previous studies have used varying combinations of periodontal measurements [82-88, 90-94] with no consistency or justification for measurements used.   There have been many diagnostic and classification systems developed [79-81, 95, 96] to help define periodontitis, however, none of these systems are used in routine practise and all have some flaws.   Thus in 2003, the Division of

Oral health at the Centres for Disease Control and Prevention in collaboration with the American Academy of Periodontology (CDC-AAP) appointed a working group to develop a standardised case definition for periodontitis [79]. Use of the CDC-AAP definition found a greater prevalence (91.8 versus 72.1%, p<0.01) and severity (43% versus 22% severe periodontitis, p<0.001) in our non-deficient COPD cohort compared to AATD patients. However, this algorithm is primarily used in American research, whereas the Basic Periodontal Examination (BPE) recommended by The British Society of Periodontology is applied in clinical practice [100] and used routinely in the UK. When BPE criteria were applied to the two cohorts, there was no difference in prevalence between the AATD and non-deficient COPD cohorts, although the latter group was still more likely to have more severe periodontal disease (27.1 versus 13.2%, p<0.05).

The variation in results may, in part, be explained by the fact that PPD is considered a reflection of current disease status, whilst CAL represents cumulative disease [94]. In younger populations, PD and CAL correlate well, but gingival recession and therefore attachment loss is a normal part of the ageing process. Increased PPD can also be seen in conditions other than periodontitis, and conversely periodontitis can exist without significant pockets, if gingival recession is also present [81]. In the current study, 66.7% of all patients were aged ≥60years and this predominance of older adults may explain why increased PPD was less apparent than increased CAL. 87.5% of non-deficient COPD patients were aged ≥60years compared to 39.7% of AATD which could also part explain why prevalence was higher in this group. Additionally, even when periodontal disease is successfully treated, the recession can remain [217], thus it could be that some of the patients in this study, (in particular the older COPD group) have had previous disease which

has been subsequently treated but increased CAL is still noted. Thus, it is important to be clear whether investigators are assessing active periodontitis or evidence of previous or cumulative disease. The use of the presence of BoP may assist in this as when evident, BoP is suggestive of active inflammation and disease. It may be practical to use a definition that utilises both PD and CAL such as the CDC-AAP criteria. This would negate some of the problems of diversity of age and ensure patients with previous co-morbid disease but not active periodontitis are not misclassified as having a healthy periodontium.

Both COPD and periodontitis share common risk factors including poor socio-economic status, smoking [143, 144] and age. In addition to this, it is possible the link seen between the two conditions also reflects poor oral health in combination with poor nutrition, poor self-care behaviours and exposure to other environmental factors such as pollutants. Many previous studies investigating relationships between COPD and periodontitis have failed to adjust for these common risk factors [83, 87, 143, 144]. The importance of this is recognised in a large meta-analysis which initially reported the odds ratio (OR) for having COPD in populations with periodontitis as 2.08, (95% confidence interval 1.48-2.91; p<0.001), yet after correction for known common risk factors the OR was reduced to 1.78 95% CI 1.23-2.58 [143].   The numbers of participants in this current study prevented correction for all possible confounders but it was possible to correct for age and smoking pack year history using a binary regression method. Before correction for age and smoking, most of the periodontal criteria indicated an increased prevalence of periodontitis in non-deficient COPD compared to AATD. However, after correction, only average CAL ≥4mm still had a greater prevalence in the non-deficient COPD group, suggesting any uncorrected data should be interpreted with caution. The adjusted data concurs with the study of Hyman and

colleagues who only found an increased risk of COPD in patients with clinical attachment loss ≥4mm [90].

Non-deficient COPD patients were of a poorer socio-economic status, had lower income and were less well educated than AATD patients, a finding in keeping with previous studies comparing COPD and healthy controls [86, 91, 92, 94]. Regression analyses confirmed these variables did contribute to the association between periodontitis in non-deficient COPD compared to AATD. The data from this study also shows non-deficient COPD patients had significantly reduced brush frequency; 46.7% brushing twice a day compared to 72.3% of AATD patients (p<0.001) which was also proven to be a significant confounder in the subsequent regression analysis. In keeping with this, other studies have shown COPD patients have significantly reduced brush frequency [83, 85, 87] compared to the general population and thus is likely to be a crucial confounder when seeking associations and/ or a common pathophysiological process.

Previous studies have reported associations with lung function and CAL in COPD [84, 87, 94]. The data in this study also shows a significant negative correlation for FEV_1: FVC ratio and average and maximum CAL in both respiratory cohorts. This association is only seen for AATD when correlating with PPD. There were no significant correlations for parameters of gas transfer and any periodontal measurement in the non-deficient groups, but some negative correlations did exist for the AATD group. The reasons for this are not clear but could reflect increased neutrophilic inflammation seen in AATD lung disease [69, 218].

Unfortunately, there are some limitations with this part of the study. When taking periodontal measurements it is possible that placement of the tips of the measurement probes may not always be optimal leading to under and over-reporting of probing depth [79]. Insufficient patient numbers prevented inclusion of all potential confounders into single regression models, but attempts were made to account for these using separate regression models. Ideally there would have been recruitment of participants with no evidence of lung disease as a healthy control group, however as an alternative, the data was compared to the results from the Adult Dental Health Survey [101] as a surrogate control.

4.2 Validation of commercially available ELISA kits for the measurement of salivary CRP and IL-8

The use of saliva as a diagnostic fluid is gaining interest due to its relative abundance and non-invasive methods of collection [182, 219, 220]. Several studies have reported the potential use of salivary CRP as a clinical biomarker in thyroid disease [183] , metabolic syndrome [184], myocardial infarction[185, 186] and atherosclerosis [187], COPD [83, 188] and periodontitis [83, 189, 221].

Most literature describing the results of biomarkers measured in saliva use commercially available ELISA kits for ease of implementation. The kits provide some data in their product literature regarding validation for use, but it is our practice to conduct our own in-house validation of kits not used previously in our laboratory.

It is generally accepted that a recovery between 80-120% is a reasonable indication that the assay is suitable for use with the type of sample being analysed [222]. However, the Abcam ELISA kit for CRP produced recovery of spiked samples 6-144%. After contacting Abcam to discuss the results of our experiment they found that their product information leaflet was incorrect and the CRP ELISA kit had not been validated for use in saliva and consequently their product information leaflet was going to be amended.

We were successfully able to confirm that the Salimetrics kit for salivary CRP was valid to be used as per the manufacturer's protocol and at dilutions up to and including 1:10. Similarly, we were also able to validate the R&D kit for measurement of salivary IL-8, although the inter-assay variability was not as good for this kit as the Salimetrics kit, and thus data should be interpreted with that in mind.

The link between local and systemic inflammation in the development and progression of inflammatory diseases is an area of great interest. However, saliva varies between individuals both in terms of its composition and viscosity, both features which can interfere with assays and alter performance and results, otherwise known as matrix effects. The dilution required is dependent on the content of proteins in the sample which is highly variable in saliva. In addition, when manufacturers provide validation data they generally use healthy controls, yet patient samples are likely to be affected by the presence of inflammation and associated mediators which could further interfere with assay accuracy.

Thus, to ensure an ELISA is suitable to analyse salivary samples, it is crucial that kits are appropriately validated to ensure results that are generated are both replicable and valid for

their experiment. It appears that it is not sufficient to rely upon the validation data including in the product literature of all commercially available ELISAs for salivary CRP measurement and we would extrapolate this to other salivary measurement kits.

4.3 Correlation of plasma with salivary CRP and IL-8

Plasma hs-CRP was detectable in 100% of samples tested and was significantly higher in non-deficient COPD patients than AATD patients. This is in keeping with other studies showing increased plasma CRP in patients with COPD in comparison to healthy controls [28, 147, 162, 163], although no studies compare it to AATD. The reason for increased peripheral CRP is thought to be due to the sustained low grade systemic inflammation [23, 223, 224] commonly reported in COPD, but this has not been examined in AATD.

Plasma IL-8 was detectable in 76.4% of samples tested, but more often detectable in COPD patients, than AATD; 93.6% versus 57.1%, p<0.001. The mean IL-8 levels were similar in both groups but all values fell below the lower limit of quantification of the standard curve and as such further analysis was not possible.   This is in keeping with previous work which reports plasma IL-8 is not always detectable in the peripheral circulation [29, 38] and significant differences are not always apparent between disease and health [38, 201]. IL-8 is an inflammatory cytokine with a primary role in neutrophil chemotaxis at the site of insult or injury [199]. Therefore, it is not surprising that its levels are often undetectable in the peripheral circulation of healthy or otherwise stable patients.

Salivary CRP and IL-8 was detectable in 100% of samples tested. The mean values were greater in COPD patients for CRP, but no significant difference was seen for IL-8. The results for salivary CRP replicate a previous study showing elevated levels in patients with COPD compared to health [188]. The lack of a difference in salivary IL-8 between health and disease has previously been reported [210]. However, the values in our patients were markedly raised in comparison to the mean values reported in other trials comparing salivary IL-8 in healthy patients to those with disease, and was comparative to the levels seen in patients with oral cancer [225, 226] which does support the hypothesis of increased local production of IL-8, a potent neutrophil chemoattractant and activator in these patients with predominantly neutrophilic mediated lung disease.

A moderately significant positive relationship between plasma hs-CRP and salivary CRP was seen; which is in keeping with previous findings [192, 193] , but when the groups were analysed separately, the relationship persisted for AATD only. The reasons for this are not clear but may reflect the greater difference in peripheral CRP between the two groups compared to the smaller although still significant difference in salivary CRP. Additionally, it has previously been shown that correlations between peripheral and salivary CRP are weaker when the peripheral CRP is < 2.0mg/L compared to when levels are higher than this [193]. This may partly explain our findings as 47.7% of our patients had peripheral CRP <2.0mg/L. A possible reason for this is that CRP is a large molecule and only transits into saliva from the peripheral circulation when it is in great abundance. Moderately positive correlations were seen in both groups for salivary CRP and salivary IL-8, suggesting that oral inflammation is characterized not only by locally produce inflammatory mediators, such as IL-8, but also by systemically produced inflammatory mediators which can travel to the site

of insult via the peripheral circulation and then enter the saliva by methods including cellular diffusion, ultra-filtration in salivary glands or through microvascular injury to the gingival epithelium [182, 227].

4.4 Correlation of plasma and salivary CRP and IL-8 with demographic data, indices of lung function and disease severity

No significant associations were seen for plasma hs-CRP, salivary CRP or salivary IL-8 and gender, age, smoking status or pack year history, co-morbid presence of diabetes mellitus, hypertension or vascular disease, age at leaving education, NS-SEC score or annual household income.   This is contradictory to previous work demonstrating peripheral [147, 160] and salivary CRP to be higher in COPD than healthy non-smokers, although healthy smokers have a similarly raised salivary CRP to COPD patients [188]. Peripheral CRP has also been shown to be higher in patients with colonized airways [228]. As we did not measure this and therefore correct for it, it could be that the presence of microbial colonization in some patients reduced associations that would otherwise have been seen for peripheral CRP and our variables of interest. Peripheral CRP is frequently raised in patients with ischaemic heart disease or atherosclerosis [153-156], but it is also raised in patients with subclinical disease [187]. The lack of relationships between measured CRP and presence of co-morbidity may reflect several possibilities; the presence of sub-clinical or yet un-diagnosed

disease and the use of statin therapy in patients with confirmed disease. Statins have been shown to reduce CRP [229], but this is not something we controlled for in this study.

Plasma hs-CRP was significantly positively correlated with BMI and IMD quintile in AATD patients, but neither was associated with COPD patients. Correlations with CRP and BMI have been seen previously [187] and are thought to occur due to the release of inflammatory cytokines from adipocytes and other inflammatory cells within adipose tissue. It is not clear why this observation was not seen in this study for non-deficient COPD patients, but the very varied levels of CRP may have masked this association. The correlation seen between increasing hs-CRP and IMD quintile suggests an increased inflammatory response in those patients living in more deprived areas as determined by their residential postcode. This relationship was only seen for AATD patients but this could reflect the wider spread of this group across quintiles, whereas this was less so for the non-deficient COPD group. This result suggests that poorer socio-economic status is associated with an enhanced inflammatory response, which is in keeping with previous observations showing patients with a lower SES are more likely to have chronic disease [230-232], although in this study the association was not seen for other measurements of socio-economic status.

A weakly positive correlation was seen in the COPD group for salivary IL-8 and highest qualification gained. The reasons for this weak relationship are unclear and no logical explanation exists, thus the association is more likely to reflect a type one error occurring with a small sample size.

No significant associations were seen for any inflammatory markers and GOLD stage of COPD severity, exacerbation frequency, presence of bronchitis, radiological evidence of

emphysema, use of inhaled therapies or any lung function parameter. This could reflect a lack of sufficient participant numbers and a larger, appropriately powered study could explore this possibility further. Previous work exploring associations of salivary CRP with lung function in COPD has also failed to find a correlation [188], although peripheral CRP is frequently reported to be inversely associated with exercise tolerance and lung function [28, 147, 164, 165]. This association is felt most likely to be as a consequence of prolonged low grade systemic inflammation causing skeletal muscle dysfunction or muscle wasting [233]. One possible theory therefore why we did not see this association in our patients could be that our patients do not have evidence of muscle dysfunction or wasting. In future work this is something that could be addressed further by functional muscle assessment. One possible reason for a lack of association with salivary inflammatory markers include the high percentage (85% and 53% of non-deficient COPD and AATD patients) using inhaled corticosteroids, which have been shown to reduce peripheral CRP levels [234] and thus potentially also in saliva.

Salivary CRP was positively correlated with mMRC score for COPD patients and AATD patients, but no association was seen for plasma hs-CRP or salivary IL-8. Finding an association between increased inflammation and worse mMRC score is not unexpected and has been reported previously for peripheral markers [161] and salivary markers, including CRP [188]. However, our data only finds an association for salivary CRP. This may reflect that patients with worse mMRC scores tend to mouth breathe rather than inhale and exhale through the nasal passages. This constant exposure of the oral mucosa to air pollutants could be the cause of the increased salivary but not peripheral CRP. Further exploration of

the use of salivary CRP as a tool to correlate with self-reported symptoms would be beneficial.

4.5 Correlation of CRP and IL-8 with dental symptoms and indices of periodontitis

No significant associations were seen for plasma hs-CRP, salivary CRP or salivary IL-8 and no. of teeth, frequency of tooth brushing, use of mouthwash or dental floss, last dental examination, previous dental disease, appearance of loss of bone, wider spaces between teeth, bleeding gums, loose teeth or previous dental extractions.

Salivary IL-8 was significantly associated with patients reporting an appearance of longer teeth in the non-deficient COPD group and with painful teeth and gums in the AATD group, but did not correlate with any periodontal measurements or diagnoses of periodontitis.

Plasma hs-CRP did not correlate with any of the periodontal measurements undertaken, although in the non-deficient COPD group, the plasma hs-CRP was significantly lower in patients with a diagnosis of periodontitis by maximum probing depth ≥4mm at any tooth and BPE score than those who were disease free. This is the opposite of the findings of other work which has shown an increase in hs-CRP in patients with chronic periodontitis [171], and correlations with clinical measurements of periodontitis [171]. Salivary CRP was positively correlated with maximum probing depth (mm) and a diagnosis of periodontitis determined by PPD ≥4mm and BPE in AATD patients only.

The lack of association of salivary IL-8 and periodontal measurements may reflect the way IL-8 was measured. We used an ELISA kit which gave an assay value for the total amount of IL-8 in the sample. However, as previous work with GCF has demonstrated, there can be great variation in results depending if the total amount or the concentration of IL-8 is measured [203-205]. To calculate concentration rather than just total amount it would be necessary to measure salivary volume collected at the sampling time.

4.6 Further Work

In addition, other markers of systemic and local inflammation could be explored using a multiplex assay method which would generate richer data for analysis without needing additional volumes of biological fluids.

Further analysis of inflammatory mediators in saliva should also include measurements of concentration rather than just total levels. This is important as previous work has shown this can produce different results, certainly for IL-8, and this feasibly applies to other mediators too. Additionally, the measurement of markers in GCF could also be advantageous rather than analysing mediators in saliva which is more easily contaminated and of which composition can change. Previous work has also confirmed GCF levels of inflammatory markers correlate well with peripheral levels of the same markers [171].

Exploring the enhanced neutrophilic inflammatory hypothesis relating chronic lung disease and periodontitis could be achieved by isolating neutrophils from patients with and without

COPD and within these groups further sub group analysis of those patients with periodontitis and those with a heathy periodontium. Neutrophil function could then be assessed by measuring chemotaxis and phagocytosis ability and determining if the neutrophils from patients with COPD behaved in a similar manner to those with periodontitis and if these neutrophils had impaired function in comparison to age matched controls without COPD or periodontitis. A further interesting angle would be to explore the microbiome present on dental plaque and compare it to the microbiome from respiratory samples such as sputum or bronchoalveolar fluid. This would help determine if there is an association between the two compartments as at least some of the same microbiome would be expected to be present in both lung and oral cavity if there was a genuine association relating to overspill theory from oral cavity to the respiratory system or from haematogenous dissemination of microorganisms.

We compared non-deficient COPD with AATD but if further work was to be undertaken it would be useful to have a healthy, age-matched cohort to be used as a control group. This would be helpful in particular if looking at the hypothesis of enhanced neutrophilic inflammation as this would not be seen in the healthy control group and as such if this was the mechanism of action, clear differences should be apparent.

Some of our results may be affected by relatively small sample sizes. If this work was to be replicated in the future, it would be beneficial to have a larger cohort recruited to minimise type 1 errors and allow for larger regression models to be used to account for the large number of confounding variables. Statistical power calculations would be useful to

determine the numbers of patients that would need to be recruited to each group to minimise Type 1 and 2 errors.

4.7 Summary

In summary, the criterion used to diagnose periodontitis in epidemiological studies has a significant impact on the prevalence and associations with other chronic diseases. Using strict criteria this data indicates an increased prevalence in non-deficient COPD compared to AATD but an association with the severity of physiological impairment is stronger in AATD suggesting the deficiency may enhance not only lung destruction but also periodontal damage irrespective of other demographic confounders.

It is important to recognise the burden of periodontal disease as co-morbidity in other chronic diseases so at risk individuals can be managed appropriately at an early stage. Ascertaining accurate prevalence data provides information on the social and economic effects of the disease and its' implication/s in developing community health education programmes. Further accurate data will assist with research into other areas of chronic comorbidity associated with oral disease and potentially the development of beneficial treatments and interventions.

The available evidence from this study still provides support for the hypothesis that COPD and periodontitis could be causally linked; raising the possibility that treatment of one could influence the severity and progression of the other. There are similarities in disease

mechanisms (that of neutrophilic inflammation and connective tissue loss) that suggest a shared pathophysiology and support epidemiological evidence of association. However, the presence and severity of periodontitis must be defined using robust criteria and second, the diagnosis of COPD must be confirmed according to international standards; this will require close collaboration between dentists and physicians. Third, known relevant confounding factors must be recorded and considered, and this will require a significant sample size to allow appropriate statistical modelling. Finally, the inflammatory profiles (systemically or locally) in groups of patients with COPD and periodontitis need to be compared with that of matched patients with COPD and no periodontitis. Such studies could then be used to design appropriately powered interventional trials to determine whether treating periodontitis is of benefit to COPD related lung disease.

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Appendix 1

This questionnaire is voluntary. All answers are kept anonymously, but you are under no obligation to disclose information you would rather withhold.

1. Current Postcode

   ………..

2. At what age did you finish your continuous full-time education at school, college or university?.............

3. Highest academic qualification held: GCSE/O-Level   ☐   A-level   ☐

                                            Degree   ☐   Post-graduate   ☐

4. Any vocational qualification?   ☐ Yes   ☐ No   Details

   …………………………………………………

5. Are you currently in a paid job?   ☐ Yes   ☐ No

6. If no, have you ever had a paid job? ☐ Yes   ☐ No   Year left last paid job……………………

7. What is the title of your job?...

8. Are you: Full Time ☐   Part Time ☐

9. Short description of work:

   ………

   ………

   ……………………………

10. What is the house-hold annual taxable income (excluding benefits) before tax?

    £0-£10k ☐   >£10k - £20k ☐   >£20k - £40k ☐   >£40k - £80k ☐

        >£80k ☐

11. Notes:

    ……

    ……

    …………………………………..

Dental Health Questions:

1. How often do you brush your teeth? ☐ Not every day ☐ Once a day ☐ >2 times a day

2. Do you regularly floss your teeth? ☐ Yes ☐ No

3. Do you regularly use mouthwash? ☐ Yes ☐ No

4. How long has it been since you last had a dental examination?

☐ Within 6/12 ☐ Within year ☐ Within 2 years ☐ Greater than 2 years ☐ Never

5. Have you ever been told that you have periodontal disease or gum disease? ☐ Yes ☐ No

6. Has a dentist ever told you that you had lost bone around your teeth? ☐ Yes ☐ No

7. Have you noticed in recent years that the space between your teeth gets wider, or that 'black triangles' have developed between your teeth? ☐ Yes ☐ No

8. Have you noticed a recession of your gums, so that teeth appear longer? ☐ Yes ☐ No

9. Have you had painful gums? ☐ Yes ☐ No

10. Do your gums ever bleed? ☐ Yes ☐ No

11. Are your teeth loose? ☐ Yes ☐ No

12. Have you had any adult teeth extracted due to gum disease? ☐ Yes ☐ No

13. If so, how many? ☐ 1 ☐ 1-5 ☐ 6-31 ☐ All