Evaluation of saliva biomarkers in chronic obstructive pulmonary disease: correlation to patient reported outcomes

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Abstract

COPD is a debilitating chronic respiratory disease with a systemic footprint. COPD is a highly heterogeneous disease but invariably its management involves a one-size fits all approach. This fails to address individual variations in disease progression, symptom burden and functional decline. There remains a need for sensitive monitoring tools that could provide personalised care based on patients' particular phenotypes and informative self-management.

This thesis has standardised collection protocols and processing for saliva, a complex body-fluid which is readily accessible and user-friendly for near-patient testing. I have modified immunoassays to work in saliva with demonstrable reproducible results for quantification of C-reactive protein, Procalcitonin and Neutrophil Elastase. Symptom assessment is crucial in longitudinal self-monitoring of COPD. I designed a novel patient wellbeing scale incorporated into an electronic self-assessment diary, which was embraced by patients as improving symptom change recognition, education and self-management. Using sophisticated analytical tools, I have attempted to cluster/phenotype disease trajectory paths driven by a compilation of symptom scores, spirometric volumes and saliva biomarker levels and produced novel patient-specific multidimensional composite scores with significant correlation to COPD disease severity. Prodromal changes in FEV_1, salivary biomarkers and self-assessment scores were reproducibly demonstrated, with potential to predict exacerbation onset.

These results could be exploited for the development of a much-needed personalised COPD monitoring eco-system, which isolates early deteriorations and prompts timely interventions, leading to beneficial disease outcomes. Patient-researcher iterative co-design has been key throughout this thesis. One outcome of this relationship is the design and production of a

bespoke integral saliva collector prototype which could substitute laboratory-based processing of saliva samples in readiness for analyte testing.

In conclusion, this thesis has created the necessary tools to improve the classification and monitoring of COPD, opening new avenues for proactive patient self-management and providing the basis for future personalised and stratified care.

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First and foremost, I would like to acknowledge my lead supervisor Professor Monica Spiteri for providing me with the guidance and support to achieve this thesis. We have discussed the works in this thesis at numerous meetings and forums and her education and encouragement have enabled me to a produce a high quality piece of work. She has provided me with the skills set to progress towards becoming a successful independent researcher. Throughout my education I have only ever achieved my full potential when supported by an excellent teacher. Presently this has only occurred twice in my life, and this is one of those occasions.

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Publications Arising

- ***Peer reviewed journals***

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- ***Intellectual property***

1. Copyright of look and content of the electronic wellbeing diary mobile application COPD-SPOC Monitor, published on Apple. [https://itunes.apple.com/gb/app/copd-spoc-monitor/id591784012]. *Work from Chapter 2.*
2. Registered trademark, NEPeSMO®, approved in classes 9, 10 and 44 in EU [CTM 011828506 (2013)] and US [4.643.112 (2014)] for near-patient electronic systems monitoring. Named Applicants: **Neil Patel** and Monica Spiteri. *Work from Chapter 4.*
3. "Improvements in and Relating to Sample Collection", patent filed 26th June 2014; UK publication GB2527516A (30th December 2015); with arising PCT Publication Numbers 2015/198029, 2015/198030 and 2015/198031. Named Inventors: **Neil Patel** and Monica Spiteri. *Work from Chapter 6.*

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Abbreviations

3D	3-dimensional
A&E	Accident & emergency
AAT	α1-antitrypsin
Ab	Antibody
ADL	Activities of daily living
ANOVA	Analysis of variance
ATS	American Thoracic Society
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BIC	Bayesian information criterion
BLRT	Bootstrap log-likelihood
BMI	Body mass index
BTS	British Thoracic Society
CAD	Computer aided design
CAT	COPD assessment test
CCL	CC chemokine ligand
CCQ	Clinical COPD questionnaire
CIBA	Company for Chemical Industry Basel
COPD	Chronic obstructive pulmonary disease
CPA	Change point analysis
CRP	C-reactive protein
CUSUM	Cumulative sum analysis
CV	Coefficient of variance
CXC	Cysteine-X-Cysteine

CXCL	Cysteine-X-Cysteine chemokine ligand
DBS	Dried blood spot
DC	Dendritic cells
DHEA	Dehydroepiandrosterone
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EBC	Exhaled breath condensate
EGF	Epidermal growth factor
ELFA	Enzyme linked fluorescent assay
ELISA	Enzyme linked immunosorbent assay
ERS	European Respiratory Society
ETC	Electronic taste chip
EU	European Union
EXACT	Exacerbation of chronic pulmonary disease tool
FEV_1	Forced expiratory volume in 1 second
FTIR	Fourier transform infrared spectroscopy
FVC	Forced vital capacity
GOLD	Global initiative for chronic obstructive lung disease
HIV	Human immunodeficiency virus
HRCT	High resolution computer tomography
HRO	Healthcare reported outcome
IBM	International Business Machines
IFN	Interferon
IL	Interleukin
IP	Inducible protein

IQR	Interquartile range
kDa	Kilodaltons
LCGA	Latent class growth analysis
LT	Leukotriene
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MI	Myocardial infarction
MLE	Master lot entry
MMP	Matrix metalloproteinase
MRC	Medical Research Council
N	No
NE	Neutrophil elastase
NES	Never-smokers
NO	Nitric oxide
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
NS	Non-significant
PBS-T	Phosphate buffer saline 0.05% - Tween 20
PCT	Procalcitonin
POC	Point of care
PRO	Patient reported outcome
RANTES	Regulated on activation, normal T-cell expressed and secreted
RNA	Ribonucleic acid
RNS	Reactive nitrogen species

ROC	Receiver operating characteristics
ROS	Reactive oxygen species
RPM	Revolutions per minute
RV	Residual volume
S	Sample
sCRP	Salivary CRP levels
SD	Standard deviation
SGRQ	St George's Respiratory Questionnaire
SLPI	Secretory leukoprotease inhibitor
Sm	Smokers
sPCT	Salivary PCT levels
SPSS	Statistical package for social scientists
sNE	Salivary NE levels
TB	Tuberculosis
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T-helper cell
TLC	Total lung capacity
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoietin
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
Y	Yes

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Dedication

Dedicated to Jaimini Patel (Mum)

"alma mater"

Chapter 1:

General Introduction

1.1. Definition of Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a common, complex disease with an insidious onset, involving debilitating breathlessness, with a progressive decline in the patient's ability to perform essential activities of daily living. It is characterised by airway obstruction that has little or no reversibility to bronchodilator agents for example, salbutamol. The airflow obstruction is due to a combination of small airways disease and parenchymal damage (emphysema); the relative contributions of these factors vary within individuals. COPD is associated with chronic inflammation due to an abnormal inflammatory response in the lung, most likely caused by noxious particles such as tobacco smoking. This causes progressive airway remodelling and destruction of the lung parenchyma (GOLD, 2016).

1.1.1. Development of the term COPD

Interest in bronchitis developed in the early 1950's when chest physicians who had traditionally focused their time on tuberculosis were freed with the advent of treatment for the disease. It was in 1952 that the Association of Physicians of Great Britain held a symposium on chronic bronchitis at which a definition in terms of expectoration was first considered (Fletcher, 1959). The Medical Research Council (MRC) established a Committee for Research into Chronic Bronchitis under the chairmanship of Professor R.V. Christie. When the Committee first met it was unable to agree on any definition of chronic bronchitis or the criteria required for its diagnosis. At the time there was considerable confusion between the diagnosis of emphysema and chronic bronchitis with emphysema assumed to present in every patient with persistent airflow obstruction even when no pathology had been found on autopsy (Cope, 1951). Mortality statistics in the 1950's revealed that the greatest mortality from bronchitis and emphysema was in the United Kingdom (UK) (Reid and Rose, 1964). This was due to a classification bias as a traditional belief among American clinicians that bronchitis was an

unimportant and trivial condition. Emphysema tended to be defined as an increase in the Residual Volume (RV)/ Total Lung Capacity (TLC) ratio; also since patients with asthma usually shared this abnormality they were often concomitantly diagnosed as having emphysema.

Two landmark meetings took place: The Company for Chemical Industry Basel (CIBA) Guest Symposium 1959, and the American Thoracic Society (ATS) Committee on Diagnostic Standards 1962; both described the components of the modern day definition. The CIBA Guest Symposium defined emphysema on a morbid anatomical basis as the enlargement of the size of air spaces in the lung peripheral to terminal bronchioles. It was suggested that a general term was needed to encompass emphysema, asthma and bronchitis and thus a term of "chronic non-specific lung-disease" was accepted. The ATS Committee on Diagnostic Standards defined chronic bronchitis in clinical terms including a chronic cough of at least three months over a minimum of two years. Emphysema was described anatomically in terms of enlarged alveolar spaces and loss of alveolar walls. Neither definition used any physiological criteria. Asthmatic bronchitis was considered an overlapping condition. Many other attempts to clarify this complex disease, whilst not significantly improving on these basic definitions did progress to include reference to functionality. As disease understanding evolved, several new terms were introduced to give greater emphasis to the cause of disability; the most widely adopted became chronic obstructive pulmonary disease, and William Briscoe is believed to be the first person to use the term COPD in discussions at the 9th Aspen Emphysema Conference 1966 (Petty, 2006).

1.2. Epidemiology of COPD

COPD poses a major public health burden worldwide with approximately 44 million confirmed cases and 160 million undiagnosed cases (Fletcher et al., 2011). It now ranks 3rd in global mortality surpassed only by ischaemic heart disease and stroke. In 2010, 2.9 million deaths were attributed to COPD (although this is a fall from 3.1 million in 1990) which represents 5.5% of all-cause mortality (Lozano et al., 2012). COPD represents 27% of deaths related to smoking, surpassed only by cancer and cardiovascular disease (Soriano and Rodriguez-Roisin, 2011).

1.2.1. Impact on the UK

An estimated 3 million people are affected in the UK, with approximately 835,000 having been diagnosed, and 2.2 million people undiagnosed which equates to 13% of the population of England over 35 years of age (Shahab et al., 2006). COPD is closely associated with socioeconomic status, with higher rates observed in lower status communities (Yin et al., 2011, Kanervisto et al., 2011). COPD ranks 5th in mortality in the UK and is the 3rd largest cause of respiratory death, accounting for 23%. This equates to approximately 30,000 deaths each year which is one of the highest rates in the European Union (EU), with more than 90% occurring in the over 65 age group in 2004. COPD accounted for 4.8% of all deaths in England between 2007 to 2009 (Health, 2011). The rate of mortality for respiratory disease in the UK is almost double the European average, although this was on a downward trend between 1994 to 2010 (Lopez-Campos et al., 2014). The Health Development Agency estimated in 2004 that around 85% of COPD-related deaths could be attributed to smoking (Hubbard, 2006). Five-year survival from diagnosis is 78% in men and 72% in women with clinically mild disease defined as not requiring continuous drug therapy, but falls to 30% in men and 24% in women with severe disease defined as requiring oxygen or nebulised therapy. Objectively-measured

physical activity is the strongest predictor of all-cause mortality in patients with COPD (Waschki et al., 2011, Hallin et al., 2011).

1.2.2. Impact on the National Health Service

Although only a small proportion of people with COPD are admitted to hospital each year, it accounts for approximately 12% (130,000) of all emergency admissions. This makes COPD the 2nd largest cause of admissions in the UK, and one of the most-costly inpatient conditions treated by the National Health Service (NHS). One million bed days per year, nearly 2% of total capacity, are used for its treatment (Halpin and Miravitlles, 2006).

Admission rates have risen in all age groups since 1994 except in the under 45s; with the highest rise in the over 85s where rates have almost doubled from 1994 to 2005. (NICE, 2010). The National COPD Audit 2013 to 2016 released results from the organisational and clinical secondary care audit in November 2014. This document revealed that since the previous audit (2008) the median number of COPD admissions had further increased by 13%. It should be noted that this audit reports data from just England and Wales; previous audits encompassed the whole of the UK. Thus results for previous audits have been reworked for just England and Wales to allow for direct comparisons (Royal College of Physicians, 2014). With respects to readmissions there are no results at present from the current National COPD Audit. Previous national audits (2003 and 2008) revealed a reduction in length of stay by one day to five days between the two time frames (George et al., 2011). This reduction continues with a recent longitudinal study conducted in London over four years between 2006 to 2010 also demonstrating a reduction by one day (Harries et al., 2015). George et al., in the 2008 data also documented an increase from 26% to 34% in the proportion of patients having a shorter stay

of at most three days compared to 2003. However, the readmission rates had risen between 2003 to 2008 from 30 to 33% with the median time to readmission being 38 days.

Health economics on COPD suggest an average annual per patient cost to the UK of £781 to £1,154 with an overall annual cost (both direct and indirect) of between £800 to £1500 million pounds (Halpin, 2006). Costs increase with disease severity and exacerbation frequency. Patients with severe disease (Global Initiative for Chronic Obstructive Lung Disease (GOLD) Stage 3 and 4) and an exacerbation frequency of two or more per annum have a per patient annual expense of £3,499 (Punekar et al., 2014). A breakdown of direct costs reveals that 54% are related to in-patient hospitalisations (Britton, 2003). The indirect costs of COPD are substantial with an impact on annual productivity amounting to an estimated 24 million lost working days per annum in sick leave and £3.8 billion pounds in direct costs from lost productivity (NICE, 2010). Overall a reduced patient requirement for hospital care could potentially ease the economic burden on the UK healthcare system.

1.3. Risk factors for COPD

1.3.1. Genetic risk factors

There is strong evidence that genetic factors can influence COPD development in response to smoking, although these genetic differences are mostly unknown. Overall, twin and familial aggregation studies suggest that genetic factors likely influence variation in pulmonary function in non-smokers, but these results do not necessarily indicate that genetic factors increase the risk of developing a clinical diagnosis of COPD (Silverman, 2006).

- *SERPINA-1:* This is the only gene with direct causality encoding α1-antitrypsin (AAT), a serine protease inhibitor which protects the lungs from oxidative stress (Nakamura, 2011). A deficiency in this enzyme results in an accelerated development of emphysema.
- *Cutis laxa*: A genetic disease related to irregular elastin fibre processing, which can result in emphysema in childhood and adolescence (Turner-Stokes et al., 1983).
- *Gender*: Female gender is associated with lung function reduction and more severe disease in patients with early onset of COPD or low smoking exposure (Sorheim et al., 2010). Smoking has a greater impact on female lung function and after adjustment for smoking females experience a higher risk of being admitted to hospital for COPD (Prescott et al., 1997).
- *Pre-existing Asthma*: Asthma and COPD are thought to share a common background, with the differentiation into one disease or the other being modulated by environmental and host factors. Airway inflammation and airflow obstruction seen in asthmatics with increased airway hyper reactivity may lead to subsequent lung remodelling due to airway wall thickening and sub-epithelial fibrosis (Vignola et al., 2000). There is sufficient evidence of an association between chronic asthma and both chronic airway obstruction and accelerated loss of pulmonary function. As airway obstruction can lead directly to COPD, it is likely that asthma, with or without additional risk factors, can predispose a person to develop COPD. Studies demonstrating radiographic evidence of emphysema among life-long non-smokers with asthma

also support the possible role of chronic asthma in the genesis of COPD (Silva et al., 2004, Eisner et al., 2010).

1.3.2. Acquired risk factors

There are numerous acquired risk factors that can increase the risk of developing COPD.

- *Cigarette smoking:* The primary risk factor for COPD is chronic tobacco smoking, with a body of evidence stretching back for over 40 years. Approximately 80 to 90% of cases are due to smoking: however, the majority of population-attributable fraction estimates are less than 80%, and COPD only affects 10 to 20% of smokers indicating that other risk factors, besides cigarette smoking, are important (Eisner et al., 2010). Numerous cross-sectional and cohort studies since 2000, using different definitions, have consistently demonstrated an association between smoking and COPD. Moreover, there is a consistent exposure–response relationship with cohort studies supporting the causal criterion of temporality, in which the exposure precedes the onset of disease (Sampsonas et al., 2006).
- *Air pollution:* Longitudinal cohort studies provide strong evidence of an association between outdoor pollution and decreased lung function growth during childhood and adolescence (Allain et al., 2010). Furthermore, decreased lung function growth early in life translates into a greater incidence of COPD in later adulthood and thus a higher likelihood of a true association between air pollution and COPD. Higher rates of COPD are also observed in large cities compared to rural communities. Supplementary studies are needed to confirm the exact effect of each component of air pollution on the lungs (Eisner et al., 2010).
- *Second hand cigarette smoke:* Second hand smoke exposure appears to cause asthma in children and adults, although its role in causing COPD has received limited attention in epidemiological studies. Cumulative home and workplace exposure is associated with a greater

risk of COPD, and a body of literature now supports an association between exposure and the development of COPD independent of personal cigarette smoking (Eisner et al., 2005).

- *Biomass fuels:* Biomass is a material derived from living or recently deceased organisms which are then used for combustion. Over half the world is exposed daily to the smoke from combustion of biomass fuels. In numerous developing countries, indoor air pollution from biomass smoke is a common cause of COPD especially in women. Amongst the different types of biomass, exposure to wood smoke presents the greatest risk (Kurmi et al., 2010).
- *Occupational exposure:* Intense and prolonged exposure to workplace dusts found in coal mining, gold mining, and the cotton textile industry and chemicals such as cadmium, isocyanates and fumes from welding have been implicated in the development of airflow obstruction, even in non-smokers (Santo Tomas, 2011). There is sufficient evidence from multiple epidemiological studies to infer a causal relationship between occupational exposures and development of COPD. The population attributable risk percentage for work-related COPD is at least 15% and emerging data also indicates that occupational exposures, at a minimum, are additive to the smoking associated risk of COPD (Blanc, 2011).
- *Nutrition:* Nutrition may affect the development and maintenance of lung function and could modulate pulmonary responses to injury. It has been demonstrated that patients with coeliac disease appear to be at a moderately increased risk of COPD (Ludvigsson et al., 2011). Currently there is little evidence of an association between antioxidant intake and pulmonary function. As there are fewer studies that evaluated COPD as a specific end-point, there is limited/suggestive evidence of an association between diet and COPD (Eisner et al., 2010).

- *Microorganisms:* An association between mycobacterium tuberculosis and COPD has been demonstrated in studies mostly undertaken in Africa, Asia and South America (Menezes et al., 2007).

1.4. Pathogenesis of COPD

1.4.1. Pulmonary inflammation

Inflammation is present in the lungs of all smokers and is thought to be a normal protective response to inhaled toxins. An enhanced or abnormal response is believed to be a characteristic feature of patients who develop COPD. The precise mechanisms of this response is not fully understood, but this abnormal inflammatory response leads to tissue destruction, consequent on an impairment of defence and repair mechanisms which would normally limit such destruction. In general, the inflammatory and structural changes in the airways increase with disease severity and persist even after smoking cessation (Rutgers et al., 2000a). Two other processes are also central to COPD pathogenesis: an imbalance between proteases/anti-proteases, and oxidants/antioxidants termed oxidative stress (Figure 1.1).

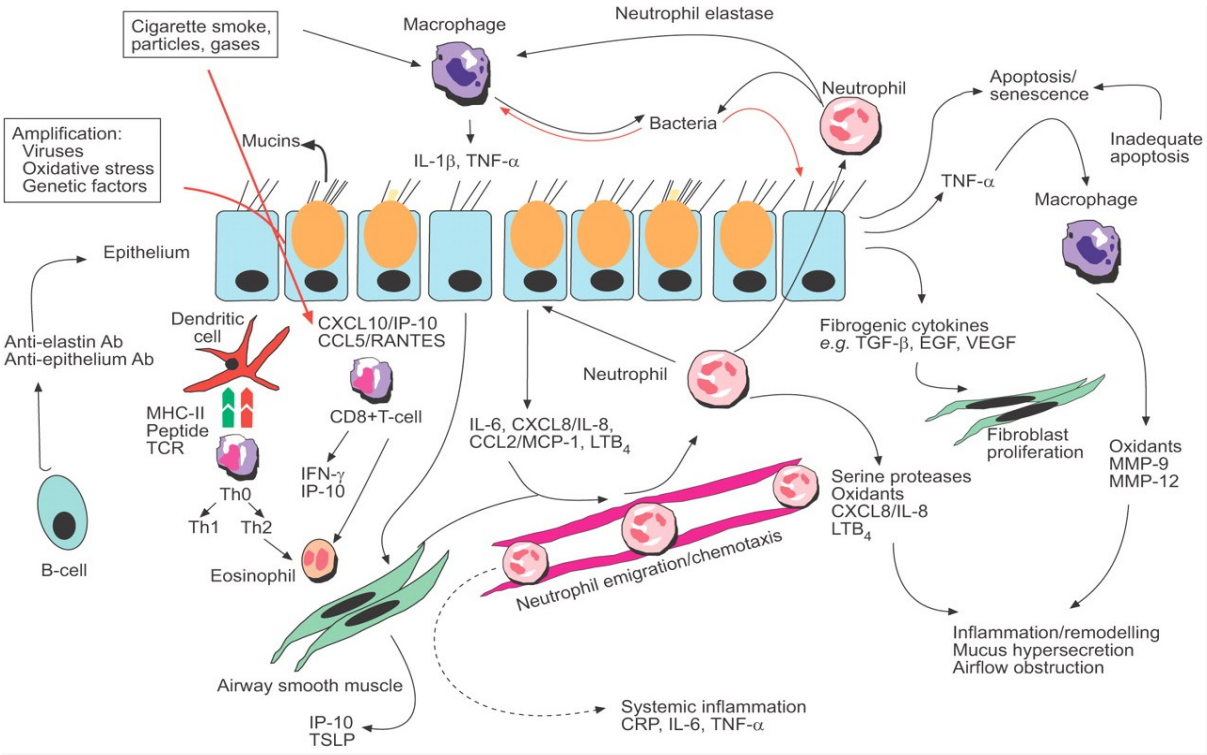


Figure 1.1: COPD pathogenesis.

This figure (adapted from Chung and Adcock, 2008) summaries key inflammatory and cellular interactions and associated amplification signals linking chronic cigarette exposure to COPD.

Studies of bronchial biopsy specimens from patients with mild to moderate COPD show increased inflammatory cell infiltrates in the central airways, compared with non-smokers or smokers who have not yet developed the disease (Di Stefano et al., 2004). The predominant cells are macrophages (greater than 80%), and T-lymphocytes (mainly CD8+ cells) which release pro-inflammatory mediators into the lungs (Finkelstein et al., 1995). Increased numbers of neutrophils are also present, particularly in the glands, which become even more prominent as the disease progresses; in some patients an increased number of eosinophils have been observed (Thompson et al., 1989). It has been hypothesised that the presence of increased CD8+ T-lymphocytes could identify those smokers who go on to develop COPD; with a correlation between T-lymphocyte numbers, alveolar destruction and airflow limitation severity (Di Stefano et al., 1998). Smokers with normal lung function also show, albeit to a

lesser extent, increased numbers of T-lymphocytes compared with non-smokers (Di Stefano et al., 2002). The mechanism which leads to CD8+ T-lymphocyte accumulation in the lungs is not fully understood. It has been shown that at least a proportion of disease-specific lymphocytes that are part of the inflammatory cascade in the lungs and regional lymph nodes, are recruited from the lymphatic and blood circulations (Lehmann et al., 2001). Studies of peripheral blood T-lymphocytes in patients with COPD have shown peripheral T-lymphocytes (particularly CD8+) are more frequently activated with increased production of various mediators; many of these T-lymphocyte abnormalities are highly correlated with disease severity (Gadgil and Duncan, 2008). CD8+ cells have also been shown to be associated with alveolar cell apoptosis in patients with emphysema (Majo et al., 2001).

Neutrophils contain the only cell products that have been shown to directly cause the pathological features of COPD. These include serum proteinases, including Neutrophil Elastase (NE), cathepsin G, proteinase-3, matrix metalloproteinase (MMP)-8 and MMP-9, which contribute to alveolar destruction (Stockley, 2002) and are also potent stimuli of mucus secretion (Kim and Nadel, 2012). A positive correlation has been demonstrated between induced sputum MMP-8 and 9 activities and the degree of airflow obstruction (Vernooy et al., 2004). The direct role neutrophils play in COPD pathogenesis is unclear. Increased numbers of neutrophils, recruited from the circulation, have been demonstrated in the bronchial tissues of some patients with COPD and have been shown to relate to airflow obstruction (Di Stefano et al., 1998). These neutrophils have a unique protein profile compared to neutrophils from healthy age-matched controls (Langereis et al., 2011). Neutrophils can migrate to the respiratory tract under the control of chemotactic factors (Traves et al., 2002), for example, leukotriene (LK)-B_4 has been shown to be increased in the airways of patients with COPD (Di Stefano et al., 2009). The percentage of neutrophils in sputum is increased in COPD patients,

and further enhanced during an exacerbation (Papi et al., 2006). Relationships have been demonstrated between increased levels of neutrophils in bronchial biopsies and sputum and the decline in forced expiratory volume in one second (FEV_1) (Singh et al., 2010).

Macrophages demonstrate a five to ten-fold increase in the lungs of patients with COPD and are localised to sites of alveolar destruction with macrophage numbers correlating to disease severity (Vlahos and Bozinovski, 2014). Cigarette smoking activates macrophages to release inflammatory mediators, as well as promoting the secretion of proteases in a variety of pulmonary diseases (Thomas, 2001). Compared with macrophages from normal smokers those from patients with COPD are more activated, secrete more inflammatory proteins, and have greater elastolytic activity which is further enhanced by exposure to cigarette smoke (Hiemstra, 2013). Increased macrophage numbers may result through increased recruitment from the circulation in response to monocytic chemokines such as monocyte chemotactic peptide (MCP)-1, which is increased in sputum and bronchoalveolar lavage (BAL) of COPD patients (Barczyk et al., 2001). Cysteine X Cysteine (CXC) chemokines also act as chemo-attractants to monocytes. Many of the inflammatory mediators that are expressed in COPD are controlled by the transcription factor nuclear factor κB, which is up-regulated in sputum alveolar macrophages in COPD patients compared to control non-smokers (Caramori et al., 2003). This up-regulation may be a key molecular mechanism involved in the on-going and evolving inflammatory process found in COPD airways.

Dendritic cells (DC) are antigen-presenting cells responsible for immune homeostasis. They initiate and orchestrate innate and adaptive immunity in responses to tissue damage or infection. DC circulate in the blood and localise to mucosal surfaces in immature form where they act as sentinels, sampling constituents of the external environment that breach the

epithelium. With internalisation of the antigen, they become activated, mature, and migrate to draining lymph nodes to induce cellular proliferation and to regulate the balance of Th1/Th2 T-lymphocytes. There is evidence in humans to show that cigarette smoking induces the recruitment of large numbers of immature DC into the small airways of COPD patients (Givi et al., 2012). It is hypothesised that chronic exposure to cigarette smoking impairs the normal maturation process of DC and subsequently alters/suppresses their normal function and interaction with naive lymphocytes, resulting in an imbalance of immunity that may increase susceptibility of patients with COPD to respiratory infections (Tsoumakidou et al., 2008).

Eosinophil levels are frequently elevated in COPD, especially during exacerbations (Vedel-Krogh et al., 2015). Eosinophils are similar to neutrophils, containing high concentrations of reactive oxygen species (ROS) and proteases both of which contribute to host defence and collateral damage to the host epithelium (Crotty Alexander et al., 2015). Recent work has revealed that blood eosinophil levels in COPD subjects correlate with elevated eosinophil levels in the bronchial submucosa as well as an increased thickness of the basement membrane (Eltboli et al., 2015). Eosinophilic airway inflammation in COPD may also be predictive of corticosteroid responsiveness during clinical stability and exacerbations (Singh et al., 2014).

Once activated these inflammatory cells release a wide variety of cytokines and inflammatory mediators that propagate/amplify the inflammatory events by modulating the behaviour of airway wall-forming cells, including epithelial cells, fibroblasts and airway smooth muscle cells (Figure 1.1). The combination and interaction of mediators attract and activate inflammatory cells and proteinases, which cause elastolysis and mucus hypersecretion culminating, in the typical pathophysiological features of COPD (Chung and Adcock, 2008).

Overall a wide range of inflammatory mediators have been demonstrated to be elevated in COPD and to amplify the inflammatory process (Figure 1.1). These include lipid mediators such as LTB_4, which is chemoattractant for neutrophils (Griffiths et al., 1995). Chemokines MCP-1 (Deshmane et al., 2009) and macrophage inflammatory protein (MIP)-1α which attract monocytes (Menten et al., 2002). Interleukin (IL)-8 (Mukaida, 2003) and GRO-α (growth related-oncogene) (Bechara et al., 2007), which attracts both neutrophils and monocytes. Interferon inducible protein (IP)-10 (Taub et al., 1993), which attracts $CD8^+$ cells, ROS and nitric oxide (NO) (Domej et al., 2014); granulocyte-macrophage colony-stimulating factor (GM-CSF) (Shi et al., 2006), which prolongs neutrophils' survival; tumour necrosis factor (TNF)-α (Ferrarotti et al., 2003), which amplifies inflammation by switching on multiple inflammatory genes and may also account for some of the systemic effects of the disease; endothelin (Spiropoulos et al., 2003) and transforming growth factor (TGF)-β (Konigshoff et al., 2009), which induce development of fibrosis.

The pro-inflammatory factors released by activated immune cells stimulate the resident cells to produce and secrete additional pro-inflammatory factors, thereby attracting more immune cells to the site of inflammation, thus leading to vicious circle which sustains the inflammatory process (Agusti, 2005). Yet despite the significant role played by inflammation in COPD, anti-inflammatory treatments do not appear to modulate disease progression, or decrease inflammatory markers in induced sputum (Culpitt et al., 1999). Although lung function improves, or at least the rate of decline decreases, after patients with COPD cease smoking, airway inflammation persists despite the removal of the assumed stimulus (Rutgers et al., 2000a). An explanation for this may be the failure to excrete particular components of cigarette smoke, or that damaged tissues cannot be repaired back to normal (Hogg, 2006). This is highlighted by the different healing responses in cells and structures. For example, epithelial

cell layers and goblet cells appear to regenerate towards their distribution and function in non-smokers; however, there is no evidence that the increased mass of smooth muscle in the bronchiolar airways shows the same pattern (Thorley and Tetley, 2007).

1.4.2. Protease/anti-protease imbalance

In the simplest sense, emphysema is caused by the imbalance between proteases and anti-proteases that results in parenchymal lung destruction (Churg and Wright, 2005). Cigarette smoking-induced inflammation is also linked to an increased expression of proteases that degrade the extracellular matrix particularly elastin and enhance lung tissue remodelling in COPD (Shapiro, 2002). Important to understanding this aspect of COPD pathogenesis were the observations of an association between AAT and the development of early onset emphysema (Fischer et al., 2011). These observations form the basis of the protease/anti-protease hypothesis, which describe that under normal circumstances the release of proteolytic enzymes from inflammatory cells migrating to the lungs to fight infection does not cause lung damage because of inactivation of these proteolytic enzymes by an excess of inhibitors. However, in conditions of excessive enzyme load, or where there is an absolute or a functional deficiency of anti-proteases, an imbalance develops between proteases and anti-proteases in favour of proteases, leading to uncontrolled enzyme activity and degradation of lung connective tissue in alveolar walls, resulting in emphysema (MacNee, 2005).

Elastin along with microfibrils are the two main components of elastic fibres which are major extracellular matrix assemblies. Elastin is formed following the assembly and cross-linking of its soluble precursor tropoelastin (Wise and Weiss, 2009). The cross-links known as desmosines are unique to elastin and they can be used as a marker of elastin degradation (Luisetti et al., 2008). The quantification of desmosines in sputum, along with increases in

plasma and an elevated free component in urine provide indexes that characterise patients with COPD and can be monitored during the course of the disease and treatments (Ma et al., 2007). After deposition, tropoelastin production is substantially reduced and there is minimal turnover of the mature, cross-linked form of the eventual elastin. The majority of elastin formation in mammals occurs during late foetal and in early neonatal periods, and at maturity the production of new elastin ceases (Swee et al., 1995). In the event of injury, the production of tropoelastin can be rapidly restarted. Production is influenced by exogenous factors such as TNF-α, IL-1β, insulin-like growth factor-1 and TGF-β (Pierce et al., 2006). A specific set of proteases, broadly grouped under the name elastases are responsible for the remodelling of elastin. The MMPs are particularly important in breakdown of elastin with MMP-2, -3, -9 and 12 explicitly shown to degrade elastin (Antonicelli et al., 2007). Interconnecting elastic fibre cables facilitate coordinated expansion and relaxation of alveoli during respiration, and emphysema progression is linked to destruction of these alveolar elastic fibres by elastolytic proteases associated with an anti-protease deficiency such as in AAT, and impaired extracellular matrix regeneration and maintenance by lung fibroblasts (Zhang et al., 2011). It has been shown that in asthma and chronic bronchitis patients, the levels of active and total elastase are inversely correlated with the degree of airway obstruction as assessed by FEV_1 (Vignola et al., 1998). Hamster lungs exposed to elastase have shown that although elastin synthesis after injury restores elastin content (Lucey et al., 1998), it does not restore normal lung architecture (Kuhn et al., 1976). Together with destruction of elastin plus the abnormality in elastin synthesis, inactivation or reduction of anti-proteases and elevation of proteases is central to the protease/anti-protease hypothesis.

1.4.3. Oxidative stress

Tobacco smoke contains high concentrations of free radicals and other oxidants. Oxidants generated in the biological fluids are efficiently scavenged by antioxidants. Sources of oxidative stress arise from inflammatory cells, such as neutrophils and macrophages, which generate reactive oxygen species (ROS) when activated. There is now considerable evidence for the increased generation of ROS in COPD (MacNee, 2001). Together with inhaled ROS and reactive nitrogen species (RNS), these endogenous and exogenous oxidants may constitute a major oxidative burden to the lung. ROS and RNS are thought to be critical in amplifying the normal inflammatory response to cigarette smoke/environmental oxidants, through the up-regulation of redox-sensitive transcription factors, and hence pro-inflammatory gene expression (Montuschi et al., 2000).

1.4.4. Systemic manifestations of COPD

Studies have shown that some of the above-described events and consequent mediators of COPD inflammation are also reflected systemically and expressed as well-documented clinical non-pulmonary manifestations of the disease (Barbu et al., 2011). Adding to the complexity of the COPD journey, major systemic consequences and co-morbidities are now recognised including: deconditioning, exercise intolerance, skeletal muscle dysfunction, osteoporosis, metabolic impact (Posluszna and Doboszynska, 2011), anxiety and depression (Yohannes and Alexopoulos, 2014), cardiovascular disease and lung carcinoma (Yang et al., 2011, King, 2015). It is unclear however whether non-pulmonary manifestations of COPD are the results of: (1) inflammatory processes in the lung parenchyma that "spill-over" into the systemic circulation; (2) tobacco smoking although sustained inflammation persists even on smoking cessation; (3) pathophysiological changes within COPD lungs leading to systemic effects for example, hypoxia, hyperinflation (Bailey et al., 2012).

1.5. Diagnosis of COPD

In the majority of cases COPD commences decades before the onset of symptoms and runs an insidious undiagnosed course over years. Globally, determinants of COPD under-diagnosis are male sex (except in Spain), younger age, never and current smoking, lower level of education, absence of reported symptoms, lack of previous spirometry, and milder severity of airflow limitation (Lamprecht et al., 2015). Identification of this undiagnosed (preclinical phase) and thus early recognition of cases may therefore have the potential to reduce the future burden of morbidity and mortality for COPD for example, by providing support for early smoking cessation initiatives (Soriano et al., 2009). The diagnosis of COPD is frequently at a relatively late stage of the disease; most patients are not diagnosed until they are over 50 years of age. Patients suffering from more severe COPD have substantial limitations in activities of daily living (ADL), leading to poor health related quality of life and disability (Gore et al., 2000).

Several studies have attempted to generate algorithms to identify populations that would be suitable for spirometry in an attempt to identify undiagnosed COPD. It has been demonstrated that early detection can be achieved by offering spirometry to adults with tobacco or relevant occupational exposure and at least one respiratory symptom such as exertional breathlessness, chronic cough, regular sputum production, frequent winter bronchitis or wheeze (Ulrik et al., 2011). Age, smoking intensity and smoking status among patients with any kinds of acute respiratory infections attending urgent primary care have also been demonstrated to be a feasible and effective way to triage those who need spirometry testing to confirm the presence of underlying COPD (Sandelowsky et al., 2011).

Overall there remains an unmet need for screening programmes to identify preclinical and clinical COPD in an attempt to improve burden of the disease. Presently however, no consensus

exists on how, when and where spirometry should be offered to screen for COPD despite the obvious advantages an earlier diagnosis would provide (Soriano et al., 2009). A recent systematic review and meta-analysis looking at the effectiveness of case finding strategies for COPD concurs with Soriano et al. This review suggests that well-conducted randomised control trials comparing case finding strategies are needed to identify the most effective target population, recruitment strategy and screening tests to identify individuals with COPD (Haroon et al., 2015).

In conclusion a diagnosis of COPD is confirmed when a patient who experiences the above symptoms is found to have non-reversible airflow obstruction on spirometry. This is defined as a post bronchodilator FEV_1/ Forced vital capacity (FVC) ratio of less than 0.70, in the absence of an alternative explanation for the symptoms for example, left ventricular failure, or other diseases that cause airflow obstruction for example, asthma (Qaseem et al., 2011).

1.5.1. Spirometry

As discussed above spirometry is required to confirm a diagnosis of COPD. Severity of the disease is then stratified according to FEV_1 (Postma et al., 2011), which is the best single correlate of mortality in COPD (Celli, 2000). FEV_1 however is not predictive of disease progression (Man et al., 2006) with the rate of decline of FEV_1 demonstrating variability in different COPD patients for example, COPD patients with persistent lower airway bacterial colonisation and frequent exacerbator status (greater than 3 per annum) (Donaldson et al., 2005).

Spirometry as a test requires large resources and is impractical to monitor patients at more than 3 monthly intervals. Also as the patient's condition deteriorates the ability to perform the test

becomes more difficult. Evidence suggests that spirometry should be used to assess for COPD in patients with respiratory symptoms but not those who are asymptomatic (Qaseem et al., 2011).

1.5.1.1. Severity of COPD

Consensus on a working classification for severity stratification of COPD patients has taken many years to achieve. Initially there was disagreement between the European Respiratory Society (ERS), ATS and British Thoracic Society (BTS) on whether spirometry should be performed post bronchodilatation; the ratio of FEV_1/FVC that defines airway obstruction and the FEV_1 thresholds for different stages of the disease (Siafakas et al., 1995, BTS., 1997, ATS., 1995).

In 1997, in an effort to streamline global approach to COPD, its management and prevention the United States National Heart, Lung, and Blood Institute, together with the World Health Organization formed the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The first step in the GOLD programme was to prepare a consensus report, Global Strategy for the Diagnosis, Management, and Prevention of COPD; this drove the evolution of the GOLD classification of COPD severity and was published in 2001 (Table 1.1). All spirometric measurements were performed post bronchodilator therapy (Pauwels et al., 2001).

Table 1.1: 2001 GOLD classification of COPD severity.

	Stage			
	0 (at risk)	**I (mild)**	**II (moderate)**	**III (severe)**
FEV_1/FVC (ratio)	normal	<0.70	<0.70	<0.70
FEV_1 (% predicted)	normal	≥ 80%	30% ≤ FEV_1 < 80% (IIA: 50% ≤ FEV_1 < 80%), (IIB: 30% ≤ FEV_1 < 50%)	FEV_1 < 30% or FEV_1 < 50% and respiratory failure* or clinical signs of heart failure
Symptoms	Chronic symptoms (cough and sputum production)	With or without chronic symptoms (cough and sputum)	With or without chronic symptoms (cough, sputum, dyspnoea)	

*Respiratory failure: arterial partial pressure of oxygen (PaO_2) less than 8.0kPa (60mmHg) with or without arterial partial pressure of CO_2 ($PaCO_2$) greater than 6.7kPa (50mHg) while breathing air at sea level

The GOLD Science Committee aimed to post a yearly update to the consensus report and revise the entire document every five years. The 2003 update maintained the framework of the original classifications but the stages of severity were adjusted (Table 1.2). This was in line with recommendations that were being proposed by the COPD Guidelines Committee nominated jointly by the ERS and ATS (Fabbri and Hurd, 2003).

Table 1.2: Updated COPD severity classification GOLD 2003.

	Stage				
	0 (at risk)	**I (mild)**	**II (moderate)**	**III (severe)**	**IV (very severe)**
FEV_1/FVC (ratio)	normal	<0.70	<0.70	<0.70	<0.70
FEV_1 (% predicted)	normal	≥ 80 % predicted	50% ≤ FEV_1 < 80%	30% ≤ FEV_1 < 50%	FEV_1 < 30% or FEV_1 < 50% predicted and chronic respiratory failure*
Symptoms	Chronic symptoms (cough and sputum production)	With or without chronic symptoms (cough and sputum)	With or without chronic symptoms (cough and sputum)	With or without chronic symptoms (cough and sputum)	

*Respiratory failure is defined as an arterial partial pressure of oxygen (PaO_2) less than 8.0kPa, with or without an arterial partial pressure of CO_2 ($PaCO_2$) greater than 6.7kPa whilst breathing at sea level.

Accordingly, in 2004, both the ATS and the ERS updated their original classification published in 1995 (Celli and MacNee, 2004). The classification now based on post-bronchodilator values mirrored GOLD although they did not incorporate an FEV_1 less than 50% predicated with respiratory failure as Stage 4 disease. In the same year the BTS guidelines (BTS, 1997) were superseded by the National Institute for Health and Clinical Excellence (NICE) published guidelines (NICE, 2004). The NICE criteria for COPD diagnosis using spirometry remained the same and the FEV_1 thresholds for staging were brought into line with GOLD, however the definition for each severity stage was different.

In 2006 GOLD released its revised consensus report (in line with its 5-year cycle) (Rabe et al., 2007). The only change to spirometric classification of COPD disease severity was the removal of Stage 0. This was in recognition that there was incomplete evidence that the individuals who met this definition were at an increased risk of progressing to Stage I of the disease (Vestbo and Hogg, 2006).

Eventually, NICE-revised guidelines on COPD stages in 2010 mirrored the GOLD classification, albeit with the emphasis that symptoms had to be present in Stage 1 to make a confident diagnosis of COPD (NICE, 2010). This guideline remains unchanged although a review decision with regards to an update (July 2014) is awaited (NICE, 2010).

GOLD released another revised consensus report in 2011 with no change in the spirometric classification of severity; however, to better reflect the complexity of COPD a multidimensional mode of classification was incorporated. The "Combined COPD Assessment" combined symptom burden based on either modified MRC score or COPD Assessment Test (CAT), exacerbation frequency per annum and spirometric classification of severity to assign patients into 4 risk groups (Figure 1.2) (GOLD, 2016).

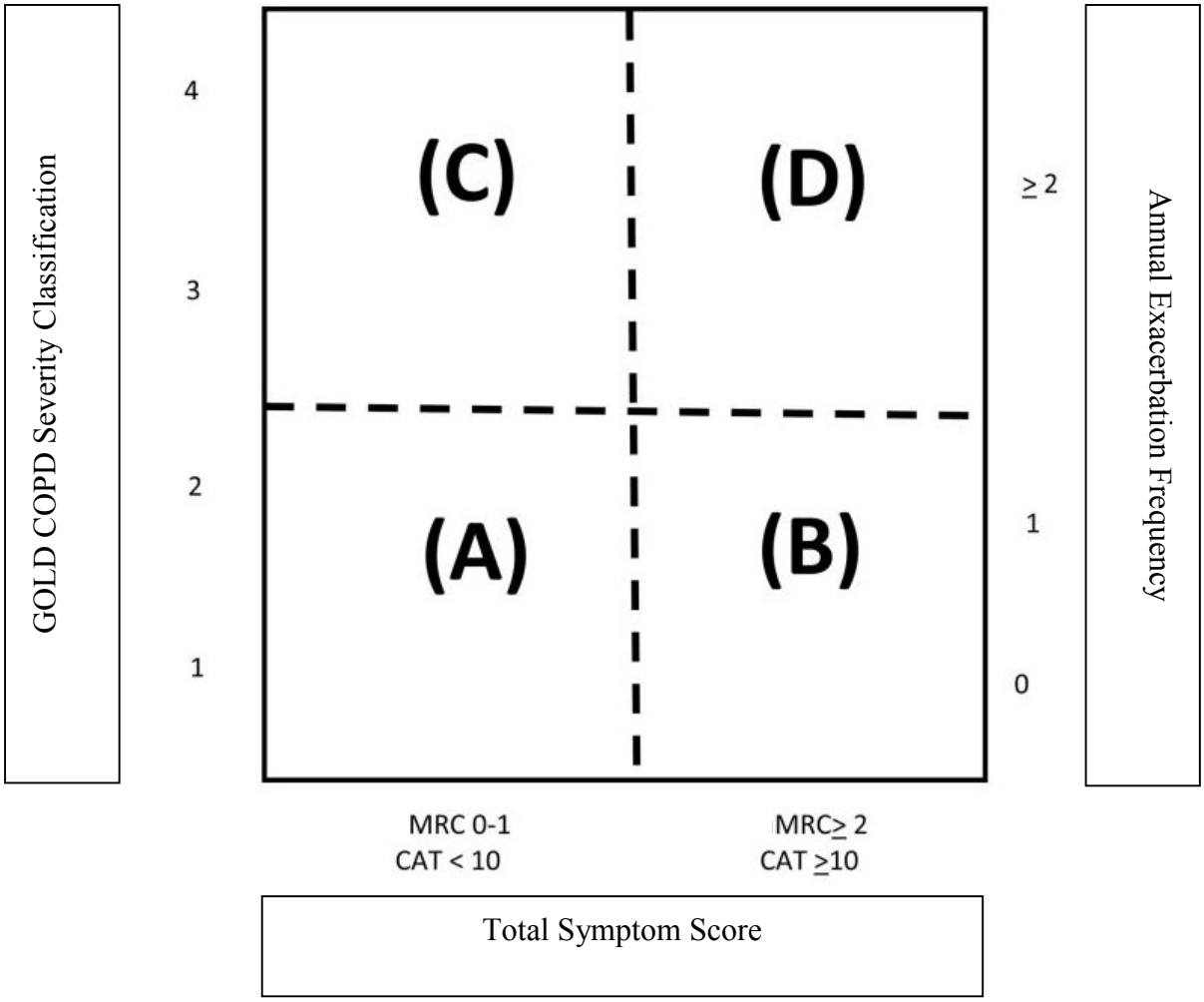


Figure 1.2: Combined COPD Assessment.

A multidimensional risk staging assessment (adapted from GOLD, 2016). A = Low risk, less symptoms, B = low risk, more symptoms, C = high risk, less symptoms, D = High risk, more symptoms. CAT = COPD assessment tool, MRC = Medical Research Council.

The most recent GOLD update 2015 maintains the spirometric severity staging, however the Combined COPD assessment (Figure 1.2) has been amended and places COPD subjects in category "D" if they have suffered with at least one exacerbation in the preceding 12 months leading to hospitalisation (GOLD, 2016). Comparison of the two classification systems reveals that spirometry alone accurately reflects prognosis whereas the Combined COPD assessment provides better diagnostic separation regarding predicting exacerbations (Lange et al., 2012).

It has also been demonstrated that classification of COPD by either the modified MRC or CAT scores is not identical and thus care should be taken when using the Combined COPD assessment to stratify patients (Kim et al., 2013). In conclusion the Combined COPD assessment appears to have a role in risk stratifying exacerbations but its role in prognosis has yet to be determined.

FEV_1 as described above remains the main index for diagnosis, disease stratification and mortality prediction. Yet it has been observed that other pulmonary and extra-pulmonary variables for example, MRC score, also predict mortality and this has led to the development of various multimodal dimensional indices (van Dijk et al., 2011). BODE (**B**ody mass index, airflow **o**bstruction (FEV_1 % predicted), **d**yspnoea (modified MRC score) and **e**xercise capacity (6-minute walking distance)) at present is the most validated and has been shown to predict survival in COPD (Marin et al., 2013). BODE was developed in the late 1990's and was found to better than FEV_1 at predicting the risk of death from any cause and from respiratory causes in COPD patients (Celli et al., 2004). Marin et al., conducted a pooled analysis on the individual data of 3633 patients from 11 COPD cohorts which revealed that whilst no COPD multicomponent index predicts short term survival up to 12 months accurately however the most valid multicomponent index to predict time of death in all COPD patients is BODE (Marin et al., 2013).

1.5.2. Patient reported outcomes

It is increasingly recognised that the frequent assessment of symptoms is a necessity in the stratification and prognostication of COPD (Vestbo et al., 2013). Spirometry (FEV_1) is a relatively poor correlate of symptoms such as breathlessness and the impact of COPD on quality of life. Furthermore, many consequences of COPD, including anxiety, depression and

the ability to perform ADL, can only be described and reported reliably by the patient (Jones et al., 2012). Patient reported outcomes (PRO) thus have an ability to provide an estimate the of all the effects of a disease on a patient (Jones et al., 2012).

Dyspnoea (breathlessness) is one of the predominant symptoms and is often the reason a patient will seek medical attention. In the 1950's when research was being undertaken to define COPD, a standardised questionnaire on respiratory symptoms was developed to help aid diagnosis. Its validity and reproducibility was tested in a survey of Post Office workers in London, although observer and subject error were present, bias could be reduced to a degree which made the method suitable for epidemiological comparison (Fairbairn et al., 1959). The questionnaire was later accepted and published by the MRC's Bronchitis Research Committee, with subsequent widespread international recognition (Fletcher, 1978). The widely used modified MRC dyspnoea score (Table 1.3) was adopted from the questionnaire's "Breathlessness" section.

Table 1.3: Modified MRC dyspnoea score.

	Degree of breathlessness related to activities
Grade 1	Not troubled by breathlessness except on strenuous exercise
Grade 2	Short of breath when hurrying or walking up a slight hill
Grade 3	Walks slower than contemporaries on level ground because of breathlessness, or has to stop for breath when walking at own pace
Grade 4	Stops for breath after walking about 100 metres or after a few minutes on level ground
Grade 5	Too breathless to leave the house, or breathless when dressing or undressing

The development of the MRC score has helped to quantify symptoms and it has been demonstrated to be a simple and valid method of categorising COPD patients in terms of their disability; although no association has been demonstrated with FEV_1 (Bestall et al., 1999). The modified MRC score (herein described as MRC score) quantifies the disability associated with breathlessness and correlates well with direct measurements of disability such as walking distance. Its main disadvantage over other more complex scales for example, St George's Respiratory Questionnaire (SGRQ) is its relative insensitivity to change (Stenton, 2008). This provides an insight between disability and physiological parameters and confirms that the phenotyping of COPD is complex. MRC score is related to 3-year mortality after hospitalisation for an acute exacerbation of COPD and has been shown to be an independent determinant of length of stay (Tsimogianni et al., 2009). More recently a study has tried to investigate whether a symptom-based case finding questionnaire and MRC score can be used to identify "at risk" individuals. In adults with known risk factors, the likelihood of having moderate to severe COPD is increased in those who report an MRC score of 3 or more common respiratory symptoms or a score of 4 or 5. However, selecting individuals for spirometry based on symptoms alone only identified less than half of those with moderate to severe COPD (Hill et al., 2011).

In conjunction with the MRC score a variety of other PRO questionnaires have been developed. Presently the clinical COPD questionnaire (CCQ), Exacerbation of Chronic Pulmonary Disease Tool (EXACT) and CAT have demonstrated some validity and responsiveness to treatment in various studies (Cazzola et al., 2015). CCQ has similar psychometric properties to CAT and exhibits reliability, validity and high reproducibility (Tsiligianni et al., 2012). EXACT appears to be an effective method at assessing the severity of acute exacerbations of COPD however doubts remain about the ability of EXACT to accurately detect exacerbations

(Mackay et al., 2014). CAT has been developed as a short, simple questionnaire that has been demonstrated to be an effective tool to measure health status in patients with COPD (Minov et al., 2015). These PRO scoring systems are summarised in Table 1.4.

Table 1.4. Summary of COPD scoring systems.

Score	Variables	Pros	Cons
MRC	Breathlessness	Simple and valid score for catergorising disability	No association with FEV_1.
CAT	Cough, Phlegm, Chest tightness, Breathlessness, Activities of daily living, Confidence, Sleep, Lethargy	Reliable, valid, high reproducibility for measuring health status.	Not validated for determining the severity of an exacerbation
EXACT	Breathlessness, Cough Sputum Chest symptoms.	Reliable and valid for monitoring respiratory symptoms.	Not validated for the detection of an exacerbation.
BODE	Body mass index, Airflow obstruction (FEV_1) Dyspnoea Exercise capacity	Better than FEV_1 for predicting all-cause mortality.	Not designed to be used as a daily scoring system.
CCQ	Breathlessness, Activities of daily living, Cough Phlegm.	Similar to CAT.	

1.5.3. Ancillary investigative tools

In an attempt to improve the understanding of COPD pathogenesis diagnosis and staging of the disease research has also focused on the role of lung-focused tools. These include radiology to directly image the lungs and the analysis of lung-exposed body-fluids for example, BAL and sputum.

1.5.3.1. Imaging Techniques

It is increasingly clear that spirometric measures of lung function alone are inadequate for a complete understanding of impact of COPD and are insufficient for the complete categorisation of COPD severity. As discussed, (Section 1.5.2, Page 27) PROs attempt to provide the patient's perspective and accompany objective spirometry in the classification and monitoring of COPD. To further characterise COPD, techniques in chest imaging and quantitative image analysis have advanced to a point where they can now provide novel *in-vivo* insights into the disease and potentially the response to its treatment (Washko, 2012). High resolution computer tomography (HRCT) is useful in determining emphysema in patients who are diagnostically challenging and can also reveal associated disease processes for example, bronchiectasis which are not picked up on conventional imaging (Patel et al., 2004, Nakano et al., 2000). HRCT can also better characterise the extent of emphysema which is known to be a primary cause of symptom-related burden and increased mortality (Haruna et al., 2010). Additionally, greater lung emphysema and airway wall thickness assessed by HRCT are associated with exacerbation frequency (Han et al., 2011).

Presently radiological modalities are not used routinely in the diagnosis or monitoring of COPD but as discussed they can play a vital role in cases that are complex and where concomitant pathologies are present. Radiology also has a place in diagnostically challenging COPD

individuals and identification of pneumonia (defined as new consolidation on chest X-ray)-driven acute exacerbations especially as the presence of pneumonia in COPD acute episodes is likely to be underestimated (Finney et al., 2015).

1.5.3.2. Bronchoalveolar lavage

BAL is used as a tool for direct sampling from the lung to explore the pathogenesis of COPD (Zheng et al., 2000, Molet et al., 2005). Importantly BAL has been shown to sample a different compartment of the lungs compared to sputum (Tsoumakidou et al., 2003). Several biomarkers have been measured in BAL, primarily eosinophil cationic protein, myeloperoxidase, TNF-α and IL-8 which have been demonstrated to be increased in COPD and healthy smokers compared with healthy non-smokers. IL-8 levels are increased in patients with frequent exacerbations (Tumkaya et al., 2007). Surfactant protein A has been suggested as a potential diagnostic biomarker with BAL results correlating to levels in induced sputum (Ohlmeler et al., 2008). BAL however cannot be widely adopted in clinical practice as it requires concurrent bronchoscopy which many COPD patients irrespective of disease state find difficult to tolerate.

1.5.3.3. Sputum

Sputum is a widely sampled body fluid in COPD as it is produced directly in the lungs and expectorated daily by the majority of patients. The analysis of sputum has improved the understanding of airway inflammation in COPD; however as not all COPD patients produce sputum, induction of sputum by hypertonic saline is also utilised (Rutgers et al., 2000b). Induced sputum has been shown to demonstrate the same inflammatory profile as spontaneous sputum (Tsoumakidou et al., 2003). Sputum induction however is not tolerated by all and questions remain about repeatability (Djukanovic, 2000). In COPD patients with chronic sputum production are found elevated levels of eosinophils, neutrophils, eotaxin (an eosinophil

chemoattractant), IL-6, MCP-1 and TNF-α compared to non-sputum producers (Khurana et al., 2014). Key exacerbation-related sputum biomarkers have also been identified including sputum IL-1β for bacterial-driven exacerbations (Bafadhel et al., 2011b), with IL-6 appearing to be raised in patients who have respiratory viruses isolated by nasal lavage (Rohde et al., 2008).

The assessment of sputum in clinical practice can be split into subjective observations of colour, volume and texture and direct culture of sputum to identify respiratory pathogens. Changes in sputum colour can reflect an acute exacerbation of COPD with the presence of green being highly sensitive (95%) for the presence of a bacterial exacerbation (Stockley et al., 2000). An increase in sputum volume (Wedzicha and Donaldson, 2003) and increased sputum viscosity (texture) (Bhowmik et al., 2009) have also been is also associated with acute exacerbations. COPD patients who have potentially pathogenic microorganisms in the sputum demonstrate an exaggerated airway inflammatory response and poor health status compared to those who have non-potentially pathogenic sputum even in the stable phase of their disease. These COPD patients may represent the frequent exacerbator sub-group (Banerjee et al., 2004). Interestingly however NICE does not recommend the routine screening of sputum in COPD patients who are stable or undergoing an acute exacerbation in primary care and only recommend purulent sputum microscopy and culture for COPD patients referred to hospital (NICE, 2010).

In summary, the current available tools for COPD monitoring provide meaningful clinically relevant information but the numerous modalities and frequency of testing places a large burden on COPD patients and consequently NHS resources. Additionally, some tests for example, sputum, cannot be performed by all patients and others for example, spirometry, are

not sensitive enough for routine monitoring and not performed in practice frequently enough other than on a 6 months to annual basis. The implications of this on COPD disease surveillance and patient self-management provides support to the argument of the need to develop more encompassing tests *or* at least to determine the positioning of currently available tools (spirometry, PROs and biomarkers) in the COPD process and care pathway (Agusti et al., 2011). This needs to be done in conjunction with the utilisation of minimally invasive and intrusive methodologies; recognising the increasing shift change in patient care away from hospital bed-side towards patient self-management programmes and treatments within the comfort of patients' homes.

1.6. Acute exacerbations of COPD

The natural course of COPD is characterised by a chronic progressively declining disease state punctuated with episodes of sudden worsening in symptoms called acute exacerbations. These exacerbations reflect a more severe, disruptive and limiting phase in the patient's illness; a period often associated with high-cost medical and support interventions as well an increased risk of death (Toy et al., 2010). Prevention, early detection, and prompt treatment would have an important impact on clinical progression and improving quality of life in patients (Wilkinson et al., 2004). COPD exacerbations are difficult to define precisely due to two main issues: fluctuating symptoms and possible presence of co-morbid conditions. Patients experience a day to day variability in their symptoms (Kessler et al., 2011) and thus to characterise an exacerbation purely based on PRO, it is necessary to pinpoint when a sustainable decline in any of these continuous measurements is worse than expected. Presence of co-morbidities such as cardiovascular disease could potentially bias interpretation as they share common symptoms such as dyspnoea and acutely can also precipitate respiratory dysfunction.

1.6.1. Definition of an exacerbation

The current definition follows on discussions of the workshop, "COPD: Working Towards a Greater Understanding" (Rodriguez-Roisin, 2000). It was agreed that an exacerbation of COPD is "a sustained worsening of the patient's condition, from the stable state and beyond normal day-to-day variations, that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD".

This general description however poses operational challenges for detailed phenotypic characterisation of COPD exacerbations. The definition lacks particular clarity in regards of

whether the reported changes should be quantitative and/or qualitative. Indeed the duration of symptom change that qualifies as an exacerbation varies within studies (Han et al., 2010).

Rodriguez-Roisin et al., proposed a further generalised sub-classification based on healthcare utilisation but this has not been widely accepted. Healthcare utilisation is recognised as an inadequate substitute for episode severity, as it depends on many social and co-morbidity factors, and is now considered an outcome in its own right (Burge and Wedzicha, 2003).

Consequently, in clinical practice it is now more generally accepted that a COPD exacerbation identifies an episode where the patient seeks medical intervention rather than a predefined change in one or more symptoms (Rodriguez-Roisin, 2000, Calverley, 2005). Attempts have been made to create a more comprehensive definition and sub-classification of COPD exacerbations, with limited significant progress (Caramori et al., 2009). New approaches continue to be explored in an attempt to identify key body-fluid biomarkers that can be used as diagnostic tools to enable better stratification of the exacerbations and to determine underlying aetiology and guide treatment (Pauwels et al., 2004, Barnes et al., 2006, Thomsen et al., 2013).

1.6.2. Aetiology of an exacerbation

COPD exacerbations are often triggered by airway infection either with bacterial or viral pathogens (Wedzicha and Seemungal, 2007) and pollution (Ling and van Eeden, 2009) (Chapter 1, Page 37). During acute COPD exacerbations there is increased bronchial wall inflammation with an influx of eosinophils, neutrophils and lymphocytes (Bathoorn et al., 2008).

- *Bacterial Infections:* The majority 50 to 69% of acute exacerbations of COPD are the result of a bacterial infection (Butorac-Petanjek et al., 2010). The most commonly isolated organism is Haemophilus influenzae (De Serres et al., 2009) accounting for 50% of all sputum isolates (King 2012) alongisde Klebsiella pneumonia, and Pseudomonas aeruginosa (Ma et al., 2015).
- *Viral Infections*: Approximately one third of acute exacerbations of COPD are associated with viruses and recent work suggests that a more severe inflammatory response is associated with viral compared to bacterial exacerbations (Clark et al., 2015). Viral pathogens are also thought to be partially related to the increased influx of eosinophils as described above (Rohde et al., 2008). The commonest viral pathogen is human rhinovirus (Wilkinson et al., 2006).
- *Co*-existing *viral and bacterial infections*: It has been demonstrated that in a sub-group of COPD exacerbations there is co-existing bacterial and viral pathogens with a corresponding increase in exacerbation severity and inflammatory response compared to single pathogen exacerbations (Wark et al., 2013).
- *Air pollution*: It has been estimated that up to 9% of admissions for acute exacerbations of COPD may be due to atmospheric pollution (Li et al., 1996). The main irritants include particulate matter, sulphur dioxide, ozone, and nitrogen oxides with evidence indicating an adverse effect of air-pollution on symptom burden and acute exacerbations of COPD (Peacock et al., 2011).
- *Unknown origin*: Approximately 30% of acute exacerbations of COPD are non-specific where no cause can be identified (Sapey and Stockley, 2006). Pulmonary embolisms have been implicated in a third of these cases (Wang et al., 2012).

In view of the mixed aetiology of acute exacerbations of COPD and the importance of pathogen interaction further work has looked to phenotype exacerbations. Using this approach four distinct clusters of exacerbations have been identified: Bacterial (55%), Viral (29%), Eosinophilic (28%) and pauci-inflammatory related to minimal changes in the inflammatory profile (Bafadhel et al., 2011b). Interestingly although increased eosinophil numbers are believed to be in response to viral pathogens as described above they also appear to have a separate cluster to viral exacerbations. This study highlights the importance of more advanced statistical techniques (such as cluster analysis) to understand the heterogeneity of COPD exacerbations and thus identify distinct exacerbation phenotypes, which appear in some COPD patients not to be driven solely by an offending pathogen.

1.6.3. Burden of an exacerbations

Acute exacerbations of COPD resulting in hospitalisation are associated with a higher mortality and morbidity than patients that can be managed at home (Steinmetz et al., 2006). They account for up to 70% of the cost of medical care for these patients, and thus identification of factors that may be associated with acute COPD exacerbations and managing them proactively could result in avoidance of hospital admission and a reduction in healthcare costs (Niewoehner, 2006). In-hospital mortality is approximately 8% (Fruchter and Yigla, 2008), with COPD patients presenting with hypercapnic respiratory failure at increased risk (11%) (Anzueto, 2010). Overall 6-month post-hospitalisation mortality is 24%; with 1-, 2- and 3-year mortality rates of 33%, 39% and 49%, respectively (Gunen et al., 2005). Importantly, however there is evidence to suggest that delays of greater than 24 hours in the initiation of treatment for an acute exacerbation of COPD in patients who subsequently present to an Accident & Emergency (A&E) department results in a higher risk of hospitalisation (Chandra et al., 2009). Hospitalisations are more frequent in winter, and weekend admissions have a higher mortality

(Jenkins et al., 2011, Barba et al., 2011). A large retrospective study looking at winter weekend admissions has shown that the opening of a dedicated medical admissions unit reduces mortality (Brims et al., 2011). Frequent acute exacerbations of COPD result in an accelerated decline in lung function (FEV_1); additionally, exacerbation frequency also increases as the disease becomes more severe (Tashkin, 2011). This results in an impaired quality of life and restricted daily activities of living.

Overall the burden of acute exacerbations needs to be improved with earlier identification and the prompt initiation of treatment (antibiotics and steroids or other as appropriate). This requires intuitive monitoring tools so that COPD patients can effectively self-manage and identify changes in their condition and initiate an appropriate course of action taken either by contacting community-based respiratory specialists or starting stand-by treatment. Indeed, NICE advocates that COPD patients at risk of exacerbations should be given self-management advice that encourages them to respond promptly to the symptoms of an exacerbation. In conjunction to this, COPD patients should be given a course of treatment (antibiotics and steroids) to keep at home for use as part of a self-management strategy (NICE, 2010). However, the majority of patient remain unclear as to when they should ideally start treatment and often delay taking action.

1.7. Development of COPD monitoring tools

Presently there remains an unmet need to develop reliable, sensitive and easily accessible monitoring tools for health status in COPD patients. These tools would ideally help with COPD patient self-management in the stable phase of their disease to predict and thus facilitate/guide the early initiation of treatment for acute exacerbations. This would greatly improve COPD outcomes with consequent socio-economic benefit. The concept of point-of-care (POC) diagnostics in medicine is rapidly expanding as technologies increasingly rely on minimally invasive sampling of body fluids which are not reliant on laboratory infrastructure for processing and analysis. Technological advances in POC testing, primarily miniaturisation and reduction in user operator variables, have also increased their practical utility (Loubiere and Moatti, 2010). POC tests have been successfully deployed in patient self-monitoring and community testing for example, hand-held glucose (Newman and Turner, 2005), International Normalised Ratio monitors (Garcia-Alamino et al., 2010), pregnancy testing (Posthuma-Trumpie et al., 2008) and urine dipsticks which can test for a wide variety of analytes (Wilson and Gaido, 2004). Within the field of respiratory medicine peak flow meters were one of the first POC tests to monitor asthma (Lockhart et al., 1960). Recent developments of POC tests in respiratory medicine are focused on viral and bacterial respiratory pathogen diagnostics (Zumla et al., 2014). Overall it is equally vital to recognise that adoption of POC technologies is not sufficient to achieve improved clinical outcomes unless there is also innovation in the process of delivering care (St John, 2010).

1.7.1. Novel monitoring approaches in COPD

One concept of developing novel POC monitoring tools is to utilise easily accessible patient-produced samples to identify disease-specific biomarkers and enable near-patient testing to determine health status. Presently within the field of COPD several patient-derived sample approaches have been undertaken in an attempt to develop the ideal POC test to monitor disease progress and identify acute episodes.

1.7.1.1. Electronic nose breath analysis

An electronic nose functions by chemically analysing vapour, using a nano-composite array with polymer sensors. When exposed to a gas mixture the sensors swell, thereby changing the electrical resistance, resulting in a unique "smell-print" of differential electrical resistances. Electronic nose technology has been shown to identify AAT deficiency in a population of COPD patients (Hattesohl et al., 2011), as well as discriminating patients with lung cancer from COPD and healthy controls (Dragonieri et al., 2009), and distinguishing between asthma and COPD (Hattesohl et al., 2011, Fens et al., 2011, Dragonieri et al., 2009). It has been shown to have acceptable within-day and between-day repeatability in patients with stable COPD and can be used to identify sub-phenotypes of mild-to-moderate COPD (Bofan et al., 2013, Fens et al., 2013b). A recent study has demonstrated that electronic nose technology can identify the presence of bacterial colonisation in clinically stable COPD (Sibila et al., 2014) and identify acute COPD exacerbations with evidence of bacterial infection (Shafiek et al., 2015). Overall electronic nose breath analysis utilises pattern recognition, without analysing individual molecular components, which has shown promise for diagnostic objectives (Dragonieri et al., 2009). However, the inability to detect specific target analytes leads to the possibility that other substances may produce similar "smell-prints" thus reducing the sensitivity of the technology (Röck et al., 2008). A high degree of variability between repeated samples has also been

demonstrated, in addition to other methodological factors such as breath exertion factor, which still need to be standardised before breath analysis can be reliably used for widespread POC testing (Phillips et al., 2014, Fens et al., 2013a).

1.7.1.2. Exhaled breath condensate

Exhaled breath condensate (EBC) is collected by cooling or freezing exhaled air. This body-fluid has several qualities which are useful when testing for target analytes; it is simple to perform, non-invasive and makes repeated sampling possible (Mutlu et al., 2001). The composition of EBC is mainly water vapour but hundreds of different compounds at trace concentrations can be found from small inorganic ions through large organic molecules to peptides, proteins, surfactants, macromolecules and volatile organic compounds (Peralbo-Molina et al., 2015). The collection procedure has been standardised (Horvath et al., 2005) and there is strong evidence that abnormalities in EBC composition may reflect biochemical changes of airway lining fluid (Bajaj and Ishmael, 2013). Multiple biomarkers have been identified as elevated in EBC of smokers and COPD patients both during stable and acute exacerbations for example, LK-β4 and 8-isoprostane (Biernacki et al., 2003), hydrogen peroxide (Dekhuijzen et al., 1996), prostaglandins (Montuschi et al., 2003) and AAT (Koczulla et al., 2011). A lower pH has also been observed in COPD (Kostikas et al., 2002, Papaioannou et al., 2011); whilst earlier studies showed no change in pH during an acute exacerbation (Antus et al., 2010), recent work has demonstrated a further decrease in pH during an acute exacerbation of COPD when pH testing is performed immediately after exhaled breath sampling. Interestingly by contrast, same samples tested after storage demonstrated a rise in pH (Warwick et al., 2013). Levels of AAT and eicosanoids have also been demonstrated to rise during acute COPD exacerbations (Antczak et al., 2012, Koczulla et al., 2011). Unfortunately, despite numerous studies, analysis of EBC has limitations regarding optimisation and

validation of quantitative analytical procedures; thus it is not possible to make direct comparisons between different laboratories (Dodig and Čepelak, 2013). This between-instrument variation is currently a major limitation in the application of EBC to a POC instrument.

1.7.1.3. Urine

Urine testing is a non-invasive and widely used POC test sample with over-the-counter tests for pregnancy. Urine test strips can quantify ascorbic acid, glucose, bilirubin, ketones, specific gravity, blood, pH, protein, urobilinogen, nitrates and leukocytes (Siemens, 2016). Urine based POC testing has also been developed for drugs of abuse for example, cocaine (Beck et al., 2014).

With respects to COPD the focus has been on detection of desmosine and isodesmosine, both by-products of elastin degradation, in urine. It has been shown that both active smoking and presence of COPD are significantly and independently associated with higher urinary excretion of elastin degradation products (Stone et al., 1995). Levels of both products are also elevated in the urine of COPD patients experiencing a more rapid decline in their FEV_1 (Gottlieb et al., 1996). A small but significant increase in desmosine has been observed during an acute exacerbation of COPD (Fiorenza et al., 2002), and a recent abstract has also identified a panel of urinary biomarkers for example, IL-6 that can detect acute exacerbations of COPD (Gita et al., 2014). Recent work has explored the metabolic signature of urine by using proton nuclear magnetic resonance and identified several biomarkers for example, acetate, which were found to be enhanced in COPD patients (Wang et al., 2013). Interestingly within this aforementioned study metabolic differences between COPD and healthy subjects were more pronounced in urine than serum counterparts. Urine appears to be an ideal sample for incorporating into a

POC test due to ease of collection. However, urine as a POC test is limited in the accuracy of quantifiable levels of analytes and there is no serum-urinary biomarker overlap in COPD. Thus well validated serum-based biomarkers for example, CRP shows no correlation to urinary CRP levels and it is hypothesised that CRP is an unlikely constituent of urine (Chuang et al., 2010). This positions urine as a potential bio-sample significantly behind serum-based testing for valid biomarkers in COPD.

1.7.1.4. Dried blood spot

Dried blood spot (DBS) samples are formed by drops of whole blood collected on filter paper from a simple finger prick. DBS testing is mainly used for large population screening of viral diseases for example, Human Immunodeficiency virus (Lehmann et al., 2013). The availability of DBS samples has also been useful for medical researchers, especially those working in remote, isolated communities with several advantages over traditional methods. DBS can be stored, packaged and even mailed without the logistical challenges that are present with whole blood, urine or saliva samples (Benyshek, 2010). However, one major disadvantage presently is consistency of storage and handling of dried samples, for example humidity and temperature which can affect the target analyte (Denniff & Spooner 2010). DBS samples for C-Reactive protein (CRP) quantification have been shown to be stable for up to 21 days prior to analysis (Cordon et al., 1991). Initial small studies have shown correlations in CRP levels between paired DBS and serum samples (Cordon et al., 1991, McDade et al., 2004). However, DBS levels of CRP are consistently lower than serum values possibly due to inaccurate sample volume estimate or CRP adhering to the testing paper (Brindle et al., 2010). These points are technical limitations that need to be overcome.

Although blood spot testing could be employed as an alternative to the dried spot method and is well established for glucose monitoring, one potential disadvantage is that patients would have to self-prick. Consequences of prolonged self-pricking include scar formation, the loss of perception and pain (Heinemann, 2008). This is not ideal for the COPD patients who are often elderly and who are frequently exposed to courses of systemic steroids with consequent thinning of their skin. Additionally, finger prick anxiety has been observed in 30% of patients with the subsequent avoidance of testing (Shlomowitz and Feher, 2014).

1.8. Saliva

Saliva possesses several functions involved in oral health and homeostasis. It originates from three pairs of major salivary glands (parotid 20%, submandibular 65 to 70% and sublingual 5%) and from a large number of minor salivary glands between 600 to 1000, which exist as small discrete aggregates of secretory tissue present in the submucosa throughout most of the oral cavity (Carranza et al., 2005) (Figure 1.3).

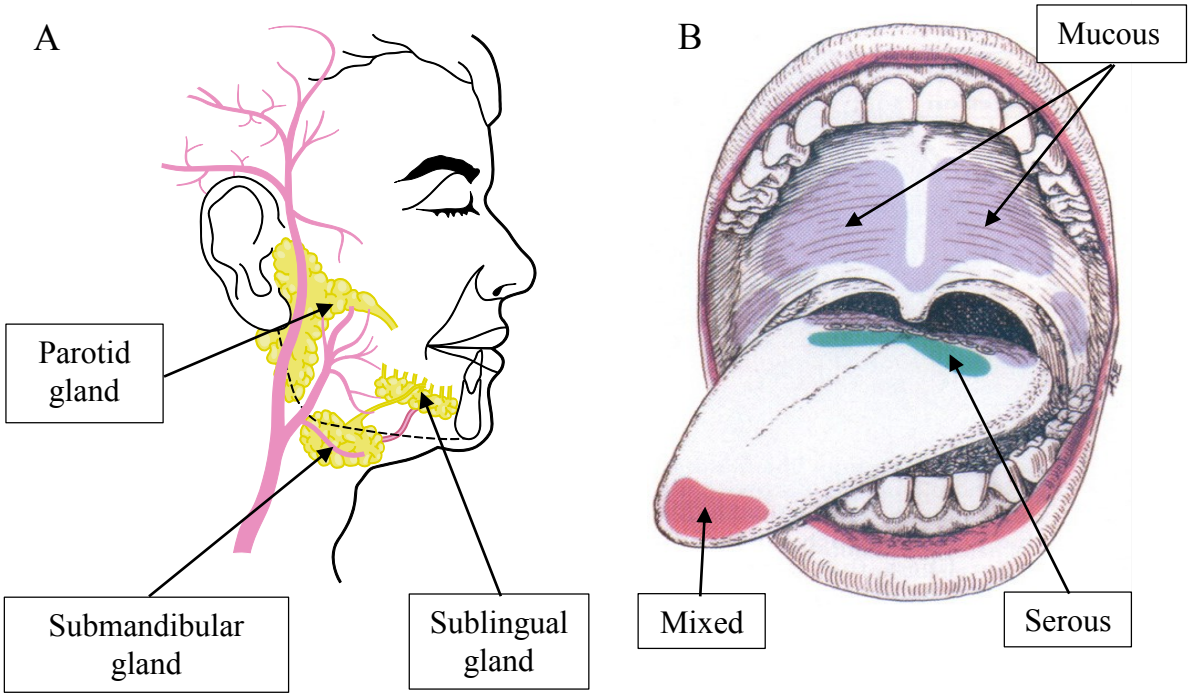


Figure 1.3: Location of major and minor salivary glands

Figure A demonstrates the anatomical location of the major saliva glands contributing 95% to total saliva flow. Parotid = 20%, Sub-mandibular = 65 to 70% and sublingual = 5%. Figure B illustrates the location of the minor salivary glands located in the oral cavity. These minor glands secrete different types of saliva: serous, mucous and mixed sero-mucous.

Saliva composition also has a non-glandular origin, with possible constituents coming from the oropharyngeal mucosa, the gingival crevice epithelia and blood with derived compounds both actively and passively transferred. With increasing emphasis on near-patient and non-invasive

diagnostics, saliva is being increasingly promoted as a suitable alternative diagnostic bio-sample to blood, providing useful information on health status and disease (Wong, 2008).

Saliva has numerous practical advantages including: non-invasive access, simple self- and POC sampling; minimal training for collection; capability and cost effectiveness for multiple sampling and large population screening (Denny et al., 2008). Saliva has been widely and reliably used to identify infectious agents (Gallo et al., 1997, Thieme et al., 1992, Li et al., 1996, Ikuta et al., 2000, Blackbourn et al., 1998), steroid hormones (Trilck et al., 2005), drug monitoring (Mandel, 1993) and disease presence (Tishler et al., 1996, Hanemaaijer et al., 1998, Kaufman and Lamster, 2002, Hu et al., 2008, Miller et al., 2010, Sugimoto et al., 2010, Xiao et al., 2012, Zhang et al., 2012).

Saliva is not just a passive "ultra-filtrate" of serum but a complex multi-factorial body fluid. Saliva contains a variety of molecules; inorganic compounds, organic non-proteins, lipids, proteins, polypeptide compounds and hormones that reflect important pathophysiological activities. The complete proteome of saliva contains approximately 2340 proteins. Major compositional differences between serum and saliva have been demonstrated with approximately 20% of whole saliva proteins found in plasma (Topkas et al., 2012) (Figure 1.4).

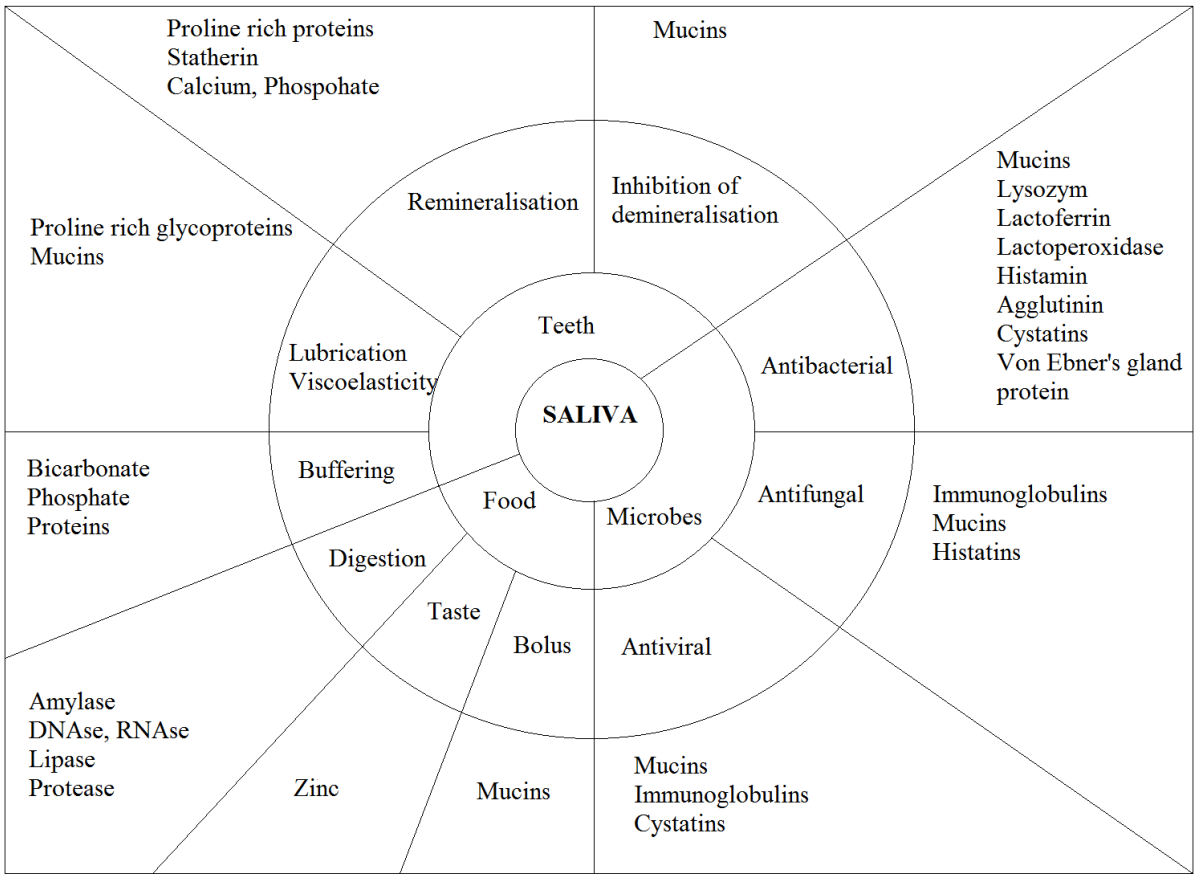


Figure 1.4: Saliva functions and composition.

This figure (adapted from A. Bardow, 2008) illustrates the main functions of saliva its multifactorial role within the oral cavity and the constituent components that drive specific functions.

However, despite these differences, the distribution found across key categories, such as molecular function, biological processes and cellular components, shows significant similarities. The major constituents of human serum are immunoglobulins and albumins which account for 60 to 80% of the total weight (Bjorhall et al., 2005). Overall 99% of total protein content is represented by 22 proteins. This makes the identification of the remaining 1%, which includes inflammatory markers for example, CRP, Procalcitonin (PCT) and NE challenging. However, in whole saliva the 20 most abundant proteins in serum represent only approximately 40% of the entire salivary proteome (Loo et al., 2010: unpublished observations) Whole saliva

has also approximately 15% less proteins than serum (Loo et al., 2010). Thus based on the premise that saliva has a comparatively lower content of "overpowering" serum high abundant proteins, identification and measurement of serum low abundant proteins such as CRP, PCT and NE should be technically feasible in saliva. The possible use of saliva as a body fluid that reflects the systemic characteristics of an individual as does blood is due to its exchange with substances that compose the plasmatic liquid (Lima et al., 2010). This is due to the presence of a thin layer of epithelial cells separating the salivary ducts from the systemic circulation, making it possible for proteins to be transferred into saliva via active carriage, ultra-filtration or passive diffusion via a concentration gradient (Catalan et al., 2009).

1.8.1. Gland specific or whole saliva sampling

A sample of saliva for protein analysis can be either a gland-specific or a heterogeneous sample made up from the individual glands (whole saliva) (Wong, 2008). Gland-specific saliva requires the direct cannulation of the target salivary gland. This is an invasive procedure and used only when there is a specific necessity to test a particular gland's function or a clinical interest in particular analytes. For example, salivary secretory-IgA and α-amylase, do show differences from one salivary gland to another (Crawford et al., 1975, Veerman et al., 1996). Whilst there is the potential to use less invasive absorbent devices to sample specific glandular or mixed rather than whole saliva, this approach would prove difficult to adopt within clinical environments such as for repeated self-sampling in patients at POC or as a method to obtain repeated samples from patients in the community. Absorbent devices can introduce bias if not correctly positioned and may also cause interference in immunoassays (Shirtcliff et al., 2001). Levels of CRP appear to be reduced in glandular compared to unstimulated whole saliva (Topkas et al., 2012); PCT and NE have not yet been explored widely in saliva. Overall whole

unfractionated saliva would appear to be more practical, expeditious and simpler to provide (Nunes et al., 2015).

Although whole saliva can be collected easily, the standardisation of pre-collection parameters (of which there are a number of different factors) need to be considered. These include the type of whole saliva to be sampled, stimulated or unstimulated; time of day of sampling; oral micro-trauma caused by for example, tooth brushing; the particular medications patients may be taking. In addition to these factors food and/or fluid intake prior to sampling may affect composition and flux of salivary components (Chiappin et al., 2007). Storage of fresh saliva samples at room temperature needs to be considered as a rapid protein degradation, has been found to start on sample collection. This can be negated by immediately collecting saliva in ice-cooled containers and also storing freshly collected saliva samples at 4^{o}C (Esser et al., 2008); however long term storage at -80^{o}C is required to maintain protein stability (Schipper et al., 2007). Provided that standardisation of saliva sampling, storage and processing can be optimised, saliva offers a readily available means for quantification of changes of specific biomarkers with potential clinical implications for the diagnosis and management of both oral and systemic diseases. It is important to recognise however that saliva contains lower concentrations of analytes that are found in blood (100 to 1000-fold lower) (Pfaffe et al., 2011). This reduced analyte concentration hinders widespread use of saliva for target analyte quantification as most analysing technology is not sensitive enough (or not available) to detect the lower salivary concentrations of many analytes compared to serum (Hart et al., 2011). Initial challenges in saliva-based analyte quantification have been met with the development of laboratory-based high sensitivity Enzyme-Linked immunosorbent assay (ELISA)s and polymerase chain reaction techniques (Malamud and Rodriguez-Chavez, 2011). Salivary POC diagnostics however are further complicated by the high viscosity and heterogeneous properties

associated with saliva (Christodoulides et al., 2005). Some of these limitations can be overcome as discussed below with for example, paramagnetic particle analyte capture (Section 1.8.3.3.1, Page 59).

The heterogeneous properties of saliva therefore require standardised collection and processing protocols that require training and education. Interestingly, studies exploring patient compliance with saliva collection protocols have demonstrated "low" adherence at 26 to 28% of the study population (Hall et al., 2011, Kudielka et al., 2003). It is unclear from these studies whether patients were involved in the practical design of said protocols to ensure maximal patient compliance.

Thus the argument for collection of whole unfractionated saliva for biomarker analysis has been made, however what type of whole unfractionated saliva to obtain needs specific consideration.

1.8.2. Differences between stimulated and unstimulated saliva

The production of whole unfractionated saliva can be split into two separate methods. A stimulated sample can be produced mechanically by mastication on an inert substance for example: paraffin wax, cotton, cellulose acetate, or chemically with a gustatory stimulus for example: citric acid on the tongue. An unstimulated sample can be provided either by passive drool thus allowing saliva to drain off the lower lip into a plastic vial (Nurkka et al., 2003, Hodinka et al., 1998), spit or absorbed via a swab placed in the oral cavity. Whilst spitting directly into a collector vial is possible, specimens collected by this method contain up to fourteen times more bacteria than those collected by passive drool; this affects storage and analysis of several compounds (Nurkka et al., 2003).

The rate of flow for unstimulated whole saliva has been demonstrated to be lower than mechanically stimulated methods (Bergdahl, 2000, Topkas et al., 2012). However, one study in asthmatic children compared to non-asthmatic controls demonstrated no significant difference in unstimulated and stimulated salivary flow rates between the two groups (Al-Dlaigan et al., 2002).

The composition of a whole saliva sample can be affected by which sampling method is chosen. No difference has been demonstrated in the total protein content between stimulated and unstimulated submandibular saliva (Dawes, 1975). Other work has also confirmed that there is no difference in total protein content between stimulated and unstimulated whole saliva collected by varying methods (Topkas et al., 2012). More recently unstimulated whole saliva has been found to have higher amino acid concentrations when compared to citric acid stimulated saliva (Rad et al., 2014). Importantly, citric acid stimulated saliva may affect immunoassay performance by creating interference with antibody binding (Bourbeau et al., 2003). With respects to salivary CRP no difference in concentrations has been demonstrated between unstimulated whole saliva via passive drool and mechanically stimulated saliva samples (Mohamed et al., 2012). Presently, no comparative studies between different forms of whole saliva have been conducted for either salivary PCT or NE.

Studies on therapeutic drug monitoring in saliva have revealed higher mean concentrations of theophylline in the unstimulated whole saliva of children taking the medication for asthma compared to stimulated (citric acid) whole saliva. Importantly, although both demonstrated statistically significant strong correlations with total serum values, unstimulated whole saliva was stronger ($r = 0.98$ in comparison to $r = 0.90$ respectively) (Siegel et al., 1990). Mixed unstimulated saliva collected via passive absorbance showed a recovery of greater than 90%

for a panel of anti-epileptic medications, caffeine and theophylline (Groschl et al., 2008). Recent work has also confirmed the benefits of unstimulated whole saliva therapeutic drug monitoring for a panel of anti-epileptic medications (Patsalos and Berry, 2013).

Absorbance of unstimulated saliva via polyester swabs has been demonstrated to produce better yields for salivary steroids compared to using cotton swabs (Groschl and Rauh, 2006). Cotton swab retrieval of saliva, compared to passive drool collection, appears to reduce the concentrations of cortisol, s-IgA (Strazdins et al., 2005) and CRP (Topkas et al., 2012). Furthermore, stimulated or unstimulated saliva collected via an oral swab can cause end-user anxiety, with subjects appearing apprehensive about having to place "foreign objects/materials" into their mouths. A study investigating cotinine levels in both stimulated and unstimulated saliva documented that 9% of participants found collection of stimulated saliva via chewing on cotton wool "not at all acceptable" compared to 1% for passive collection of unstimulated saliva (Binnie et al., 2004).

Overall unstimulated compared to stimulated saliva provides a higher total protein content in the sample, for example, higher CRP concentrations. Whilst a reduction in saliva flow is noted during unstimulated-sampling, the case for not using oral stimulants or passive absorbance with oral swabs when collecting saliva samples is well made; furthermore, current immunoassays are generally designed to work with small sample volumes which do not prove to be a problem for most patients to produce. Importantly, as previously described saliva stimulants may also cause immunoassay interference or alteration of levels of some analytes (Granger et al., 2007b).

1.8.3. Technological approaches to salivary analysis

Currently there is a wide variety of technological approaches to saliva-based testing; all of which seek to produce a sensitive and reproducible diagnostic that can efficiently and repeatedly be used to provide meaningful clinical information.

1.8.3.1. Mass spectrometry

The first step is to categorise the entire saliva proteome. Mass spectrometry (MS) has been used to analyse salivary proteins in minute detail and thus generate a comprehensive proteome (Denny et al., 2008). This technique is extremely sensitive for high accuracy measurements of proteins and peptides, with unbiased results as no prior knowledge of protein composition is required (Gillette and Carr, 2013). There are four different MS techniques used in salivary biomarker screening and identification. These include: 2-dimensional gel electrophoresis, 2-dimensional liquid chromatography-MS, matrix assisted laser desorption ionisation-time of flight-MS and surface enhanced laser absorption/ionisation time-of-flight-MS. It should be noted that different yields of total proteins have been identified with the various MS techniques. (Al-Tarawneh et al., 2011). Initial biomarker fractionation is required for MS-based techniques (Esser et al., 2008). Each fractionation is then subject to the MS techniques as described above of which the resulting data is then used to search protein databases for identification. As an example from a list of 1058 proteins reported as potential cancer biomarkers, 34% are found in the whole saliva proteome and 12% are found in both unstimulated whole saliva and serum proteomes (Anderson, 2010). Saliva has a lower composition of over-powering serum abundant proteins (Section 1.8, Page 49). Thus saliva-based testing for these proteins should be more fruitful than serum (Loo et al., 2010). Overall the completion of the saliva proteome by MS will help to establish saliva as a validated diagnostic fluid. However, the adoption of MS techniques as a POC test is hindered by sample preparation (fractionation). Newer

techniques for example, paper spray MS, have been shown to be effective in the analysis of complex biological fluids and allows for a reduced sample volume and minimal sample pre-treatment compared to traditional MS. This approach coupled with a bio-fluid sampler could provide for an effective POC test in the future (Wang et al., 2013).

1.8.3.2. Lateral flow assays

Early saliva-based POC involved lateral flow assays (Figure 1.5) which had the advantage of simplicity, but lacked the capability for carrying out complicated operations for example, nucleic acid diagnostics. Lateral flow tests are routinely used to detect: pregnancy, infectious diseases, myocardial infarction and substances of abuse. Samples may include urine, saliva, blood, and stool. Typical lateral flow tests however lack signal amplification, and thus, sensitivity falls short of ELISA-based methodologies. Several engineering solutions have been developed to overcome problems with autonomously transporting liquids, controlling fluid flow in small devices, facilitating mixing, thermal control, dry store reagents for prolonged periods of time (greater than 1 year) and means for biomarker quantification (Hart et al., 2011).

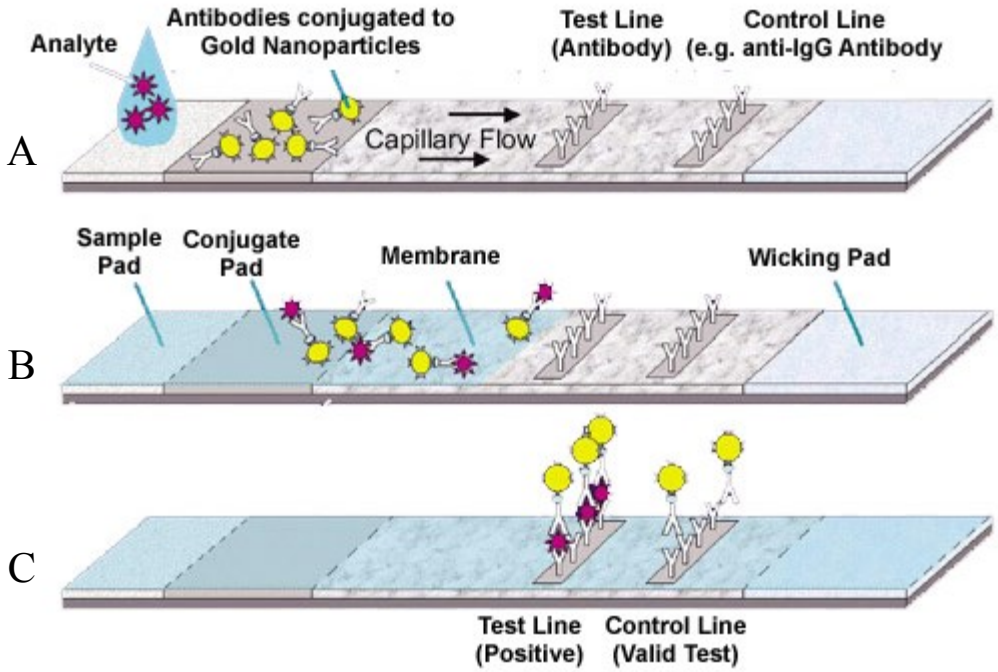


Figure 1.5: Lateral flow assay architecture.

This diagram (adapted from Cytodiagnostics, Canada) represents a typical lateral flow assay test-strip. (A) Sample containing target analyte is deposited onto the sample pad which by capillary flow migrates through the test strip. (B) It initially encounters the conjugate pad where target analyte binds to a target antibody. The remaining antibodies in the conjugate pad which are unbound continue to migrate with the antigen-antibody complexes. (C) At the test line the analyte-antibody complexes attach to an analyte specific "capture" antibody to create a coloured line. Antibodies that contain no analyte continue to migrate to the control line where they bind to create a control line.

Lateral flow test strips have been developed to semi-quantitatively determine nitrous oxide and uric acid in saliva by using chromatography paper impregnated with nitrous oxide and uric acid detection chemistries. Brief emersion of the test strip in saliva produces a change in colour, which is proportional to the levels of the target biomarkers. These test strips have been shown to accurately monitor nitrous oxide and uric acid concentrations in patients undergoing dialysis

(Walt et al., 2007). Quantum dots which have superior signal brightness and higher photostability compared to organic fluorophores when combined with a lateral flow test strip can result in a test that is capable of rapid, sensitive and quantitative detection of nitrated ceruloplasmin, a significant biomarker for cardiovascular disease, lung carcinoma and stress response to smoking (Li et al., 2010). A recent study demonstrated that a gold nano-particle-based immunochromatographic test strip could detect organophosphate exposure, providing a simple, accurate and qualitative tool that may be adapted to test for other biomarkers (Zhang et al., 2013). Presently however, lateral flow assays are limited when highly quantitative and reproducible results are demanded (Sajid et al., 2015).

Research continues on the integration of a lateral flow strip combined with a microfluidic cassette, which for sample processing would significantly expand the range of applications and tasks that could be performed by immunochromatographic methods. Recent advances in microfluidics make it possible to miniaturise, integrate, and automate various bench-top procedures into credit card-sized cassettes or chips. Microfluidics offers greater functionality and more sophisticated flow control than lateral flow devices, and potentially could expand the range of assay technologies that can be performed in formats similar to that of a test strip. The field is also often referred to as micro-total analysis or lab-on-a-chip. Although progress with developing a lab-on-a-chip concept has been slow, commercial devices using molecular assays to detect infectious agents are starting to appear on the clinical market (St John and Price, 2014).

1.8.3.3. Microfluidic technology: Lab-on-a-chip

Lab-on-a-chip has two sub-disciplines: (1) automating laboratory procedures with the aim of enhancing discoveries through high throughput parallel processing which may be needed for combinatorial biology and chemistry, drug screening, and other biological studies (Paegel and Joyce, 2010); and (2) creating autonomous, fully integrated POC devices, which are often disposable and aimed at assaying one or a few more analytes within a relatively short period of time (Hart et al., 2011). Microfluidic devices can be further classified as instrumented or un-instrumented (Weigl et al., 2008). Instrumented devices typically consist of a disposable cassette accompanied by a portable analyser. The disposable cassette hosts a microfluidic circuit with reaction chambers and interconnecting channels as well as the required reagents for measurement of the target analytes. The analyser incorporates functionality for pumping, thermal control for incubation, enzymatic amplification and detection via optical signals, electrochemical signals or added mass. Un-instrumented devices are updated lateral flow strips. The actuators, such as finger-actuated pouches and exothermic reaction chambers, are integrated into the disposable cassette and no external processor is needed. At present these devices provide little flexibility in terms of reconfiguration and scalability. Handling of real physiological samples has also been a problem (Srinivasan et al., 2004).

Another classification of these devices is to look at capture of target analytes using magnetic particles which can help with mixing, washing and buffer exchange, both in fluid flow and in stationary microfluidic device (van Reenen et al., 2014). Proteins are then detected via immunoassay and nucleic acids via molecular diagnostics which are generally more complicated than immunoassays (Hart et al., 2011). Recently an all-polymer microfluidic system which is highly sensitive and can provide acute virus detection within 15 minutes has been developed and tested on influenza virus identifying clinically relevant concentrations

(Kiilerich-Pedersen et al., 2013). A microfluidic device has also been produced which can detect cocaine in saliva (Wagli et al., 2013).

1.8.3.3.1 Microspheres

Lab-on-a-chip assay systems have been developed in which assays are performed on chemically-sensitised beads populated into etched silicon wafers with embedded fluid handling and optical detection capabilities. This electronic taste chip (ETC) approach allows complex assays to be performed with small sample volumes, short analysis times and markedly reduced reagent costs. Both ETC- and ELISA-based testing produce similar results (Christodoulides et al., 2007). The ETC system can provide an ultra-sensitive test for the measurement of salivary CRP, and has detected significant differences in concentrations between periodontally healthy individuals and those with chronic gingival inflammation and periodontitis (Christodoulides et al., 2005). Further work is being performed using microspheres to create a multiplexed protein biomarker assay to distinguish the severity of acute exacerbations of COPD (Walt et al., 2007). Blicharz et al., published an update to this work in 2009 demonstrating the fibre-optic microsphere-based antibody array that can simultaneously measure a panel cytokines implicated in pulmonary diseases (Blicharz et al., 2009). Currently the technology is used as a laboratory-based platform for inflammatory disease research and diagnostics; however due to its small footprint microsphere-based cassettes are being increasingly developed for use in POC (Blicharz et al., 2009). A microsphere-based cassette for the detection and quantification of IL-8 provides good repeatability and reasonable sensitivity (Qiu et al., 2009).

1.8.3.4. Immunoassays

An immunoassay tests for the presence or concentration of a macromolecule through the use of an antibody or immunoglobulin. In addition to the binding of an antibody to an antigen the other important feature is the production of a measurable signal in response to the binding by the linkage of a detectable label.

1.8.3.4.1. Immunoassay labels

1.8.3.4.1.1. Enzyme

One of the most popular labels employed is enzymes for example, horseradish peroxisade, alkaline phosphatase or glucose oxidase. These enzymes allow for analyte detection because they produce an observable colour change in the presence of specific reagents. ELISAs have the ability to detect small concentrations of analytes with excellent sensitivity and specificity. However current ELISAs are laboratory-based and require numerous steps including sample preparation, microtitre plate wash and incubation these; in turn can result in a 4-hour test time for a normal laboratory-based ELISA through to result feedback. An ultrasensitive portable immuno-sensor has been developed using an enzyme immunoassay for the quantification of tauroursodeoxycholic acid in saliva to assess oro-ileal transit time. Results from this portable assay are accurate, with strong correlation to serum concentrations (Simoni et al., 2013). A POC ELISA test kit for serum canine viruses has also been shown to yield accurate results under field conditions (Litster et al., 2012). More recently a micro-a-fluidics ELISA for rapid and reliable CD4 cell count has been developed with the entire ELISA process complete in under 9 minutes; however a portable optical detection system is still needed to be used separately to the ELISA process (Wang et al., 2014).

1.8.3.4.1.2. Radioactive isotope

A radioimmunoassay is analogous to an ELISA but utilises a radioisotope label.

1.8.3.4.1.3. Fluorophore

This method is analogous to an ELISA but utilises a fluorophore label which is a fluorescent compound that can re-emit light upon light excitation.

1.8.3.4.1.4 Chemiluminescence

This method is analogous to an ELISA but utilises a chemiluminescent substance as a label, which is a compound that emits light.

1.8.3.4.1.5. Liposome

This method involves a liposome encapsulated marker either coupled to an analyte or antibody.

Despite significant technological advances, a number of challenges remain in developing an ideal POC test based on an immunoassays concept, with as described above refinements on sample preparation and time of incubation (which for some immunoassays can be several hours) as well as the need, to incorporate some form of optical or radioactive detection.

Overall saliva-based testing offers numerous advantages to POC testing with a number of technologies demonstrating promise (Table 1.5). However, there is still considerable research that needs to be performed to establish saliva sampling in routine respiratory disease management, ranging from standardised sampling protocol and saliva-based biomarker quantification to, the ability of saliva to reflect disease status change. This thesis will attempt

to address these gaps in knowledge and provide the foundation upon which new technologies can be developed to enable POC testing of saliva to better manage COPD.

Table 1.5: Technological approaches to salivary analysis.

Technology	Pros	Cons
Mass Spectrometry	Extremely sensitive and accurate	Sample preparation (fractionation)
Lateral Flow Assays	Simple; well established for other body fluids as a point of care test.	Lack signal amplification and thus sensitivity falls short of immunoassays
Microfluidics/ Lab-on-a-chip	Small sample volumes; have been shown to produce similar results to enzyme immunoassays	Stability of reagents on the chip; accuracy of chip manufacturing.
Immunoassays	Extremely sensitive and accurate	Requires appropriate label detection machinery.

1.9. Salivary analysis in COPD

Analysis of saliva in COPD patients has focused on therapeutic drug monitoring and biomarkers of pathogenesis. Strong correlations have been demonstrated between serum and saliva levels of theophylline (Salamzadeh et al., 2008) and saliva oxidative-stress related changes (Yigla et al., 2007). More recent work has identified a significant negative correlation between FEV_1, salivary IL-8 and MMP-9 levels in COPD patients (Ji et al., 2014). Whilst the study of saliva to identify COPD-centric biomarkers is in its infancy, the above-mentioned studies all acknowledge the benefits that saliva sampling provides for ease of collection and testing.

Within in the field of COPD a range of biomarkers have been extensively investigated in a variety of bio-fluids with the identification of relevant biomarkers continuing to expand. Although, access to samples remains a major issue, it is increasingly recognised that a panel of biomarkers may provide a more comprehensive model for COPD: (1) diagnosis, (2) disease status monitoring, (3) progression and (4) guide to therapy (Shaw et al., 2014). For this thesis work 3 COPD-relevant biomarkers have been selected that will be investigated in the saliva of COPD patients and healthy subjects. These biomarkers were selected based on a systematic review of the literature over the past 30 years (web of science: COPD, biomarkers). Biomarkers were selected that were well researched with respects to COPD pathogenesis, disease state monitoring and had been extensively investigated in numerous body-fluids. In conjunction to this the availability of saliva based or validated “body-fluid” immunoassays were also considered. The target biomarkers selected were CRP (Zhang et al., 2012), PCT (Tokman et al., 2011) and NE (Lucas et al., 2013).

1.9.1. C-Reactive Protein (CRP)

CRP is a pentraxin protein (Figure 1.6) discovered in the 1930's by Oswald Avery during his research with streptococcus pneumoniae infection. He demonstrated that CRP levels were increased during the acute stage of an infection (Abernethy and Avery, 1941).

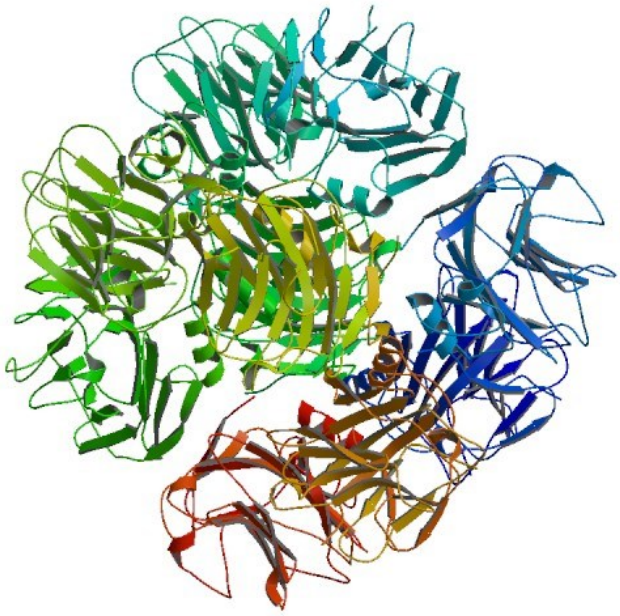


Figure 1.6: Molecular structure of C-Reactive Protein (CRP).

This figure (ribbon diagram) represents the 3-dimensional crystal structure of human CRP. CRP is a 206 amino acid polypeptide that has a variable molecular weight of approximately 115 kilodaltons (kDa) (Black et al., 2004).

CRP is synthesised in the liver and regulated by circulating levels of IL-6. Its physiological role is to bind to phosphocoline in order to activate the compliment system with levels of CRP rising rapidly in response to trauma, inflammation and infection (Du Clos, 2000). In healthy subjects, median levels of serum CRP range between 0.96 to 1.5mg/L (Ridker, 2003a, Jones et al., 2009, Yudkin et al., 1999). These levels have been shown to increase with age, body-mass index (BMI), smoking status and the co-existence of diabetes (Koenig et al., 1999). Although historically serum CRP levels less than 10mg/L have been considered clinically insignificant

(Black et al., 2004), it has now become established that levels above 1.5mg/L are associated with an increased risk of cardiovascular disease (1.5 to 3mg/L: moderate risk and greater than 3mg/L: high risk) (Ridker, 2003b). Baseline CRP levels in healthy subjects have also been associated with metabolic syndrome and type 2 diabetes (Devaraj et al., 2009).

1.9.1.1. CRP body fluid analysis in COPD

A literature review conducted over the past 30 years (web of science: COPD, biomarkers, C-reactive protein, serum, sputum, BAL, exhaled breath, electronic nose, urine, saliva) of studies using various body fluids to measure CRP levels in COPD patients these are described below under their particular medium.

1.9.1.1.1. Serum

The most extensively investigated body-fluid for CRP analysis in COPD patients is blood. Baseline median serum CRP levels in COPD subjects have been shown to range between 3.00 to 8.75mg/L (Aksu et al., 2013, Pinto-Plata et al., 2006, de Torres et al., 2006, Chan et al., 2010, Silva et al., 2015). COPD patients have been shown to have significantly higher serum CRP concentrations compared to healthy controls in the stable phase of their disease and serum CRP levels appear to increase from moderate to severe disease (Zhang et al., 2012). The evidence for increased baseline serum CRP levels resulting in an accelerated longitudinal decline in FEV_1 is conflicting. Several studies have demonstrated that serum CRP levels are associated with an accelerated decline in FEV_1 (Gan et al., 2004, Man et al., 2006, Higashimoto et al., 2009). However, a large cross-sectional analysis of approximately 1000 patients in another study showed no significant association between baseline serum CRP and the rate of decline in FEV_1 (Fogarty et al., 2007). Increased serum CRP levels of greater than 3mg/L in all COPD patients is a strong long term predictor of COPD hospitalisation and mortality (Dahl

et al., 2007, Deng et al., 2014). Relationships have also been demonstrated between PROs and serum CRP levels in stable COPD patients; with increasing CRP levels significantly correlating to a worsening in MRC score (Garrod et al., 2007).

Several studies have demonstrated an increase in serum CRP (greater than 10ng/ml) during an acute exacerbation of COPD (Kostikas et al., 2013, Hurst et al., 2006, Stolz et al., 2007b), with simultaneously elevated serum levels of CRP, fibrinogen and leucocytes associated with an increased risk of frequent exacerbations (Thomsen et al., 2013). Further elevated levels of serum CRP (greater than 15mg/L) also appear to distinguish community acquired pneumonia from an acute exacerbation of COPD (Huerta et al., 2013). Serum CRP levels greater than 50mg/L during an acute exacerbation in conjunction with a positive smoking history, at least 2 co-morbidities and confusion may assist in the identification of patients with a higher risk of mortality (Ruiz-Gonzalez et al., 2008). Significantly higher serum CRP concentration (8.8 mg/L compared to 3.4mg/L) 14 days after an index exacerbation have also been demonstrated in a group of COPD patients who had a re-exacerbation, compared with those who remained exacerbation free over 50 days (Perera et al., 2007). A relationship however has not been established between annual exacerbation frequency and stable baseline serum CRP levels in COPD patients (Gompertz et al., 2001).

1.9.1.1.2. Sputum

Two studies have investigated the levels of CRP in sputum. Out of these studies one is written in Chinese although the English abstract concludes that CRP may be secreted from the local respiratory tract (Wu et al., 2005). The other study demonstrates no correlation between sputum and serum CRP levels in same-subjects (Broekhuizen et al., 2005).

1.9.1.1.3. Exhaled breath condensate (EBC)

As described in Section 1.7.1.2, Page 42, it is not possible to make comparisons between different laboratories for the levels of biomarkers quantified in EBC. Only one study has investigated CRP in EBC of COPD patients and the text is in Russian. However the abstract highlights elevated levels of EBC CRP in COPD patients compared to healthy subjects (Dotsenko et al., 2008).

1.9.1.1.4. Saliva

No studies have yet been published investigating salivary CRP levels in COPD patients. However numerous works are now being published for salivary CRP in other disease states. There have been studies on unstimulated saliva that demonstrate elevated levels in patients with periodontal disease (Pederson et al., 1995, Christodoulides et al., 2005); however this conflicts with another small cohort study showing reduced CRP levels in patients with chronic periodontitis compared to healthy controls (Aurer et al., 2005). More recent work supports a significant association between salivary CRP concentrations and periodontitis (Shojaee et al., 2013). Salivary CRP has also been investigated in haemodialysis patients (Pallos et al., 2015) and acute urticaria (Rao et al., 2011).

Salivary levels of CRP have been found to be elevated in patients who have suffered an acute myocardial infarction (Floriano et al., 2009). Within this study a panel of salivary biomarkers including an electrocardiogram were found to have an excellent diagnostic accuracy comparable to the best serum multi-marker panels. This demonstrates saliva's reflection of systemic status. Recent work has also demonstrated that salivary CRP accurately reflect serum levels (Byrne et al., 2013). The correlation between salivary CRP and serum is important as it supports the concept that testing of salivary CRP can be used as a direct surrogate to serum.

The current consensus is that whilst salivary CRP levels could mirror systemic events, design of meaningful clinical studies such as in patients with COPD should consider the co-existent presence of periodontitis as this could affect measured CRP levels and thus interpretation of salivary CRP should account for the co-variate effect of periodontitis (gum disease).

1.9.2. Procalcitonin (PCT)

PCT is a peptide precursor of the hormone calcitonin (Figure 1.7) discovered in 1975 by Moya et al. It is composed of 116 amino acids and is produced by the neuroendocrine parafollicular cells of the thyroid gland during health.

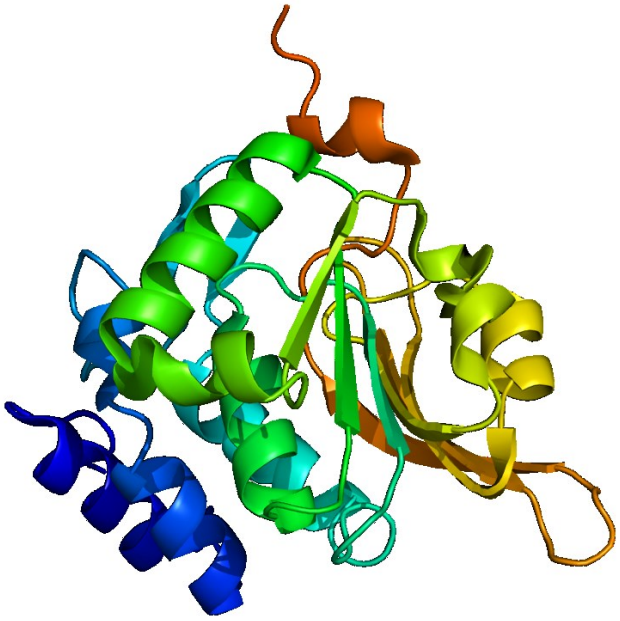


Figure 1.7: Molecular structure of Procalcitonin (PCT).

This figure (ribbon diagram) represents the 3-dimensional structure of PCT. PCT is composed of 116 amino acids with a molecular weight of 13kDa (Maruna et al., 2000).

Circulating levels of PCT in healthy subjects are undetectable (Maruna et al., 2000). Elevated levels in bacterial infection were first reported by Assicot et al. (Assicot et al., 1993) and since then it has become an important protein in the detection of bacterial inflammatory states (Maruna et al., 2000). Importantly, localised bacterial infections do not result in a significant increase in PCT alongside viral and non-infectious inflammation for example, autoimmune disorders (Oberhoffer et al., 1999). The production of PCT during inflammation is linked with bacterial endotoxin and inflammatory cytokines TNF-α, IL-1β and IL-6, with the site of

production believed to switch to the neuroendocrine cells of the lungs and/or intestine. (Müller et al., 2001).

The most elevated levels of serum PCT levels are invariably found in acute severe bacterial infections, sepsis, and severe inflammation (Becker et al., 2010). PCT has been demonstrated to be more accurate than CRP in differentiating bacterial from non-infective causes of inflammation with a sensitivity of 88% compared to 75% and a specificity of 81% compared to 67% (Simon et al., 2004). In the same study Simon et al., also found PCT superior in distinguishing between bacterial and viral aetiology causes of inflammation with a sensitivity of 92% compared to 86% but a more comparable specificity of 73% compared to 70%. A 2012 Cochrane review found no increase in mortality or treatment failure when serum PCT levels were used to guide initiation and duration of antibiotic treatment in patients with acute respiratory infections compared to healthy controls (Schuetz et al., 2012). Importantly serum PCT time-dependent decay is not affected by steroids (Perren et al., 2008).

1.9.2.1. PCT body-fluid analysis in COPD

A literature review conducted over a 30-year time frame (web of science: COPD, biomarkers, C-reactive protein, serum, sputum, BAL, exhaled breath, electronic nose, urine, saliva) for the quantification of PCT in various body-fluids in COPD patients; these are described below under the particular medium.

1.9.2.1.1 Serum

As discussed in Section 1.6.2, Page 36, the predominant cause of acute exacerbations in COPD is bacterial. There are several studies exploring the level of serum PCT during an acute exacerbation of COPD. Median serum PCT levels at exacerbation range from 0.09 to 0.10ng/ml

(Stolz et al., 2007b, Lacoma et al., 2011). Stolz et al., also demonstrated that that higher serum PCT levels were observed in patients who died in the 1 month following their acute exacerbation of COPD. In COPD patients with acute exacerbations requiring endotracheal intubation and mechanical ventilation median serum PCT levels (greater than 0.24ng/ml) are independently associated with an increased risk of mortality (Rammaert et al., 2009). Median levels of serum PCT (greater than 1.27ng/ml) can also been used to distinguish the presence of pneumonia from acute exacerbations of COPD (Bafadhel et al., 2011a). Current literature demonstrates that the use of PCT is associated with a reduction in antibiotic treatment initiation and duration without an increase in the rates of adverse patient outcomes including death, admission to an intensive care unit, re-exacerbation and hospital readmission (Blasi et al., 2010). One study has demonstrated that antibiotic usage guided by serum PCT levels (greater than 0.25ng/ml) in COPD patients presenting to a hospital emergency department for an acute exacerbation resulted in an initial reduction in antibiotic exposure and interestingly a sustained reduction in total antibiotic exposure for up to 6 months after the hospitalisation event (Stolz et al., 2007a). However, other studies have shown that COPD patients with serum PCT levels less than 0.25ng/ml at presentation to hospital for an acute exacerbation of COPD may still benefit from treatment with antibiotics (Daniels et al., 2010, Falsey et al., 2012).

Thus, serum PCT-guided antibiotic therapy has the potential to decrease unnecessary antibiotic use in non-bacterial acute exacerbations of COPD (Clark et al., 2014), thereby helping to reduce episodes of antibiotic resistance (Hayashi and Paterson, 2011), antibiotic-related adverse reactions (Soni et al., 2012), and potentially episode-associated healthcare costs (Heyland et al., 2011).

1.9.2.1.2. Saliva

To date there are no reports in the literature on saliva-analysed PCT levels in COPD. A lone study demonstrated that salivary PCT levels in patients with periodontitis and poorly controlled diabetes (HbA1c greater than 7.0%) were not significantly higher than those for healthy control subjects, although a separate sub-analysis did demonstrate a significantly higher level in patients with severe periodontitis compared to healthy individuals (Bassim et al., 2008). In this study, salivary PCT levels did not significantly correlate with serum levels. A more recent study has also demonstrated no difference in salivary PCT levels between patients with generalised chronic periodontitis and healthy controls (Yousefimanesh et al., 2015). In contrast, another study has found significantly elevated median salivary PCT levels in individuals with chronic periodontitis (0.15ng/ml) compared to healthy controls (Hendek et al., 2015). Although the evidence is conflicting only Hendek et al., stored saliva samples at -80^{o}C prior to analysis (the other studies stated storage of saliva samples at -20^{o}C prior to analysis). Thus it is possible that chronic periodontitis has some effect on salivary PCT levels however presently the literature is inconclusive. It is thus prudent to document the presence of periodontitis when sampling saliva for PCT and include this co-variate in any statistical analysis for meaningful result interpretation.

1.9.3. Neutrophil Elastase (NE)

NE is a 218 amino acid glycoprotein (Figure 1.8) which functions as a powerful serine proteinase, with the majority of NE found in the azurophil granules in the neutrophil cytoplasm. In humans, NE is encoded by the ELANE gene which resides on chromosome 19. NE hydrolyses proteins within the azurophil granules as well as proteins of the extracellular matrix following release from activated neutrophils (Takahashi et al., 1988). NE has a role in normal tissue turnover and host defence, but it also plays a role during acute and chronic inflammation (Lucas et al., 2013).

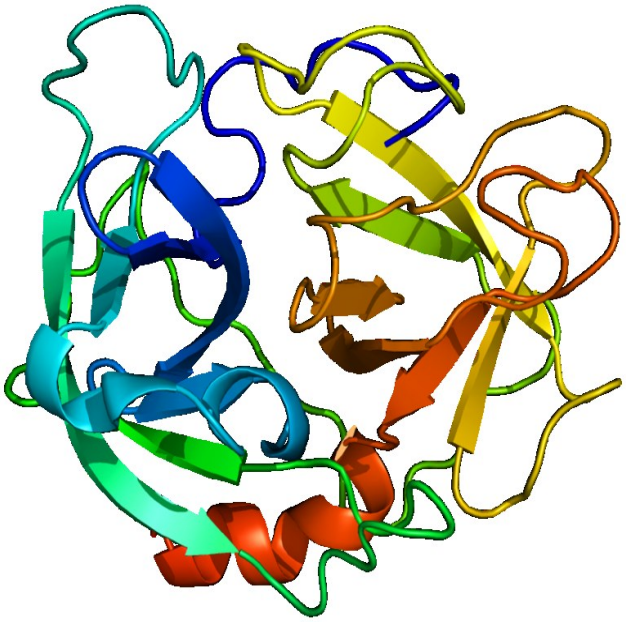


Figure 1.8: Molecular structure of Neutrophil Elastase (NE).

This figure (ribbon diagram) represents the 3-dimensional structure of NE. NE is composed of 218 amino acids and has a molecular weight of NE 29.5kDa (Korkmaz et al., 2010).

1.9.3.1. NE body-fluid analysis in COPD

A literature review conducted over a 30-year period (web of science: biomarkers, elastase, serum, sputum, BAL, exhaled breath, electronic nose, urine, saliva) of studies using various body fluids to measure NE levels in COPD patient; these are described below under the particular medium.

1.9.3.1.1. Serum

Serum NE/AAT ratio and FEV_1 adjusted by pack years appears to be a reliable predictor of the development of COPD (Hoshino et al., 2000). Elevated serum levels of NE can identify acute exacerbations of COPD (Carter et al., 2013) and distinguish COPD patients from normal healthy adults (Yan and et al., 1988). It has also been shown that serum NE levels negatively correlate with FEV_1 in stable COPD patients (Bizeto et al., 2008). Recent work however, has unexpectedly identified a reduction of serum NE at the onset of an acute exacerbation of COPD (Chillappagari et al., 2015).

1.9.3.1.2. Sputum

Sputum NE concentrations are elevated in COPD patients compared to healthy non-smokers (Baines et al., 2011), with concentrations of 3.3ug/ml compared to healthy smokers (1.45ug/ml) with higher levels of sputum NE observed in severe states (4.60ug/ml) compared with mild to moderate disease (2.4ug/ml) (Paone et al., 2011). Sputum NE levels significantly increase during an exacerbation and upon clinical resolution levels return back to their pre-exacerbation baseline levels (Ilumets et al., 2008). Sputum NE can distinguish bacterial from viral and pathogen-negative acute exacerbations of COPD, and also correlates with clinical severity of the exacerbation (Sethi et al., 2000). Even during stable phase COPD, the sputum of moderate to severe patients with potentially pathogenic micro-organisms contains a significantly higher

level of NE compared to those without potentially pathogenic micro-organisms in their sputum (Banerjee et al., 2004, Parameswaran et al., 2009).

1.9.3.1.3. Bronchoalveolar lavage (BAL)

Higher levels of NE in the BAL of COPD patients have been associated with an accelerated decline in FEV_1 (Betsuyaku et al., 2000) and increasing COPD severity (Vlahos et al., 2012). Although BAL NE has been demonstrated to be elevated in COPD patients with frequent exacerbations the results were not statistically significant (Tumkaya et al., 2007).

1.9.3.1.4. Saliva

Salivary NE has not been investigated in COPD. Elevated NE levels have been demonstrated in the saliva of patients with periodontitis compared to healthy controls, although cigarette smoking appears to reduce salivary NE levels in patients with periodontitis (Pauletto et al., 2000). A more recent study has also demonstrated a rise in salivary NE in subjects with chronic periodontitis (9.79ng/ml) compared to healthy subjects (1.35ng/ml) (Nizam et al., 2014).

1.10. Thesis Hypothesis and Objectives

COPD is a common chronic progressive illness, the 2nd leading cause of chronic disability and currently ranks 3rd in global mortality rankings (Lozano et al., 2012). Exacerbations are a major feature of the disease process. These acute, as yet unpredictable episodes, severely and progressively impair the lung function and quality of life of COPD patients, leading to inability to work and unscheduled visits to secondary care. COPD exacerbations remain the second most common cause of emergency hospital admissions; with 1 in 3 of discharged patients being re-admitted within 3 months. Thus exacerbations are important events in a COPD patient's journey and are duly recognised in national and international COPD management guidelines (GOLD, 2016, NICE, 2010). Early diagnosis and treatment of COPD exacerbations can reduce their severity and limit the associated lung damage, yet often treatment is delayed because early "worsening of symptoms" goes unrecognised by COPD patients. Specifically, as COPD symptoms can vary from day to day, patients have no means of judging the significance of such changes and so exacerbations remain unreported and untreated (Langsetmo et al., 2008). Therefore, there remains an unmet need to identify COPD exacerbations earlier ideally as part of proactive self-management initiatives so that prompt treatment can be initiated. This would improve COPD outcome as early intervention has been shown to halt COPD deterioration and prevent hospitalisation (Wilkinson et al., 2004).

There is still no consistent test for practical COPD self-monitoring. With ever-increasing emphasis on the potential of inflammatory biomarkers (Thomsen et al., 2013) in enabling personalised disease monitoring and treatment (Agusti, 2014) and the merits of saliva as a plausible POC test medium, the overall ambition of my thesis is to establish a standardised and practical protocol for saliva self-sampling and quantitative reproducibility of a panel of three COPD-related biomarkers: CRP, PCT and NE in saliva. However, any clinical usefulness of

these biomarkers for portrayal of individual COPD status and early exacerbation alerts will require correlation with patient-defined events. Accordingly, this thesis will also develop and explore PRO, their reliability and relationship with the above-mentioned salivary target biomarkers.

To address these concepts, the studies in this thesis will:

- Determine the "best" type of saliva sample for near-patient testing.
- Determine the factors required for near-patient sampling of saliva and the develop bespoke end-user sampling protocols.
- Optimise and modify conventional assays to identify whether the target salivary biomarkers can be reproducibly quantified.
- Determine the effects of blood contamination in saliva on target biomarker levels.
- Create a simple COPD-PRO score and bespoke instruments to capture this score.
- Correlate PRO data to salivary biomarker levels and spirometric volumes and develop a multidimensional health status score.

The results of these experiments will provide the foundation for two community-based clinical studies, the objectives of which are:

1. To evaluate whether target biomarker levels in saliva differentiate between health and COPD states.
2. To establish whether the selected panel of salivary biomarkers can be used to longitudinally monitor COPD and identify onset of exacerbations.

These studies are crucial, as they will be the first to establish the role of salivary biomarkers and a novel PRO instrument in COPD, their ability to predict acute exacerbations and the relationship with PRO. Additionally, the longitudinal study will allow the identification of stable and exacerbation phenotypes. Furthermore, all work will be underpinned by qualitative studies utilising focus groups of patients with COPD as "experts by experience" to understand for self-management issues as well as determine end-user validity of saliva sampling and the PRO instrument. As management of long term chronic diseases is shifting towards community-based personalised patient monitoring guided by subjective and objective measurements of health, outputs from my thesis could provide the necessary proof-of-concept required to drive development of novel POC bio-clinical tools for practical COPD surveillance.

Chapter 2:

General Materials

And

Methods

2.1. Introduction

Presently within COPD there are no studies, which demonstrate that random sampling of saliva provides accurate, reproducible data that can be used to monitor disease status (Chapter 1, Page 63). This thesis will evaluate whether there is a role for saliva as a viable medium for the measurement of COPD-related target biomarkers, to determine clinical status and to enable prediction of acute exacerbations. However this will first require a standardisation of the methods for sampling, collection and processing of saliva to minimise confounding factors and possible contamination and to ensure optimal quality information (Chapter 1, Page 50). Presently, only CRP has a commercially available saliva based assay; albeit calibrated in healthy subjects, whilst PCT and NE will require modification of non-saliva based assays. Additionally, in recognition that biomarkers in isolation are not sensitive or specific enough to monitor COPD disease state without symptom assessment (Hurst et al., 2006). As discussed in Chapter 1, page 27 symptoms or PRO are now being recognised as important in the management of COPD with a variety of different instruments having been developed. However there remains an unmet need to develop a simple, effective PRO that can be possibly used as a daily diary within self-management protocols. In this thesis a novel PRO composed of clinically relevant COPD metrics was developed and incorporated into a purposeful diary for patient evaluation.

2.1.1. Saliva

As discussed in Chapter 1, Page 47, saliva is not just a passive "ultra-filtrate" of serum (Williamson et al., 2012), but a complex multi-factorial body fluid that is derived from numerous "salivary" glands located in and around the mouth. The argument for collection of whole unfractionated saliva for biomarker analysis in this thesis has been made (Chapter 1, Page 51), with specific consideration to what type of whole saliva to obtain (Chapter 1, Page

53). Unstimulated whole saliva via passive drool appears to be the more acceptable approach and thus will be the chosen method for saliva sampling throughout this thesis. Next consideration was given to a collection device for saliva sample deposition and subsequent storage of the said sample.

2.1.2. Saliva collection devices

A wide variety of commercially available saliva collection devices exist both for unstimulated and stimulated whole saliva. Saliva samples post production and deposition, are then shipped to the respective manufacturer's laboratory for target analyte (protein, RNA or DNA) quantification. Unstimulated whole saliva via passive drool who the chosen method for saliva sampling. A review of commercially available containers that would aid this approach was undertaken.

SalivaBio (Salimetrics, USA) has both a bespoke saliva collection aid and sample storage container (cryovial) for unstimulated whole saliva samples collected via passive drool. Saliva is channelled into the storage cryovial using the bespoke saliva collection aid which is placed into a user's mouth and slots onto the top of the cryovial; thus forming a continuous pathway for saliva to flow from the user's mouth into the cryovial (Figure. 2.1).

UltraSAL-2 (Oasis Diagnostics, USA) provides a product for the collection of unstimulated whole saliva via passive drool. This collector consists of two plastic pre-marked vestibules attached to a mouth piece (which acts as a saliva collection aid) and is designed to channel saliva into the vestibules (Figure. 2.2).

Figure 2.1: SalivaBio (Salimetrics, USA) passive drool collection device.

A = collection aid; B = sample storage container (cryovial).

Figure 2.2: UltraSal-2 passive drool saliva collection device.

A = collection aid; B = sample storage container

A whole host of home saliva collector systems have been developed for genetic analysis of DNA and/or RNA, for example: Salivagene (Stratec Biomedical AG, Germany), Isohelix (Cell Projects, UK), Oragene (DNAgenotek, Canada), Saliva DNA Collection, Preservation, & Isolation (Norgen, Canada) and DNAgard (Biomatrica, USA). Saliva samples are retrieved by passive drool, spitting or stimulation. All samples are combined with a stabilisation buffer and shipped to the respective company's laboratory. These devices merely provide a container for the deposition of saliva with no additional collection aid to help the passage of passive drool into a collector.

Overall the wide array of collectors for unstimulated whole saliva via passive drool essentially function as a standard collection tube. Although both the SalivaBio kit (Salimetrics, USA) and UltraSAL-2 (Oasis Diagnostics, USA) provide a collection aid to channel saliva into a collector, the size of the cryovial (width: 10mm by length: 46mm) in the SalivaBio kit (Salimetrics, USA) was not felt to be ideal for COPD patients who would find difficultly in handling the vial either because of tremor (iatrogenic or caused from commonly prescribed treatments such as salbutamol (Committee, 2015)), and/or co-existing co-morbidities that affect grip, for example, osteoarthritis. In respects of both the SalivaBio (Salimetrics, USA) and UltraSAL-2 (Oasis Diagnostics, USA) it is unclear as to whether the insertion of a collection aid into the mouth and the pursing of lips around this aid would of itself actually stimulate saliva. Thus for collection of saliva samples in this thesis a standard 15ml centrifuge tube (Nunc, Denmark) was utilised. Target marked at 2mls (Figure 2.3) with a length of 120mm and an apex diameter of 15mm; hereon known as the saliva collector.

Figure 2.3: A marked 15ml centrifuge tube (Nunc, Denmark).

This collector would reduce saliva sample handing prior to biomarker analysis as centrifugation of saliva (a pre-requisite in the methodology of salivary biomarker analysis) could be performed without the need to transfer the saliva into another container. In addition this collector provided an acceptable grip for COPD patients and was pre-marked to enable all subjects to be directed to the volume of saliva required; a collection aid device to facilitate saliva transfer was not provided. Finally, consideration was given to storage of the produced saliva sample.

2.1.3. Saliva storage

In Chapter 1, page 50 it is discussed that saliva sample proteins degrade at room temperature on sampling. Thus for saliva that is not analysed immediately post sampling these samples will need to be maintained at 4^{o}C (Esser et al., 2008) and long term stored as soon as possible (within 4 hours) post collection at -80^{o}C (-20^{o}C storage still results in protein degradation after 6 months) prior to biomarker analysis (Schipper et al., 2007). Thus to incorporate these factors, all saliva samples were to be passively drooled by patients into an ice-cooled marked 15ml centrifuge tube (Nunc, Denmark) and transported on ice for storage until use in a -80^{o}C freezer Guy Hilton Research Centre Freezer Room (Keele University, UK) within 2 hours of collection.

2.2. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics Version 21 (IBM, USA) unless specifically stated. All data were processed using Microsoft Excel (Microsoft, USA). Parametric data are expressed as mean ± standard deviation (SD) while non-parametric as median, inter-quartile range (IQR). Non-parametric data where highlighted are logarithmically (base 10) transformed to allow for univariate analyses or other statistical techniques. Saliva samples for each biomarker that test below the lower limit of assay quantification (negative) will be assigned with the next number below and not zero for data analysis. Thus for salivary CRP concentrations below the lower limit of assay quantification (0.10ng/ml) were assigned as 0.09ng/ml for mathematical purposes; salivary PCT levels below the lower limit of assay quantification detection (0.10ng/ml) were assigned as 0.09ng/ml; salivary NE levels below the lower limit of assay quantification (2.2ng/ml) were assigned as (2.1ng/ml). This approach will enhance statistical accuracy and not result in a downward bias for the negative data (Muir K, 2004). A Wilcoxon signed-rank test was used to compare non-parametric paired data across 2 groups. A paired t-test was used to compare parametric data across 2 groups. A repeated measures ANOVA is used to compare parametric data repeated in the same sample or individual more than twice. A Greenhouse-Geisser correction was applied to correct against violations of sphericity where it arose for the repeated measures analysis of variance (ANOVA). Friedman's test was used to compare non parametric data repeated in the same sample or individual more than twice. Spearman's rank correlation co-efficient was used to correlate parametric data and a Pearson's test was used to correlate non-parametric data. A percentage CV was calculated for each sample by finding the standard deviation of results 1 and 2 for that sample (if tested in duplicate). This result would be divided by the mean of result 1 and 2 for that sample and multiplied by 100. Thus if 40 samples are analysed on 1 assay plate or kit a CV will be generated for each of the 40 samples. The intra-assay CV is then calculated

by the mean of all individual sample CVs (in this example 40 samples) tested on that plate or kit. An inter assay CV is calculated by first calculating a CV for the high and low controls tested on each assay or kit and then calculating the mean CV for each control sample CV across all individual assays or kits that are used. A p-value of less than 0.05 was considered statistically significant. A Bonferroni correction ($) was also applied to reduce the incidence of a Type 1 statistical error, which can occur when undertaking multiple comparison testing (Benjamini and Hochberg, 1995).

2.3. Patient recruitment

All subjects who provided saliva samples in this thesis had to meet certain inclusion criteria for entry into one of 3 cohorts. Healthy never-smokers, healthy smokers and COPD. Healthy individuals were only accepted if they were never smokers and had no known co-morbidities, healthy smokers were only accepted if they had no known co-morbidities and a minimum 20 pack year (a pack year is defined as smoking 20 cigarettes per day for 1 year) smoking history and COPD patients were only accepted if they had previously confirmed COPD on spirometry (Chapter 1, Page 21) according to the GOLD criteria and a minimum of 20 pack year smoking history. All COPD subjects were recruited independent of disease severity and co-morbidities, if any had to be stable prior to enrolment. Subjects in all 3 cohorts also had to be free of active infection at the point of recruitment. These individuals were recruited from known healthy volunteers, members of pulmonary rehabilitation groups in the Stoke-on-Trent area, UNHM outpatient department clinics and UHNM research groups. All demographic data was inputted into an appropriately secure database herein referred to as the Directorate of Respiratory Medicine's (University Hospital of North Midlands NHS Trust) research and outpatient clinic database. Demographic data, allocation of these individuals to the various experiments/studies in this thesis, and ethics approval numbers are described in Table 2.1, Page 89. All subjects were sent an information sheet and were subsequently invited to take part in the studies for this thesis and provided informed written consent.

Table 2.1. Thesis subjects' demographics and allocation.

Demographics	Ethics Approval	Healthy Non-Smokers (n = 28)	Healthy Smokers (n = 32)	COPD (n = 158)
Age, [a] years		43.4 ± 18.2	40.0 ± 12.9	67.7 ± 7.9
Gender, Male, (Female), n		9,19	20, 12	27, (28)
BMI, [a](kg/m^2)		24.7 ± 6.1	25.2 ± 3.3	26.4 ± 6.3
GOLD Stage		n/a	n/a	I: 23, II: 56, III: 58, IV: 16
Chapter 2	09/H1203/77	Randomly selected: multiple experiments	Randomly selected: multiple experiments	Randomly selected: multiple experiments
Chapter 3	09/H1203/77	Consecutively selected (n = 20)	Consecutively selected (n = 25)	Consecutively selected (n = 98)
Chapter 4	12/NW/0623	n/a	n/a	Consecutively selected. No overlap with Chapter 3 population (n = 60)
Chapter 5	12/NW/0623	n/a	n/a	Randomly selected. Chapter 4 population (n = 10)
Chapter 6	09/H1203/77 & 12/NW/0623	Randomly selected: multiple experiments	n/a	Randomly selected: multiple experiments

a = mean ± SD.

2.4. Optimisation of biomarker assays for saliva analysis

Of the three target analytes being studied in this thesis only CRP has a commercially available kit for detection in saliva. Measurement of PCT and NE require the adaptation of conventional kits that have previously been validated on other biological fluids for example: blood (serum and or plasma), urine and sputum.

2.4.1. Pre-analytical factors

As discussed there are multiple factors to consider when undertaking saliva analysis. Collection and storage of saliva has been standardised with unstimulated whole saliva collected via passive drool (Chapter 1, Page 53) into ice-cooled centrifuge tubes (Section 2.1.2, Page 84). The transport of said sample for long-term storage at -80^{o}C (Section 2.1.3, Page 85) unless immediate analysis is to occur with a transport time of no greater than 2 hours. Samples that required thawing were left to thaw at ambient room temperature. This protocol ensures that each saliva sample produced will be handled in the same way prior to biomarker analysis. All saliva samples would be tested in duplicate.

2.4.2. Immunoassay factors

Validation of the immunoassays will involve experiments to understand:

1. Precision: The reproducibility of results within (intra) and between (inter) assays. This will be assessed by co-efficient of variation (CV). Published acceptable levels for immunoassays are an intra-assay CV of less than 10% and an inter-assay CV of less than 15%. (Reed et al., 2002). These values served to act as a baseline minimum CV whilst conducting the initial experiments for the 3 immunoassays. They would be reconsidered as acceptable CVs if during the clinical studies described in Chapter 3, Page 213; Chapter 4,

Page 251, clinically significant differences in biomarker levels were noted at values less than 10%.

2. Accuracy: This will be assessed via spike-and-recovery (spiking of unadulterated samples with fixed concentrations of all three biomarkers) and linearity-of-dilution experiments (effects of dilution on biomarker levels).
3. Limit of detection: These will initially be based upon the limits documented in the kit insert for each immunoassay and further defined based on the optimal saliva dilution factor selected for each assay.

2.4.2.1. Measurement of salivary C-Reactive Protein (CRP) levels

To quantify salivary CRP levels in this thesis the Salivary CRP ELISA kit (Salimetrics, United States of America (USA)) was used; this immunoassay is specifically designed and validated for the quantification of salivary CRP. It is important to note however, that this ELISA is not intended for diagnostic use at the moment and is classified "for research use" in humans.

Immunoassays can be classified either as "research use only" or "for diagnostic use". The difference in the two classifications means that "research use only" assays are not able to represent an effective *in-vitro* diagnostic product, and manufacturers cannot make performance claims or give reference values; thus it is recommended when using "research use only" immunoassays that each researcher establishes their own reference range, standard curves, control levels and assay result reproducibility (Burd, 2010).

Each kit consists of a 96 well microtitre plate coated with mouse anti-human CRP antibodies, lyophilised CRP standard: 3000pg/ml (reconstituted in 1ml of distilled (d)H_2O), lyophilised CRP controls: low and high (reconstituted with 500ul of dH_20), CRP antibody-enzyme

conjugate, CRP sample diluent, CRP assay diluent, ELISA wash buffer concentrate (diluted 1:10 with dH_20), Tetramethylbenzidine (TMB) substrate, 2-molar (M) sulfuric acid stop solution and an adhesive plate cover (Figure 2.4). Pre-analysis saliva samples need to be vortexed and centrifuged at 3000 revolutions-per-minute (rpm) for 15 minutes, then diluted (1:10) in the CRP sample diluent provided by the manufacturer. Sample dilution reduces the concentration of CRP into the working assay range: 0.01 to 3ng/ml. Thus at a 1:10 dilution the quantifiable salivary CRP range is 0.10 to 30ng/ml. For any sample above the upper limit of quantification for the ELISA, the manufacturer recommends dilution at a higher denominator; this theoretically means that the kit has no upper limit of quantification for CRP if the appropriate dilution is incorporated.

Figure 2.4: Salivary CRP ELISA kit (Salimetrics, USA).

This figure displays the entire kit: A = microtitre plate; in front of this and from left to right: Wash buffer concentrate, assay diluent, TMB substrate solution, 2M Stop solution, Sample diluent, CRP antibody conjugate, CRP standard 3000pg/ml, CRP High Control, CRP Low control.

To perform an assay 50ul of diluted saliva, standards and controls are added to the wells in duplicate; thus 39 saliva samples can be tested in duplicate per microtitre plate (Table 2.2). Next 150ul of diluted CRP antibody-enzyme conjugate; (80ul of antibody-enzyme conjugate diluted 1:250 in 20mls of CRP assay diluent) is immediately added to each well using a multichannel pipette (VWR, USA). The microtitre plate is then covered and incubated at room temperature (20 to 23.3^{o}C) for two hours on a shaker horizontal plate mixer (Medigenix Diagnostics, Germany) at 500rpm. The microtitre plate is subsequently washed and 200ul of TMB substrate is added to each well, after which there is a further incubation in the dark (achieved by enveloping the microtitre plate in aluminum foil (Wrap Film Systems, UK)) for 30 minutes on the horizontal plate mixer at 500rpm. Finally, 50ul of 2M sulfuric acid stop solution is added turning the TMB yellow; the microtitre plate is placed on the horizontal mixer at 500rpm for a further three minutes or until all the wells have turned yellow. The microtitre plate is read (within 10 minutes) on a BioTek Synergy 2 ELISA plate reader (BioTek, USA) at 450nm and 620nm; the latter is used as a secondary filter correction to eliminate absorption from the microtitre plate. Total analysis time including sample preparation is approximately four hours.

Table 2.2. Generic salivary CRP ELISA plate layout.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	3000 pg/ml	3000 pg/ml	Low Control	Low Control	S8	S8	S16	S16	S24	S24	S32	S32
B	1500 pg/ml	1500 pg/ml	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
C	750 pg/ml	750 pg/ml	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
D	375 pg/ml	375 pg/ml	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
E	187.5 pg/ml	187.5 pg/ml	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
F	93.75 pg/ml	93.75 pg/ml	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
G	0 pg/ml	0 pg/ml	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
H	High Control	High Control	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39

S = sample

To calculate the levels of sample salivary CRP, a standard "curve" is created by plotting the standard concentrations against the corresponding average optical densities (Figure 2.5). This standard "curve" is generated via the equation: "$y = mx + c$" where "y" is the optical density, "x" is the salivary CRP level; "m" is the slope of the fitted line and "c" is the intercept of the "y" axis. Thus a sample of saliva processed on this ELISA generating an optical density of "y" would have a salivary CRP concentration of: "$x = a(\frac{y-c}{m})$", where "a" represents the dilution factor of the saliva sample.

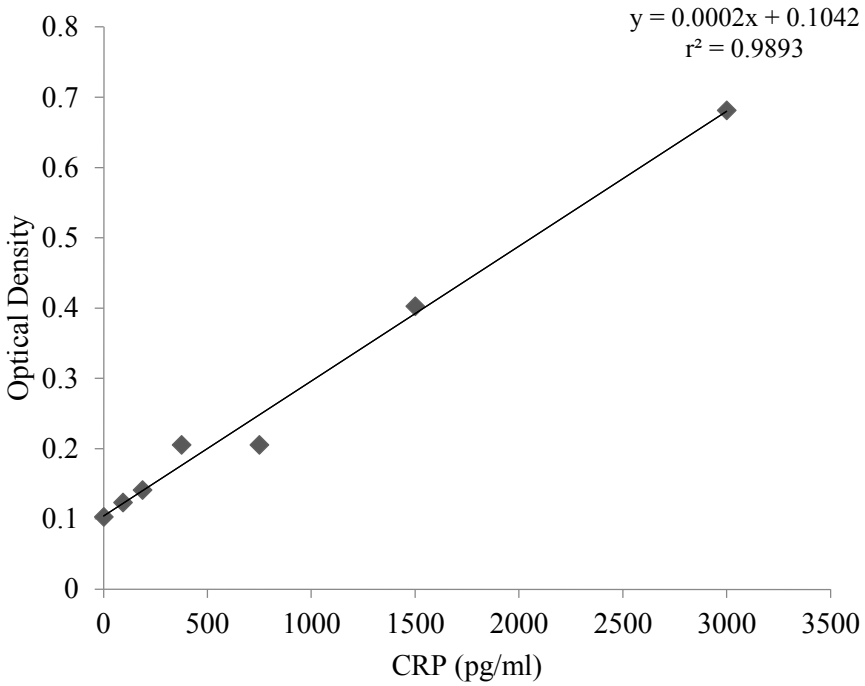


Figure 2.5: Typical CRP "line-of-best-fit" for a salivary CRP ELISA.

This scatter plot with line-of-best-fit represent each fixed concentration standard (3000, 1500, 750, 375, 187.5, 93.75, 0pg/ml) plotted against its corresponding optical density generated by aliquoting 50ul of lyophilised standard onto an ELISA microtitre plate and then performing the ELISA. The completed microtitre plate is then read on a plate reader generating an optical density for the fixed concentration standards. A line-of-best-fit is then placed through these values to generate an equation. In this example an equation for the line-of-best-fit: $y = 0.0002x + 0.1042$. This equation can be used to calculate CRP levels (x) in saliva samples with an unknown concentration but a known optical density (y). The r^2 value (99% in this example) represents the accuracy of the line-of-best-fit equation.

2.4.2.2. Salivary CRP ELISA calibration experiments in COPD saliva samples.

The manufacturer reports the Salivary CRP ELISA kit (Salimetrics, USA) performance characteristics in a manual (supplied as a kit insert) with a set of spiked CRP recovery and dilution experiments performed on the saliva of healthy subjects aged between 20 to 55 years. As the Salivary CRP ELISA (Salimetrics, USA) is classified as "research use only", the recovery and dilution experiments reported by the manufacturer in the Salivary CRP ELISA kit (Salimetrics, USA) manual were replicated to establish standard curves, control levels and reproducibility in saliva of COPD patients, who invariably are outside the above targeted age range of the immunoassay.

2.4.2.3. Aim

1. To determine the intra-assay precision.
2. To ascertain whether the recovery of CRP in COPD patients' saliva spiked with a fixed concentration of CRP (provided as part of the ELISA kit control) is consistent and comparable to healthy human saliva.
3. To ascertain the matrix effect of saliva on the recovery of endogenous CRP in five different (linear) dilutions of COPD patients' saliva.
4. To assign a limit of detection based on a selected saliva dilution.

2.4.2.4. Methods

Six COPD patients (Table 2.3) recruited from the Directorate of Respiratory Medicine's research and outpatient clinic database (Section 2.3, Page 88) in the stable phase of their disease were randomly selected, gave informed written consent (Figure 2.21, Page 176) and provided six individual 2ml unstimulated whole saliva samples via passive drool into ice-cooled collectors (Nunc, Denmark).

Table 2.3: COPD patient demographics.

Demographics	COPD Patients (n = 6)
Age, [a] years	71.7 ± 9.8
Gender, Male, (Female), n	2 (4)
***Smoking Status, Current (Ex), n**	0 (6)
Duration of COPD, [a] years	4.3 ± 1.2
FEV_1, [a] % predicted	39.7 ± 18.8
FVC, [a] % predicted	65.1 ± 9.2
BMI, [a] (kg/m^2)	26.5 ± 4.3
Exacerbations in the last 6 weeks, [a]n	0
Exacerbations in the last 1 year, [a]n	4 ± 2
Co–morbidities, n	
None	2
Cardiovascular	0
Type 2 Diabetes Mellitus	0
Hypertension	4
Gum Disease	0
Other	2
COPD Treatment, n	
Inhaled β_2 agonists, Short Acting, (Long Acting)	6, (6)
Nebulised β_2 agonists (Short Acting)	0
Inhaled Anti-cholinergic, Short Acting, (Long Acting)	2, (2)
Nebulised Anti-cholinergic (Short Acting)	0
Inhaled Steroid	6
Oral Theophylline	2

*All COPD patients had a greater than 20-year smoking pack year history. Data are presented as: a = mean ± SD; n = total number; SD = standard deviation.

Each sample was transported on ice to be stored at -80ºC in the Guy Hilton Research Centre Freezer Room (Keele University, UK). Prior to analysis samples were thawed at ambient room temperature; vortexed and subsequently centrifuged at 3000rpm for 15 minutes prior to analysis. Saliva samples were stored for no longer than 1 week prior to biomarker analysis. The recovery experiment was performed by spiking 1:10 diluted saliva samples with three different concentrations of CRP: 50, 200 and 1000pg/ml respectively (Table 2.4). Saliva was spiked with the 3000pg/ml standard supplied in the Salivary CRP ELISA kit (Salimetrics, USA). These saliva dilution factors and spiking concentrations of CRP were selected as they were identical to the experimental protocol provided in the Salivary CRP ELISA kit (Salimetrics, USA) manual. The linear dilution experiment was performed in unadulterated saliva samples analysed at five different dilutions (1:2, 1:4, 1:8, 1:10 and 1:16) (Table 2.5). All samples were tested in duplicate and only one microtitre plate was required to perform both experiments; the ELISA microtitre plate layout is summarised in Table 2.6.

Table 2.4: Saliva spiking experiment protocol.

	Reagent/Sample Volume (ul)					Total Volume
Target Spiked Concentration	**Neat Saliva**	**CRP Sample Diluent**	***3000pg/ml Standard A**	****1000pg/ml Standard B**	*****250pg/ml Standard C**	
1000pg/ml	15ul	85ul	50ul			150ul
200pg/ml	15ul	105ul		30ul		150ul
50pg/ml	15ul	105ul			30ul	150ul

* Standard A = Supplied lyophilised in the Salivary CRP ELISA kit (Salimetrics, USA).

** Standard B = 50ul of Standard A combined with 100ul of CRP sample diluent.

*** Standard C = 50ul of Standard B combined with 150ul of CRP sample diluent.

Table 2.5: Saliva dilution experimental protocol.

	Reagent/ Sample Volume (ul)						
Target Saliva dilution	**Neat Saliva**	**CRP Sample Diluent**	**1:2 diluted neat saliva**	**1:4 diluted neat saliva**	**1:8 diluted neat saliva**	**1:16 diluted neat saliva**	**Total Volume**
1:2	150ul	150ul					300ul
1:4		150ul	150ul				300ul
1:8		150ul		150ul			300ul
1:10	15ul	135ul			150ul		300ul
1:16		150ul				150ul	300ul

Table 2.6: The dilution and recovery experiment protocol for each COPD patients' sample of saliva.

COPD Patients (1 - 6)					
Patient 1 Saliva Sample 1	**Patient 2 Saliva Sample 2**	**Patient 3 Saliva Sample 3**	**Patient 4 Saliva Sample 4**	**Patient 5 Saliva Sample 5**	**Patient 6 Saliva Sample 6**
Sample 1 1:2 dilution	Sample 2 1:2 dilution	Sample 3 1:2 dilution	Sample 4 1:2 dilution	Sample 5 1:2 dilution	Sample 6 1:2 dilution
Sample 1 1:4 dilution	Sample 2 1:4 dilution	Sample 3 1:4 dilution	Sample 4 1:4 dilution	Sample 5 1:4 dilution	Sample 6 1:4 dilution
Sample 1 1:8 dilution	Sample 2 1:8 dilution	Sample 3 1:8 dilution	Sample 4 1:8 dilution	Sample 5 1:8 dilution	Sample 6 1:8 dilution
Sample 1 1:10 dilution	Sample 2 1:10 dilution	Sample 3 1:10 dilution	Sample 4 1:10 dilution	Sample 5 1:10 dilution	Sample 6 1:10 dilution
Sample 1 1:10 dilution **1000pg/ml spike**	Sample 2 1:10 dilution **200pg/ml spike**	Sample 3 1:10 dilution **50pg/ml spike**	Sample 4 1:10 dilution **1000pg/ml spike**	Sample 5 1:10 dilution **200pg/ml spike**	Sample 6 1:10 dilution **50pg/ml spike**
Sample 1 1:16 dilution	Sample 2 1:16 dilution	Sample 3 1:16 dilution	Sample 4 1:16 dilution	Sample 5 1:16 dilution	Sample 6 1:16 dilution

2.4.2.5. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, for the recovery experiment a recovery percentage was calculated using the formula: "$recovery\ percentage = 100(observed\ CRP\ level \div expected\ CRP\ level)$". For the dilution experiment a recovery percentage of CRP was calculated by assigning the unadulterated 1:10 diluted saliva sample's CRP level as 100% recovery: "$recovery\ percetage = 100(saliva\ CRP\ level \div saliva\ CRP\ level\ (1{:}10)\ dilution)$"

2.4.2.6. Results

2.4.2.6.1. Precision

The intra-assay CV for the microtitre plate was 5.6% an inter-assay CV could not be calculated as only one microtitre plate was used.

2.4.2.6.2. Recovery: salivary CRP in spiked saliva sample experiment

Accounting for the small sample size there was no significant difference in the levels of salivary CRP in the observed spiked COPD samples compared to the expected spiked COPD samples ($p=0.248$ by Wilcoxon Signed Rank Test). The recovery percentage of CRP for the spiked COPD saliva samples was greater than 90% (117 ± 26%) for all patients (Table 2.7). The recovery is greater than 100% at the lower spiked concentrations (samples 3, 5 & 6). The difference in recovery percentage of CRP (across all concentrations) between the COPD and healthy population saliva was not statistically significant ($p=0.154$ by paired t-test); thus sub-analysis between the three individual concentrations cannot be performed (Figure 2.8).

Table 2.7: Salivary CRP levels and recovery across increasing spiked concentrations.

Saliva Sample	Endogenous CRP (pg/ml)	Added Fixed Concentration CRP (pg/ml)	Expected CRP (pg/ml)	Observed CRP (pg/ml)	Recovery (%)
1	268 1554.63	1000 1000	1268 2544.63	1228 2685.88	97% 105.6%
2	516 1463.34	200 200	716 1663.34	661 1523.24	92% 91.6%
3	93 1463.34	50 50	143 1513.34	166 1389.34	116% 91.8%
4	205 1266.43	1000 1000	1205 2266.43	1303 2423.10	108% 106.9%
5	348 1199.78	200 200	516 1399.78	678 1352.03	124% 96.6%
6	163 1299.76	50 50	213 1349.76	348 1326.27	163% 100.9%
	234, 155 **1381.55, 188.80**		**616, 794** **1588.34, 687.49**	**670, 664** **1456.29, 836.78**	**117 ± 26%** **99 ± 7%**

CRP levels (pg/ml) are expressed as median, IQR; percentage recovery as mean ± SD. As saliva samples were tested in duplicate each value in black represents a mean of the duplicates. The data in red is the manufacturer's quoted figures for their recovery experiments published in the Salivary CRP ELISA kit (Salimetrics, USA) manual on saliva of healthy individuals aged 20 to 55 (Appendix 2, Page 488). This manual doesn't state whether samples were tested singularly, in duplicate or in triplicate.

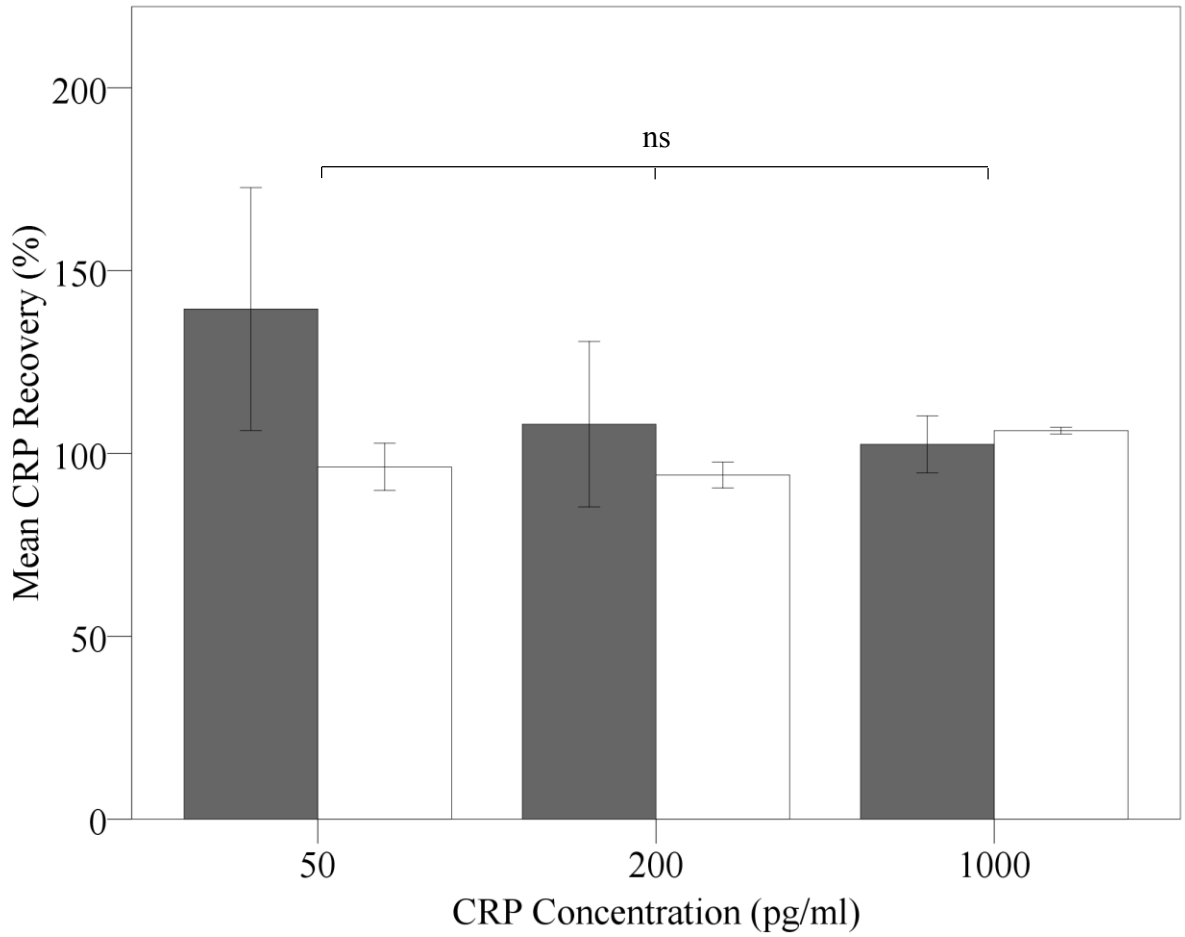


Figure 2.6: Salivary CRP recovery with increasing CRP concentrations.

Bar chart representing mean salivary CRP percentage recovery at increasing spiked concentrations (50, 200, 1000pg/ml) with error bars ± SD (n = 2 per CRP concentration). The dark grey bars represent COPD saliva the white bars healthy subjects' saliva data presented in the Salivary CRP ELISA kit (Salimetrics, USA) manual (Appendix 2, Page 488). There is no statistically significant difference in recovery between the two groups (ns: p=0.154) thus post-hoc analysis between the individual concentrations cannot be performed. ns = no significance.

Overall there is no significant difference in CRP percentage recovery and actual concentration of salivary CRP in spiked saliva samples between COPD patients and healthy subjects' saliva at a 1:10 saliva dilution.

2.4.2.6.3. Linear dilution: endogenous salivary CRP across increasing saliva dilutions

The recovery of endogenous CRP (recovery was compared to the 1:10 dilution as this is manufacturer recommended) was analysed to understand the effects of saliva sample dilution. Overall percentage recovery of endogenous CRP significantly improved in unadulterated saliva samples as the dilution factor increases ($p<0.025$ by repeated measures ANOVA with a Greenhouse-Geisser correction) (Table 2.8). Post-hoc analysis only demonstrated a significant improvement in recovery from 1:2 to 1:8 ($p<0.003$($) by paired t-test) and 1:2 to 1:16 ($p<0.002$($)) dilutions. Interestingly unlike the manufacturers sample dilution recovery experiments (Table 2.9, Page 108) recovery of salivary CRP at dilutions lower than 1:10 was reduced. It is important to note the small sample size however as the manufacturer only tested saliva sample dilution CRP recovery on two saliva samples. All COPD patients' saliva samples demonstrated an improvement in salivary CRP recovery as the dilution factor increased up to 1:8. This recovery improved at 1:16 dilution for all saliva samples except for sample 5 where CRP recovery fell markedly when the dilution increased from 1:8 to 1:16. A potential explanation could be attributable to the low endogenous CRP levels in the saliva sample; however a similar finding was not observed for saliva sample 6. Half the saliva samples at 1:16 dilution demonstrated a "super" recovery (Figure 2.7, Page 106). It is not possible to compare the manufacturers dilution recovery data to the COPD patients' salivary CRP recovery data due to the manufacturer's small sample size ($n = 2$) in their healthy dilution experiments (Table 2.9, Page 108).

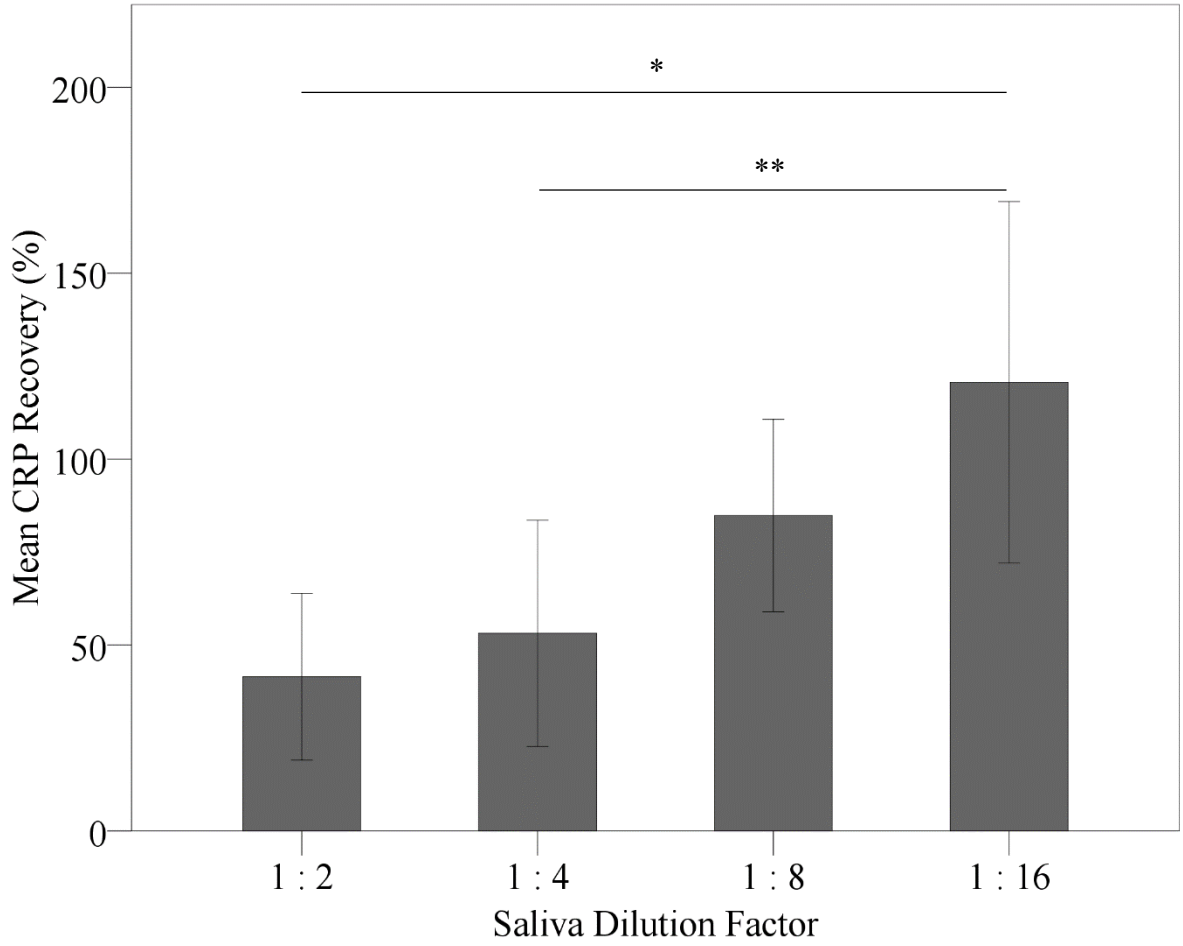


Figure 2.7: Salivary CRP recovery across increasing dilution.

Bar chart representing salivary CRP percentage recovery at increasing salivary dilution (1:2, 1:4, 1:8, 1:16) with error bars ± SD (n = 6). Overall there is a significant improvement in recovery with an increased dilution ($p<0.025$ by repeated measures ANOVA with a Greenhouse-Geisser correction). Post-hoc analysis identified a significant improvement in CRP recovery from 1:2 to 1:8, (**$p<0.003$($) by paired t-test) and 1:2 to 1:16 (*$p<0.002$($)) dilution.

Table 2.8: Endogenous salivary CRP levels and recovery percentage at increasing dilutions.

		Salivary CRP (pg/ml)			
		Saliva Sample Dilution			
Saliva Sample	**Observed CRP (1:10 dilution)**	**1:2**	**1:4**	**1:8**	**1:16**
1	268 (100%)	219 (82%)	278 (104%)	404 (151%)	348 (130%)
2	516 (100%)	184 (36%)	347 (67%)	352 (68%)	684 (130%)
3	93 (100%)	15 (16%)	18 (20%)	70 (76%)	132 (143%)
4	205 (100%)	91 (45%)	58 (28%)	146 (71%)	284 (139%)
5	348 (100%)	87 (25%)	134 (43%)	218 (69%)	252 (73%)
6	163 (100%)	64 (40%)	88 (54%)	140 (86%)	136 (84%)
Recovery (%) mean ± SD	**100% ± 0%**	**40% ± 23%**	**52% ± 31%**	**86% ± 33%**	**117% ± 30%**

Salivary CRP levels are multiplied by the dilution factor to allow standardisation of the results. The figures in brackets represent CRP recovery percentage when compared to the 1:10 dilution CRP level which is assigned as 100% recovery.

Table 2.9: Manufacturer endogenous salivary CRP levels and recovery percentage at increasing dilutions.

	Salivary CRP (pg/ml)				
	Saliva Sample Dilution				
Saliva Sample	**Expected CRP**	**1:2**	**1:4**	**1:8**	**1:16**
1	*1259.61 (100%)	1219.36 (96.8%)	1152.32 (91.5%)	1269.44 (100.8%)	1226.56 (97.4%)
2	*1627.90 (100%)	1577.64 (96.9%)	1461.96 (89.8%)	1569.12 (96.4%)	1623.52 (99.7%)
Recovery (%) mean ± SD	**100% ± 0%**	**97% ± 0.1%**	**91% ± 1%**	**99% ± 3%**	**99% ± 2%**

* The manufacturer does not state at what saliva dilution factor this value was obtained. These values were obtained from the Salivary CRP ELISA kit (Salimetrics, USA) manual (Appendix 2, Page 488). The levels of quantified CRP were multiplied by the dilution factor to allow standardisation of the results.

Overall an increased saliva dilution beyond 1:8 improves recovery of salivary CRP in unadulterated saliva samples. Lower percentage recoveries were demonstrated at 1:2 and 1:4 saliva dilutions for all COPD patients' saliva compared to their respective 1:8 and 1:16 dilution although the sample size is small.

2.4.2.7. Discussion.

The above experiments replicated the recovery and linear dilution experimental protocols provided in the manual accompanying every Salivary CRP ELISA kit (Salimetrics, USA). COPD saliva samples spiked with CRP demonstrate a consistent recovery. An enhanced recovery (greater than 100%) was noted in samples 3 and 6 both of which were spiked with

50pg/ml. This may potentially be explained by considering the working range of the immunoassay (0.01 to 3ng/ml). It is possible that the accuracy of the immunoassay is reduced when approaching the lower limit of this working range. Thus a low CRP spike for samples with low levels of endogenous CRP when diluted 1:10 will require the quantification of CRP at the lower limit of the working range (0.01 to 3ng/ml), which may have a higher variability.

This hypothesis may also provide an explanation for why the manufacturer only recommends further dilution of saliva beyond 1:10 only if the levels of salivary CRP are above the higher limit of the immunoassay working range (that is above the higher limit of quantification); as opposed to setting a higher minimum dilation level. If we considered a saliva sample diluted at 1:10, and which has a CRP level above the higher limit of quantification for the immunoassay, a further dilation (1:20) for this saliva sample would result in the diluted sample (1:20) containing 50% less endogenous CRP than (1:10) sample. However as the 1:10 dilution was above the higher limit of quantification this increased dilution would result in the saliva sample being quantified in the mid-high working range (1.5 to 3ng/ml) of the immunoassay. This argument may support the hypothesis that the immunoassay is more prone to errors in salivary CRP quantification when the diluted saliva sample is approaching the lower limit of the assay's working range. This is further supported by the enhanced recovery of salivary CRP that is apparent in unadulterated saliva samples diluted 1:16. The reduced recovery at the lower dilutions (1:2, 1:4, 1:8) possibly demonstrates the matrix effect of saliva and supports the manufacturer's recommendation of a minimum sample dilution of 1:10 for saliva samples tested with this particular immunoassay.

Interrogation of the manufacturer's experimental data (Table 2.7, Page 103) demonstrated that the median endogenous levels of CRP in the healthy saliva samples are higher than in the

COPD saliva samples used for these experiments. This would result in a positive bias for the recovery of spiked samples. For example, the manufacturer's quoted figures for sample 6 are below:

*Saliva Sample	Endogenous CRP (pg/ml)	Added CRP (pg/ml)	Expected CRP (pg/ml)	Observed CRP (pg/ml)	Recovery (%)
6	1299.76	50	1349.76	1326.27	100.9%

*extract from Table 2.7, Page 103.

This sample was spiked with 50pg/ml of CRP. However as the endogenous level is 1299.76pg/ml a recovery percentage using this value alone as an observed value would be 98%. It should also be noted that healthy saliva samples 2 and 3 have the same concentration of endogenous CRP and are thus likely to be the same individual. It would be more prudent to spike saliva samples with absent or “low” levels of endogenous CRP to better understand the recovery of a fixed concentration of CRP and reduce the positive bias introduced by higher endogenous levels.

2.4.2.8. Overall conclusion

The intra-assay precision (CV) of 5.6% was below the maximum (10%) accepted error percentage that had been selected. This will continue to be calculated for all salivary CRP ELISAs performed throughout this thesis to ensure that both the intra- and inter-assay CV's remain below the accepted and clinical thresholds. The recovery of salivary CRP is greater than 90% for all spiked samples of saliva. Serial dilution of unadulterated saliva samples appears to improve the recovery of endogenous CRP with the most significant improvement observed across 1:2 to 1:8 dilutions. A minimum dilution of 1:10 appears to be appropriate; however further dilutions cannot be recommended in samples that are not above the higher limit of the assay working range (0.01 to 3ng/ml). When salivary CRP levels are above the higher limit of the assay working range it appears that there is a large variability in recovery when using a dilution of 1:16. Thus the results from higher dilutions would be discarded if the salivary level falls below what would have been quantified at a 1:10 dilution. The minimum limit of detection will be 10pg/ml as quoted in the Salivary CRP ELISA kit (Salimetrics, USA) manual.

Whilst understanding the above caveats, the Salivary CRP ELISA kit (Salimetrics, USA) will be used throughout this thesis for COPD salivary CRP quantification, maintaining the same pre-analysis sample preparation; duplicate sample testing; minimum sample dilution (1:10) and test procedure as recommended by the manufacturer.

2.4.3. Optimisation of a serum-based Procalcitonin (PCT) assay for use with saliva

A variety of ELISAs are commercially available for the quantification of human PCT for example: RayBiotech, (USA), Abcam (UK) and Sigma-Aldrich (USA); these are all labelled for "research use only". The different classifications have previously been described (Section 2.4.2.1, Page 91) and an immunoassay classified "for diagnostic use" was selected for modification. It is assumed that as no saliva-based PCT assay is commercially available, the choice of a serum-based PCT assay classified "for diagnostic use" would be more robust for modification to saliva.

The VIDAS B.R.A.H.M.S PCT (bioMérieux, France) is an automated "for diagnostic use" quantitative assay processed on the bench-top VIDAS instruments (bioMérieux, France) for the determination of human PCT in serum or plasma using an enzyme-linked fluorescent assay (ELFA). The analyser used in this thesis is the mini VIDAS (bioMérieux, France) which comprises two analytical sections containing 6 testing slots per section. Thus 12 samples can be processed in unison. The mini VIDAS requires a monthly calibration where a quality control (QCV) test strip is analysed by the machine in each of the 12 test slots. If any value on each of the 12 test strips fall outside of the range quoted on the QCV kit insert (provided by the manufacturer) the QCV needs to be analysed again for that entire section. If the QCV fails for a second time the mini VIDAS needs to be serviced by a BioMérieux engineer. As this immunoassay has to date not been applied to saliva, experiments were designed to modify its application to suit PCT measurement in saliva.

The steps involved in measuring serum PCT using the above automated quantification are discussed. Each PCT kit is supplied with a master-lot-entry (MLE) card, 60 ready-to-use multi-well test-strips, 60 solid phase receptacles (SPR) pre-coated with mouse anti-human PCT and

two sets of lyophilised standards (S1 and S2) and controls (C1: low range; C2: high range); both standards and controls are reconstituted with 2mls dH_2O (Figure 2.8). The purpose of the MLE, standards and controls is to calibrate the mini VIDAS analyser for PCT quantification in serum or plasma samples.

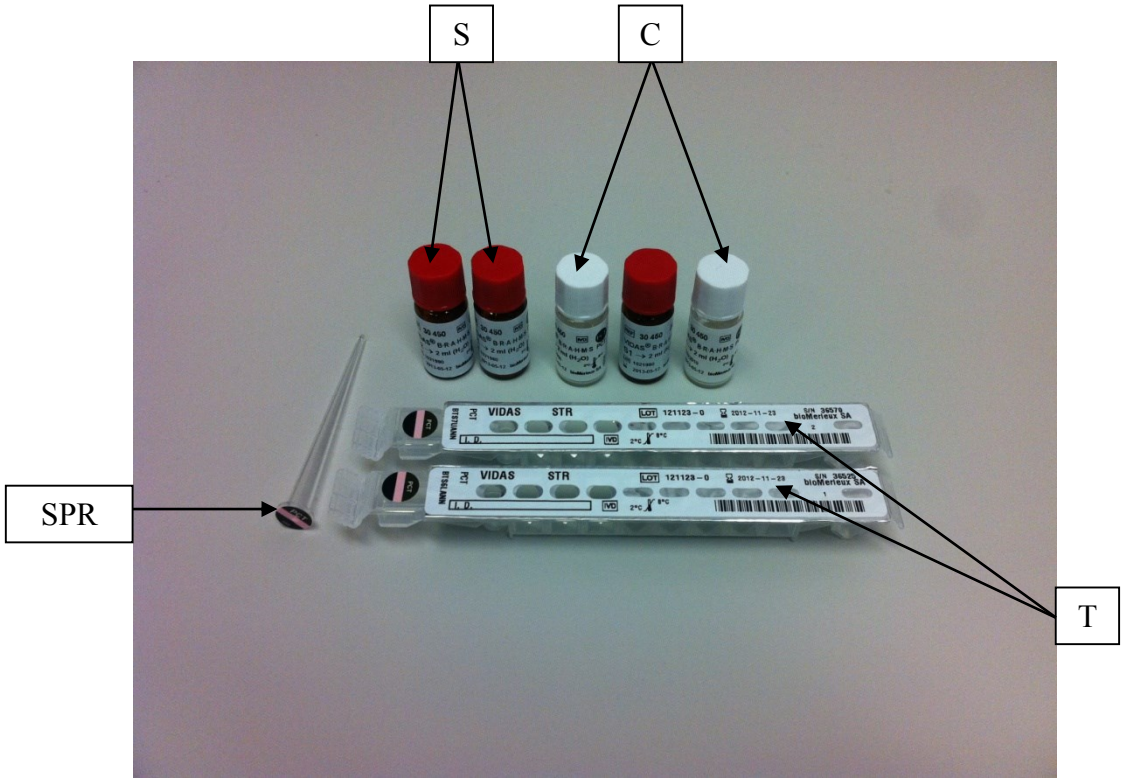


Figure 2.8: VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France).

S = standard, C = control vial, SPR = solid-phase receptacle, T = test-strip

Calibration starts first with entry of the MLE card then six test-strips containing 200ul of both standards (S1 and S2: tested in duplicate) and 200ul of high range (C1) plus low range (C2) control reagents (tested singularly) as well as 6 SPRs are inserted into the mini VIDAS (bioMérieux, France). The machine is then programmed to analyse the samples. Total analysis time is 25 minutes after which a set of results is printed (these values are used to calculate the inter-assay CV). This printout is compared to the reference data on the MLE card with

agreement between the two indicating that the mini VIDAS has been successfully calibrated. Any disagreement between the two requires the calibration process to be repeated. If the repeat calibration process fails, then the machine requires servicing by an BioMérieux engineer. The printout from the calibration can be used to calculate an inter-assay CV to determine the precision of the machine. Calibration is required monthly or on the opening of a new kit with a different batch number to the previously calibrated kit. In total each kit can analyse 27 samples in duplicate and 30 samples in duplicate if calibration is not required. All assay steps are performed internally within the mini VIDAS (bioMérieux, France) machine; results are calculated automatically from two standard calibration curves with the working assay range being 0.05 to 200ng/ml. Once calibrated for sample processing, each test-strip requires 200ul of sample; the test-strip and one SPR are then loaded into the mini VIDAS (bioMérieux, France) and the machine is programmed to analyse the sample. Each assay takes approximately 25 minutes. There are no established references to date regarding the use of the mini VIDAS (bioMérieux, France) system for processing of saliva samples.

2.4.3.1. Optimisation of a serum PCT immunoassay

To investigate whether the mini VIDAS (bioMérieux, France) could quantify PCT in saliva, experiments on the recovery of PCT in spiked saliva samples were conducted. The VIDAS B.R.A.H.M.S PCT (bioMérieux, France) kit does not contain a sample diluent as neat serum is analysed by the instrument. As saliva contains mucin, making it a viscous body fluid (Rantonen and Meurman, 1998), experiments were also conducted to investigate the effects of dilution on PCT recovery in saliva. The saliva diluent selected was Phosphate Buffer Saline - Tween 20 0.05% (Sigma, USA). Phosphate Buffer Saline - Tween (PBS-T) has a (pH), which is similar to human physiology and thus prevents protein denaturation; it also acts as an antibody-antigen staining stabiliser.

2.4.3.2. Aims

1. To determine the intra- and inter-assay precision.
2. To determine the effects of PBS-T on the levels of a fixed concentration of PCT provided as part of the PCT kit control.
3. To establish the matrix effect of saliva on the recovery of a fixed concentration of PCT provided as part of the PCT kit control; specifically, whether there is a difference in PCT recovery in saliva at low and high PCT ranges.
4. To explore this matrix effect in neat human saliva and at three different linear dilutions of saliva.
5. To assign a limit of detection based on a selected saliva dilution.

2.4.3.3. Methods

Ten healthy non-smokers and six healthy smokers (Table 2.10) were randomly selected from the Directorate of Respiratory Medicine's research and outpatient clinic database (Section 2.3, Page 88); gave informed written consent and provided 4mls of unstimulated whole saliva via passive drool (Figure 2.21, Page 176) into ice-cooled collector tubes. Saliva samples were then transported on ice and stored in the Guy Hilton Research Centre Freezer Room (Keele University, UK) at -80^{o}C. Prior to analysis all saliva samples were thawed at room temperature. All saliva samples were stored for no longer than 1 week prior to biomarker testing. The VIDAS B.R.A.H.M.S PCT (bioMérieux, France) assay is validated for serum and no protocol exists for pre-analysis saliva sample preparation. The same methodology as for salivary CRP quantification (Section 2.4.2.1, Page 92) was utilised, with all saliva samples vortexed and subsequently centrifuged at 3000rpm for 15 minutes.

Table 2.10: Healthy subject demographics.

Demographics	Healthy Subjects	
	Non-smokers (n = 10)	**Smokers (n = 6)**
Age, [a] years	30.9 ± 9.6	36.2 ± 13.8
Gender, Male, (Female), n	4 (6)	3 (3)
BMI, [a] (kg/m^2)	23.2 ± 3.5	25.9 ± 2.9
Co–morbidities, n		
None	10	6
Total Number of Oral Medications, [a]n	0	0

*co-morbidities include the presence of gum disease. Data are presented as: a = mean ± SD.

Each VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France) provides two control reagents (C), C1: High Range (16 to 20ng/ml) and C2: Low Range (1.5 to 2.0ng/ml). Each saliva sample was assigned to an individual vial of the low and high range controls; thus 32 individual vials (16 low range and 16 high range) were used. These controls were then used to spike both PBS-T and saliva.

The first step involved reconstituting all control vials with 2mls of dH_2O. PBS-T was then prepared by dissolving (1.44g Na_2HPO_4, 0.211g NaH_2PO_4, and 8g NaCl) pre-prepared pouch (Sigma, UK) in 1000 ml of dH_20. Subsequently 200ul of both the low and high range PCT controls were aliquoted into separate eppendorfs and diluted (1:2) with 200ul of PBS-T. The next step involved 200ul of both the neat low and high range control plus 200ul of the diluted low and high range control (1:2) aliquoted into test-strips and analysed in duplicate on the mini VIDAS (bioMérieux, France).

After this control experiment, 200ul of each unadulterated saliva sample were analysed neat and at three dilutions (1:2, 1:4 and 1:8) to determine endogenous PCT levels (Table 2.11). The saliva samples were then spiked with both the low and high range PCT control vials and

analysed neat (low range PCT only) and at three different dilutions (Tables 2.12, 2.13). These samples were analysed in duplicate using the mini-VIDAS (bioMérieux, France) to determine the recovery of PCT in saliva at the two concentrations and three saliva dilutions. The lower limit of quantification for PCT using a neat sample with the mini VIDAS (bioMérieux, France) is 0.05ng/ml. However as the target sample is diluted this would change (1:2 = 0.10ng/ml, 1:4 = 0.20ng/ml, 1:8 = 0.40ng/ml). The volume of all samples aliquoted onto the test strips was kept constant at 200ul.

Table 2.11: Unadulterated saliva dilution experimental protocol.

	Reagent/Sample Volume (ul)				
Target Saliva dilution	**Neat Saliva**	**PBS-T**	**1:2 diluted neat saliva**	**1:4 diluted neat saliva**	**Total Volume**
1:2	400ul	400ul			800ul
1:4		400ul	400ul		800ul
1:8		400ul		400ul	800ul

Table 2.12: Low range PCT spiking experimental protocol.

	Reagent/Sample Volume (ul)					
Target Dilution: Low Range Spiked Saliva Solution	**Neat Saliva**	**Low Range PCT Control**	**High Range PCT Control**	***Sample A**	****Sample B**	**Total Volume**
95% neat	190ul		10ul			200ul
1:2	100ul	100ul				200ul
1:4		100ul		100ul		200ul
1:8		100ul			100ul	200ul

* Sample A = 200ul neat saliva combined with 200ul of PBS-T.

** Sample B = 200ul Sample A combined with 200ul of PBS-T.

Table 2.13: High range PCT spiking experimental protocol.

	Reagent/Sample Volume (ul)					
Target Dilution: High Range Spiked Saliva Solution	**Neat Saliva**	**High Range PCT Control**	**PBS-T**	***Sample A**	****Sample B**	**Total Volume**
1:2	100ul	100ul				200ul
1:4		100ul		100ul		200ul
1:8		100ul			100ul	200ul

* Sample A = 200ul neat saliva combined with 200ul of PBS-T.

** Sample B = 200ul Sample A combined with 200ul of PBS-T.

2.4.3.4. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, for these experiments the level of PCT in the controls was assigned as a 100% recovery.

2.4.3.5. Results

2.4.3.5.1. Precision

The intra and inter-assay co-efficient of variance was 8.2 and 10.1% respectively utilising 4 PCT kits for these experiments.

2.4.3.5.2. Saliva diluent

As discussed earlier Section 2.4.3.1, Page 114 PBS-T was chosen as a diluent for saliva. It was considered important to establish whether PBS-T would affect the level of PCT in both the low and high range controls. There was no statistically significant difference when comparing both the low range (n = 16) and high range (n = 16) PCT control levels to the low and high range PCT control levels when diluted 1:2 in PBS-T (low range p=0.08 by paired t-test and high range p=0.15 respectively), (Figure 2.14, Table 2.15, Page 123; Table 2.16, Page 126).

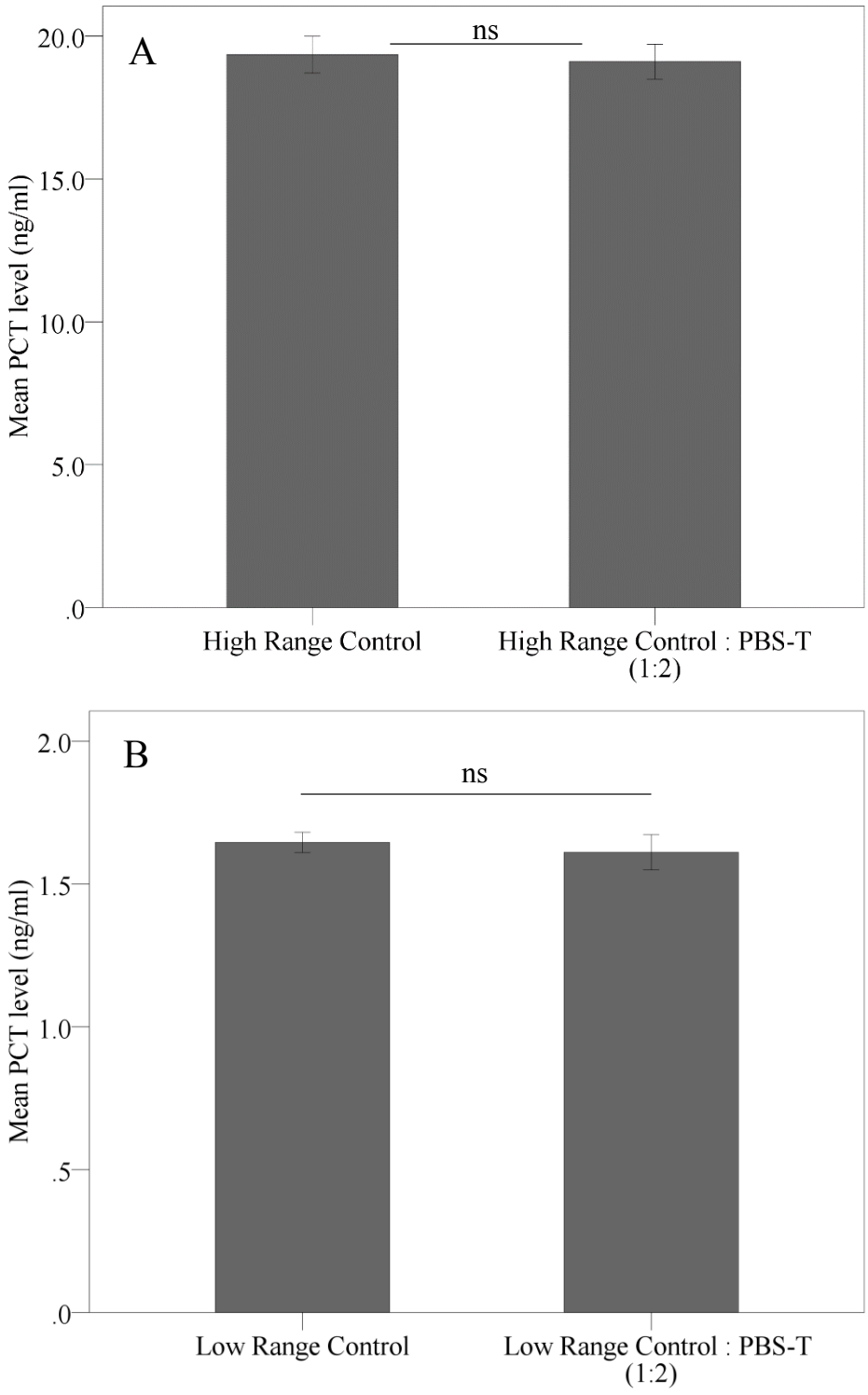


Figure 2.9: Comparison between PBS-T and Control levels.

Bar chart with error bars ± SD for (A) high range (n = 16) and (B) low range control (n = 16) respectively. Both controls were diluted with PBS-T (1:2). Overall there was no statistically significant difference in the levels of PCT between both the low range and high range controls when compared to PBS-T diluted controls (ns: p=0.08, p=0.15 respectively by paired t-test).

In conclusion PBS-T as a diluent did not appear to interfere with the quantification of PCT in this immunoassay.

2.4.3.5.3. Endogenous salivary PCT

The next step was to investigate the endogenous levels of PCT in all saliva samples (n = 16) neat and at three dilutions (1:2, 1:4 and 1:8) (Table 2.14). This would allow for endogenous levels to be included in the recovery calculation of samples spiked with PCT so that a false enhanced recovery was not calculated.

Table 2.14: Endogenous PCT levels in saliva from healthy individuals.

	Saliva Sample Dilution (PBS-T)			
Healthy Subject	**Neat Saliva**	**1:2**	**1:4**	**1:8**
1	<0.05	<0.10	<0.20	<0.40
2	<0.05	<0.10	<0.20	<0.40
3	<0.05	<0.10	<0.20	<0.40
4	<0.05	<0.10	<0.20	<0.40
5	<0.05	<0.10	<0.20	<0.40
6	<0.05	<0.10	<0.20	<0.40
7	<0.05	<0.10	<0.20	<0.40
8	<0.05	<0.10	<0.20	<0.40
9	0.065	<0.10	<0.20	<0.40
10	<0.05	<0.10	<0.20	<0.40
***11**	0.15	0.15	<0.20	<0.40
***12**	0.14	0.14	<0.20	<0.40
***13**	<0.05	<0.10	<0.20	<0.40
***14**	<0.05	<0.10	<0.20	<0.40
***15**	<0.05	<0.10	<0.20	<0.40
***16**	<0.05	<0.10	<0.20	<0.40

*current smokers.

2.4.3.5.4. Healthy non-smoker saliva studies

2.4.3.5.4.1. Low range recovery and linear dilution PCT experiments

Saliva samples were spiked with low range PCT control to determine whether the levels of PCT were affected as the dilution ratio is increased (neat, 1:2, 1:4, 1:8). The recovery of PCT was calculated by assigning the levels of PCT in the low range control diluted 1:2 with PBS-T as 100%. Thus percentage recovery at each dilution ratio was derived from the following formula where x is the diluent factor for saliva:

"$percentage\ recovery = 100\left(\frac{2(PCT\ level\ in\ Low\ Control\ diluted\ 1{:}2\ with\ PBS-T)}{x(PCT\ level\ in\ saliva\ diluted\ with\ PBST-endogenous\ PCT\ level)}\right)$"

Mean PCT recovery increased as the dilution of saliva increased (neat saliva = 56 ± 26%; 1:2 = 87 ± 15%; 1:4 = 91 ± 10%; 1:8 = 94 ± 6%) (Table 2.15; Figure 2.10).

Table 2.13: Low range PCT levels and recovery in healthy non-smokers.

Subject	*Control Level (ng/ml)	*Control PBS-T 1:2 dilution (ng/ml)	Neat Saliva Recovery (%)	**Diluted Saliva 1:2 Recovery (%)	**Diluted Saliva 1:4 Recovery (%)	**Diluted Saliva 1:8 Recovery (%)
1	1.64	1.56	17%	52%	69%	82%
2	1.65	1.56	86%	86%	90%	91%
3	1.63	1.60	51%	102%	101%	102%
4	1.69	1.62	42%	72%	83%	89%
5	1.69	1.54	45%	83%	86%	92%
6	1.67	1.68	33%	96%	95%	95%
7	1.64	1.68	76%	96%	95%	88%
8	1.66	1.64	68%	88%	96%	100%
9	1.66	1.66	100%	98%	103%	101%
10	1.63	1.60	45%	92%	94%	98%
Mean ± SD	**1.66 ± 0.02**	**1.62 ± 0.05**	**56 ± 26%**	**87 ± 15%**	**91 ± 10%**	**94 ± 6%**

*Control refers to low range PCT control, **Diluted with PBS-T. The levels of quantified PCT were multiplied by the dilution factor to allow standardisation of the results.

This increase in low range PCT recovery was statistically significant across the 3 dilutions ($p<0.002$ by a repeated measures ANOVA with a Greenhouse-Geisser correction). A separate post-hoc analysis showed a statistically significant improvement in PCT recovery between neat saliva and the 1:2 dilution ($p<0.037$($) by paired t-test), 1:4 dilution ($p<0.014$($)), and 1:8 dilution ($p<0.020$($)). Comparing 1:2 dilution with 1:4 and 1:8 respectively, the improvement in mean recovery is not statistically significant ($p=0.161$ and $p=0.374$ respectively). Comparing the 1:4 and 1:8 dilution samples, the improvement in mean recovery was also not statistically significant ($p=0.998$).

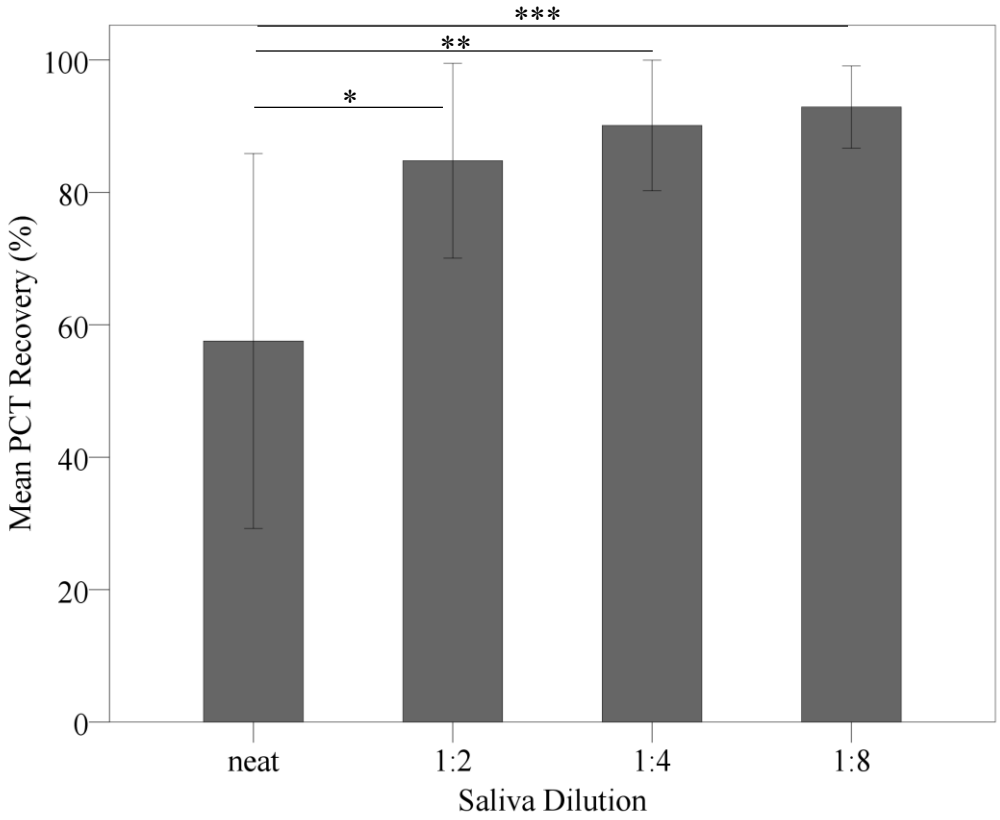


Figure 2.10: Low range salivary PCT recovery in healthy non-smokers.

Bar chart representing mean salivary PCT recovery at increasing dilution factor with error bars ± SD (n = 10). Saliva was diluted with PBS-T. Across the three dilutions there is a significant increase in recovery $p<0.002$ by repeated measures ANOVA. A post-hoc analysis shows that there is a significant difference in PCT recovery between neat and all 3 dilutions of saliva 1:2 ($p<0.037$($)) by paired t-test), 1:4 ($p<0.014$($)) and 1:8 ($p<0.02$($)) saliva dilution (*$p<0.002$($)) by paired t-test), and between 1:4 and 1:8 saliva dilution (**$p<0.004$($)) respectively. No significant difference in PCT recovery is demonstrated between 1:2 and 1:4 dilutions ($p=0.087$) respectively. The large SD error bar for the neat sample demonstrates that in some healthy subjects the matrix effect of saliva is large, these error bars narrow as the dilution ratio increases demonstrating that the matrix effect has been overcome.

In summary, diluting neat saliva improves PCT recovery; however there is no advantage in increasing the dilution from 1:2 to 1:4 or 1:8. Thus a 1:2 dilution appears to be optimal for recovery of low range PCT with further improvements in recovery not statistically significant.

2.4.3.5.4.2. High range recovery and liner dilution PCT experiments

Next it was determined whether PCT levels were affected by increasing the dilution of saliva with PBS-T (1:2, 1:4 and 1:8) in samples spiked with the high range PCT control. A neat sample of saliva was not spiked with the high range control as the concentration range is not high enough. *The low range PCT spike for neat saliva (95% pure) required the saliva sample to be spiked with the high range control (Table 2.12*). The results from the low range recovery experiment (Table 2.15) indicated a significant reduction in recovery in the neat sample. To generate a low range spike for neat saliva the high range PCT control was utilised. Thus it would not be possible using the controls in the VIDAS B.R.A.H.M.S PCT (bioMérieux, France) to generate a high range spike in neat saliva. Spiked high range PCT levels in neat saliva were investigated with recombinant PCT (Prospec, Israel) as a further experiment later in this chapter (Section 2.4.3.7, Page 134).

The recovery of PCT was calculated by assigning the levels of PCT in the high range control diluted 1:2 with PBS-T as a 100%. Thus percentage recovery at each dilution was derived from the following formula where x is the diluent factor for saliva.

"$percentage\ recovery = 100\left(\frac{2(PCT\ level\ in\ High\ Control\ diluted\ 1{:}2\ with\ PBS{-}T)}{x(PCT\ level\ in\ saliva\ diluted\ with\ PBST{-}endogenous\ PCT)}\right)$"

Overall mean PCT recovery percentage increased as the dilution of saliva with PBS-T increased (1:2 = 86 ± 8%; 1:4 = 90 ± 6%; 1:8 = 95 ± 4%) (Table 2.16, Figure 2.11).

Table 2.16: High range PCT levels and recovery in healthy non-smokers.

Subject	Control* Level (ng/ml)	Control* PBS-T 1:2 dilution (ng/ml)	Neat Saliva PBS-T 1:2	Neat Saliva PBS-T 1:4	Neat Saliva PBS-T 1:8
1	18.26	18.08	78%	87%	94%
2	19.49	18.46	86%	88%	95%
3	20.27	18.64	68%	76%	87%
4	19.32	19.14	91%	88%	94%
5	19.41	19.28	92%	93%	96%
6	19.13	18.84	91%	94%	94%
7	19.77	19.84	88%	94%	96%
8	20.25	20.02	85%	95%	100%
9	18.51	18.86	95%	94%	99%
10	19.98	20.04	89%	90%	91%
Mean ± SD	**19.44 ± 0.67**	**19.12 ± 0.67**	**86 ± 8%**	**90 ± 6%**	**95 ± 4%**

*Control refers to high range PCT control. The levels of quantified PCT were multiplied by the dilution factor to allow standardisation of the results.

There was a statistically significant increase in percentage scores for high range PCT recovery across the three dilutions ($p<0.001$ by repeated measures ANOVA). A post-hoc analysis shows that there was a significant difference in PCT recovery between 1:2 and 1:8 saliva dilution ($p<0.002$($) by paired t-test), and between 1:4 and 1:8 saliva dilution ($p<0.004$($)). No significant difference in PCT recovery was demonstrated between 1:2 and 1:4 ($p=0.087$) this result was also observed in the low range PCT recovery experiments between the same dilutions.

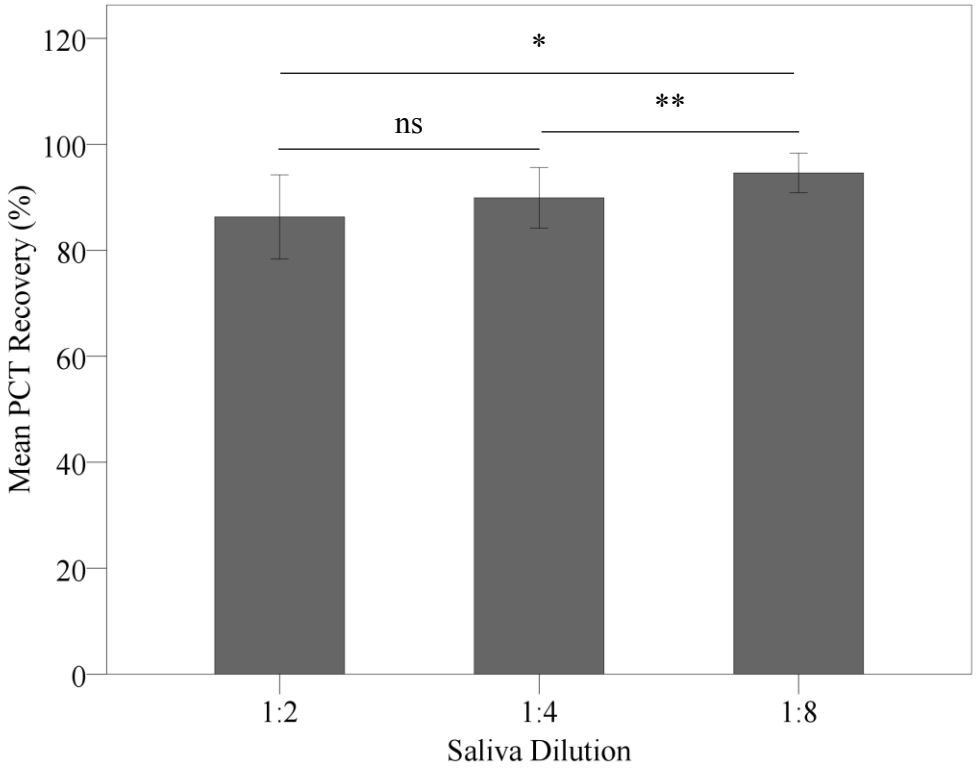


Figure 2.11: High range salivary PCT recovery in healthy non-smokers.

Bar chart representing mean salivary PCT recovery at increasing dilution factor with error bars ± SD (n = 10). Saliva was diluted with PBS-T. The figures in brackets represent the dilution factor. Across the three dilutions there was a significant increase in recovery ($p<0.001$ by repeated measures ANOVA). A post-hoc analysis shows that there was a significant difference in PCT recovery between 1:2 and 1:8 saliva dilution (*$p<0.002$($) by paired t-test), and between 1:4 and 1:8 saliva dilution (**$p<0.004$($)). No significant difference in PCT recovery was demonstrated between 1:2 and 1:4 ($p=0.087$).

It can be concluded that the possible matrix effect of saliva on high range PCT recovery can be overcome with increasing linear dilution of the saliva samples, from 1:2 to 1:8, 1:4 to 1:8 respectively but not 1:2 to 1:4.

Taken together the results for both the low and high range PCT experiments demonstrated a significant improvement in recovery of PCT in saliva with increasing linear dilution. However to add validity that the mini-VIDAS (bioMérieux, France) can be reliably used for the quantification of salivary PCT, a further sub-analysis was conducted to determine whether there was a difference in PCT recovery between the samples spiked with low range and high range PCT control.

Overall there was no statistical difference between low range and high range PCT recovery across all dilutions ($p=0.365$ by two-way repeated measures ANOVA with Greenhouse-Geisser correction). Thus accounting for the small sample size the matrix effect of saliva on the recovery of salivary PCT appears not to be increased when the concentration of PCT is lower.

2.4.3.5.5. Healthy smoker saliva studies

It has been established that using the saliva of healthy non-smokers for validating serum based ELISA's for use with saliva is appropriate (Jaedicke et al., 2012). However as the mini VIDAS (bioMérieux, France) is a fluorescence-based immunoassay salivary PCT recovery was investigated in healthy smokers.

2.4.3.5.5.1. Low range recovery and linear dilution PCT experiments

Mean PCT recovery increased as the dilution of saliva increased (neat = 75 ± 43%, 1:2 = 81 ± 23%; 1:4 = 87 ± 15%; 1:8 = 92 ± 9%) (Table 2.17, Figure 2.12). This increase in PCT recovery was not significant in different saliva dilutions for smokers ($p=0.177$ by repeated measures ANOVA with a Greenhouse-Geisser correction). Thus post-hoc analysis between the different saliva dilutions could not be performed.

Table 2.17: Low range PCT levels and recovery in healthy smokers.

Subject	*Control Level (ng/ml)	*Control PBS-T 1:2 dilution (ng/ml)	Neat Saliva	Neat Saliva PBS-T 1:2	Neat Saliva PBS-T 1:4	Neat Saliva PBS-T 1:8
11	1.59	1.50	45%	56%	71%	79%
12	1.58	1.56	12%	63%	71%	82%
13	1.71	1.66	114%	102%	101%	98%
14	1.67	1.66	51%	62%	81%	93%
15	1.66	1.62	85%	94%	95%	96%
16	1.64	1.56	113%	110%	104%	100%
Mean ± SD	**1.64 ± 0.05**	**1.59 ± 0.06**	**75 ± 43%**	**81 ± 23%**	**87 ± 15%**	**92 ± 9%**

*Control refers to low range PCT control. The levels of quantified PCT were multiplied by the dilution factor to allow standardisation of the results.

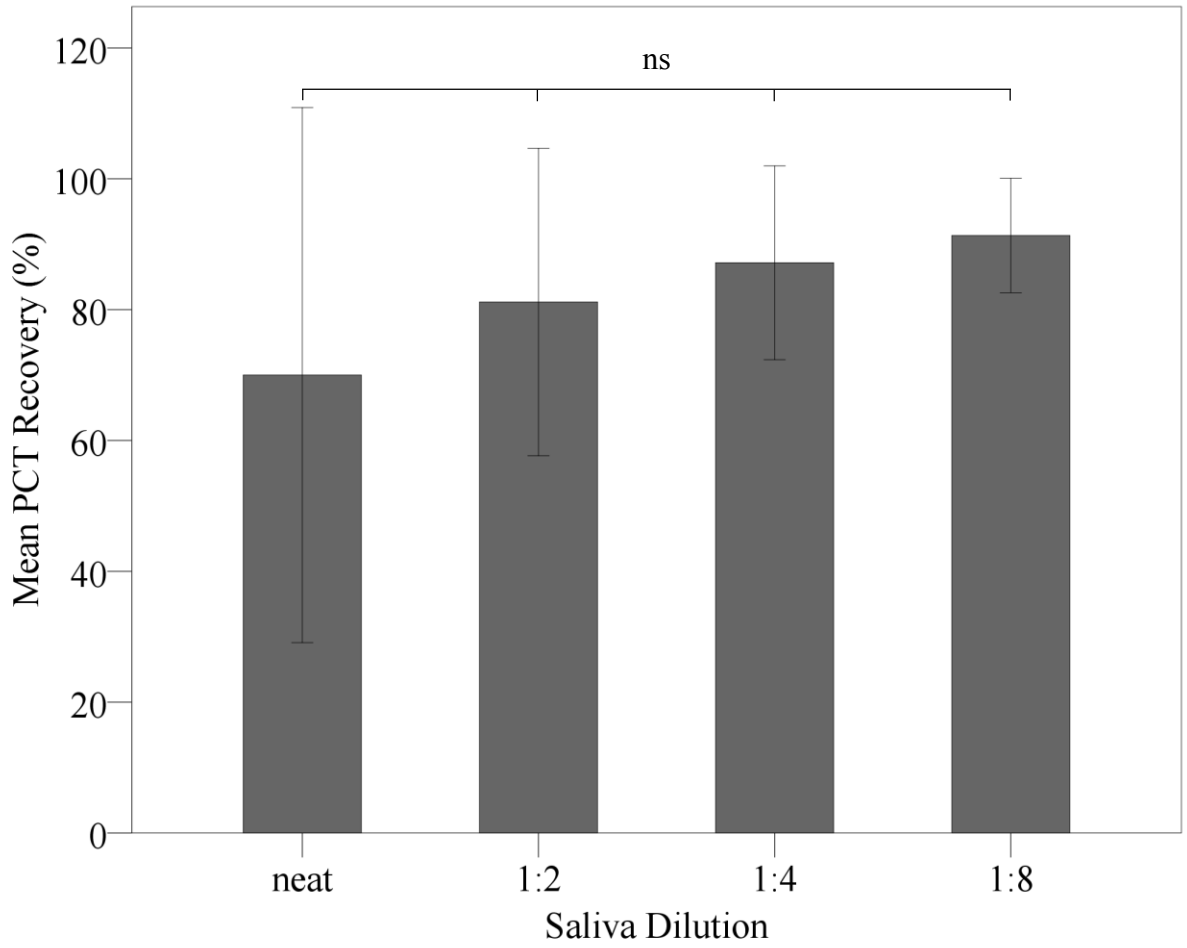


Figure 2.12: Low range salivary PCT recovery in healthy smokers.

Bar chart representing mean salivary PCT recovery at increasing dilution factor with error bars ± SD (n = 6). Saliva was diluted with PBS-T. Overall there was an increase in the recovery of PCT as the dilution factor increased, but this was not statistically significant (ns: p=0.177 by repeated measures ANOVA). The large SD error bar in neat saliva can be explained by the variability in PCT recovery for neat saliva samples. Subjects 13 and 16 had a recovery percentage of greater than 100% for neat saliva whilst subject 12 had recovery percentage of only 12%. This demonstrates the matrix effect of saliva but also the variability of this effect in different healthy smokers. The SD error bars then narrow as the dilution increases indicating that the matrix effect of saliva and thus variability of PCT recovery is being reduced.

2.4.3.5.5.2. High range recovery and linear dilution PCT experiments

Mean PCT percentage recovery increased as the dilution of saliva increased (1:2 = 86 ± 8%; 1:4 = 90 ± 6%; 1:8 = 95 ± 4%) (Table 2.18, Figure 2.13). Using a repeated measures ANOVA with a Greenhouse-Geisser correction, there was no statistically significant improvement in high range PCT recovery in different saliva dilutions for smokers (p=0.138).

Table 2.18: High range PCT levels and recovery in healthy smokers.

Subject	*Control Level (ng/ml)	*Control PBS-T 1:2 dilution (ng/ml)	Neat Saliva PBS-T 1:2	Neat Saliva PBS-T 1:4	Neat Saliva PBS-T 1:8
11	18.86	19.04	67%	80%	92%
12	18.99	18.99	64%	76%	85%
13	16.78	17.12	102%	100%	98%
14	17.05	16.03	79%	84%	89%
15	16.84	16.84	92%	97%	99%
16	16.61	16.43	106%	99%	100%
Mean ± SD	**17.66 ± 1.17**	**17.28 ± 1.41**	**86 ± 8%**	**90 ± 6%**	**95 ± 4%**

*Control refers to high range PCT control. The levels of quantified PCT were multiplied by the dilution factor to allow standardisation of the results.

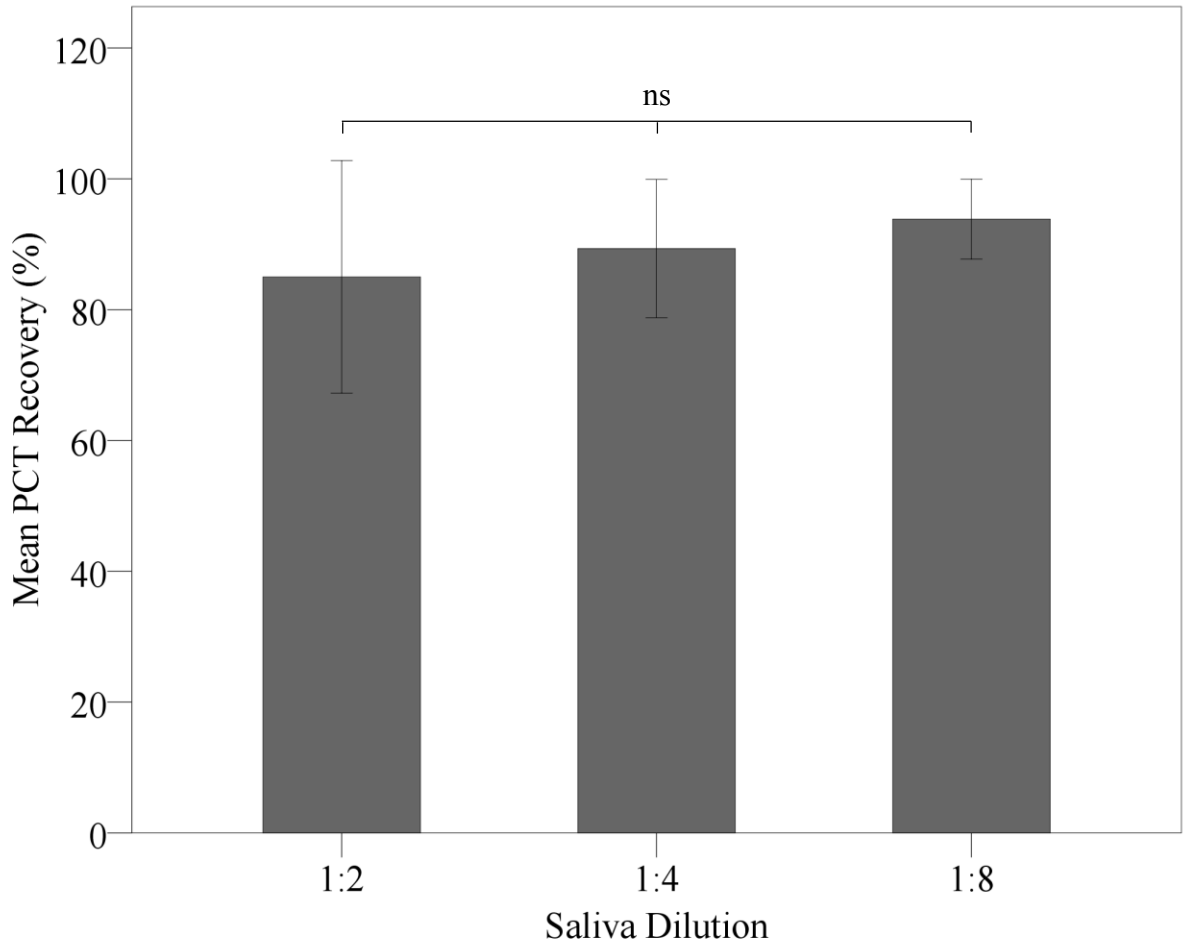


Figure 2.13: High range salivary PCT recovery in healthy smokers.

Bar chart representing mean salivary PCT recovery at increasing dilution factor with error bars ± SD (n = 6). Saliva was diluted with PBS-T. Overall there was an increase in the recovery of PCT as the dilution factor increased, but this was not statistically significant (ns: p=0.138 by repeated measures ANOVA). The larger SD error bars noted at the 1:2 dilution are due to subject 13 and 16 having a recovery percentage of greater than 100% for saliva this is consistent with what was observed for these healthy smokers in the low range experiment. Subject 12 also demonstrates the lowest PCT recovery at 1:2 dilution once again consistent with the results observed for this subject in the high range recovery experiment. The SD error bars narrow as the dilution factor increases and the matrix effect of saliva is reduced.

A further sub-analysis was conducted between low and high range results to determine whether there is a statistical difference in PCT recovery percentage between the two concentrations. Overall there is no significant difference between low and high range PCT recovery in the saliva of healthy smokers across all dilutions ($p=0.220$, two-way repeated measures ANOVA with Greenhouse-Geisser correction). Thus accounting for the small sample size, there does not appear to be a matrix effect of smokers' saliva on the recovery of salivary PCT across the range of tested PCT levels.

A final exploratory analysis compared the data between healthy non-smokers and smokers' saliva. There was no statistical difference in low and high range PCT recovery between the two populations ($p=0.623$ by two-way mixed model ANOVA). Interestingly the low range recovery of PCT in the saliva of smokers appears to be better than healthy non-smokers although this is not statistically significant. This may be explained by understanding the role of mucins in the matrix effect of saliva. Mucins can cause suppression of antibody binding in some immunoassays (Fulton et al., 1989); however the saliva of smokers has been shown to have a reduced and altered level of mucins (Taniguchi et al., 2013). Thus saliva matrix effect of smokers may be reduced compared to non-smokers resulting in a possible improvement in PCT recovery even at lower saliva dilutions.

2.4.3.6. Conclusion

Overall the recovery of PCT appears to be enhanced as the linear dilution of saliva is increased at both low and high ranges. The recovery of PCT in the saliva of healthy smokers is enhanced at lower dilutions albeit with a greater variability; but there is no statistically significant difference in recovery between healthy non-smokers and healthy smokers' saliva at either low or high concentrations of PCT.

2.4.3.7. Recombinant human PCT recovery

The previous experiments have established that PCT recovery in saliva spiked with both low and high range PCT controls is consistent with no significant difference between the two concentrations. However, the level of PCT in the low range control is between 1.58 to 1.71ng/ml. Physiologically these represent "high" levels reflective of severe systemic inflammation (Castelli et al., 2004). Accordingly, the recovery of PCT was investigated at levels that approach the lower limit of quantification for the assay (0.05ng/ml) and if recovery was affected to determine whether these recovered levels were linear.

2.4.3.7.1. Aims

1. To ascertain the recovery of recombinant PCT levels in PBS-T at increasing spiked concentrations.
2. To determine the recovery of recombinant PCT levels across a range of concentrations in spiked neat human saliva samples from healthy non-smokers.

2.4.3.7.2. Methods

Firstly, PBS-T was spiked with recombinant PCT (Prospec, Israel) to act as a control across a range of PCT concentrations: 0.625 to 100ng/ml (Table 2.19A-B). The recombinant PCT is supplied lyophilised as a 2000ng powder and was reconstituted in 1ml of dH_2O. All samples were tested in duplicate using the mini-VIDAS (bioMérieux, France). Based on the results of this control experiment, three separate saliva samples were spiked with a known quantity of recombinant PCT (Prospec, Israel) across a range of concentrations: 0.625 to 20ng/ml (Figure 2.19B). Saliva samples were chosen from three healthy non-smokers in whom I had previously demonstrated no detectable endogenous salivary PCT (Table 2.14, Page 121: Healthy Subjects 1 to 3). All samples were tested in duplicate on the mini-VIDAS (bioMérieux, France). The

lower limit of quantification for PCT on the mini-VIDAS was 0.05ng/ml for neat saliva samples.

Table 2.19A: Protocol for PBS-T spiked with recombinant PCT experiment.

		Reagent/Sample Volume (ul)						
Target PBS-T Spike	**Recombinant PCT (2000ng/ml)**	**PBS-T**	**100ng/ml Spiked PBS-T Solution**	**50ng/ml Spiked PBS-T Solution**	**25ng/ml Spiked PBS-T Solution**	**12.5ng/ml Spiked PBS-T Solution**	**6.25ng/ml Spiked PBS-T Solution**	**Total Volume**
100 ng/ml	20ul	380ul						400ul
50 ng/ml		200ul	200ul					400ul
25 ng/ml		200ul		200ul				400ul
12.5 ng/ml		200ul			200ul			400ul
6.25 ng/ml		200ul				200ul		400ul
3.125 ng/ml		200ul					200ul	400ul

Table 2.19B: Protocol for PBS-T spiked with recombinant PCT experiment.

		Reagent/Sample Volume (ul)						
Target Saliva Spike	**Recombinant PCT (2000ng/ml)**	****Neat saliva**	**20ng/ml Spiked Saliva**	**10ng/ml Spiked Saliva**	**5ng/ml Spiked Saliva**	**2.5ng/ml Spiked Saliva**	**1.25ng/ml Spiked Saliva**	**Total Volume**
***20 ng/ml**	8ul	392ul						400ul
***10 ng/ml**		200ul	200ul					400ul
***5 ng/ml**		200ul		200ul				400ul
***2.5 ng/ml**		200ul			200ul			400ul
***1.25 ng/ml**		200ul				200ul		400ul
***0.625 ng/ml**		200ul					200ul	400ul

*Spiked saliva is a minimum 98% pure. **This same protocol was adopted with PBS-T instead of unadulterated saliva to achieve a concentration range of (0.625 to 20ng/ml) which would act as a control.

2.4.3.7.3. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, a recovery percentage of PCT in PBS-T was calculated from the following formula: $percentage\ recovery = 100\left(\frac{observed\ PCT\ level}{expected\ PCT\ level}\right)$. Salivary PCT levels below the lower limit of quantification were not included in any analysis.

2.4.3.7.4. Results

2.4.3.7.4.1 Recombinant PCT levels in PBS-T

The purpose of this experiment was to understand the actual recovery of recombinant PCT levels in spiked PBS-T samples so that a cross comparison with spiked saliva samples could be made (Table 2.20).

Table 2.20. Recovery of PCT in spiked PBS-T samples.

***Added Fixed Concentration Recombinant PCT (ng/ml)**	**Observed PCT (ng/ml)**	**Recovery (%)**	***Added Recombinant PCT (ng/ml)**	**Observed PCT (ng/ml)**	**Recovery (%)**
100	58.64	59%	**20**	4.23	21%
50	27.27	55%	**10**	1.98	20%
25	11.58	46%	**5**	0.82	16%
12.5	5.29	42%	**2.5**	0.35	14%
6.25	2.35	38%	**1.25**	0.13	10%
3.125	0.96	31%	**0.625**	**<0.05	n/a
Mean ± SD		**45 ± 10%**			**16 ± 5%**

*Recombinant PCT concentration added to PBS-T, **below the lower limit of quantification for the immunoassay.

Overall there was a significant reduction in the observed recombinant PCT levels compared to the fixed (expected) concentration ($p<0.008$ by Wilcoxon signed rank test). The recovery at reducing concentrations was also reduced. However the reduction in the observed levels of salivary PCT compared to expected fixed concentration recombinant PCT levels was consistent across the range of PCT concentrations as determined by the r^2 value of 99.8% for the line-of-best-fit (Figure 2.14). This value indicates a 99.8% agreement for the line-of-best-fit between the observed compared to fixed concentrations of recombinant PCT.

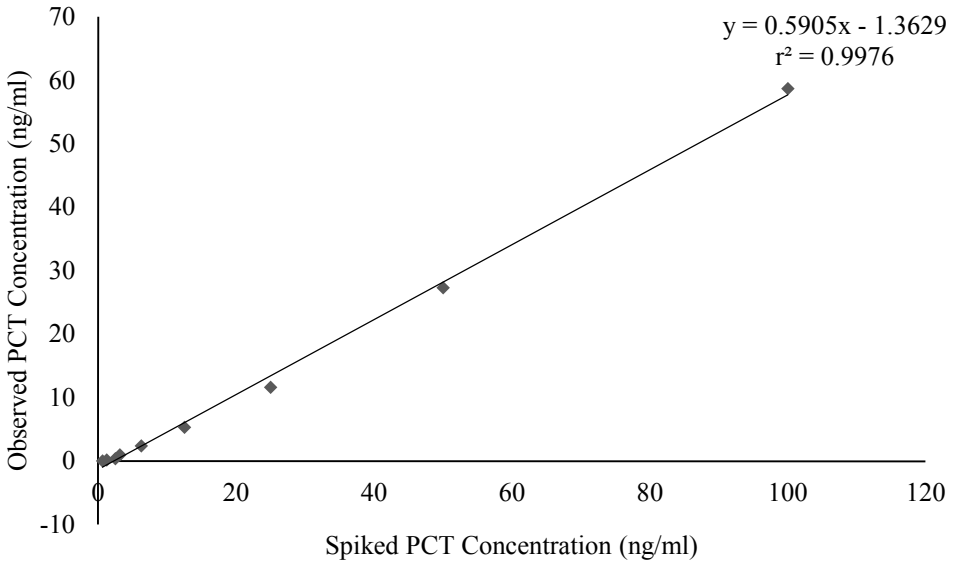


Figure 2.14: Recombinant PCT levels in PBS-T.

This scatter plot with line-of-best-fit demonstrates observed PCT levels in PBS-T spiked with a fixed concentration of recombinant PCT (0.625, 1.25, 2.5, 3.125, 5, 6.25, 10, 12.5, 20, 25, 50, 100ng/ml). 200ul of each spiked PBS-T sample was aliquoted onto a VIDAS B.R.A.H.M.S PCT test strip (bioMérieux, France) and analysed on the mini VIDAS instrument. Although the recovered levels of fixed concentrations of recombinant PCT in PBS-T were reduced, this reduction is linear across the spiked concentration range 0.625 to100ng/ml. The r^2 value was 0.998 for the line-of-best-fit: $y = 0.5905 – 1.3629$.

Overall the recovered levels of a fixed concentration of recombinant PCT in PBS-T is reduced on the mini VIDAS (bioMérieux, France) the reduction in these levels however is constant (linear). This experiment adds support that using the VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France) can give a consistent quantification of salivary PCT levels. It also supports a minimum of dilution of saliva samples with PBS-T of 1:2, as further higher dilutions (1:4 and 1:8) will reduce the lower limit of quantification (1:4 = 0.20ng/ml and 1:8 = 0.40ng/ml) for the immunoassay resulting in more saliva samples testing negative for PCT.

2.4.3.7.4.2. Recombinant PCT levels and recovery in saliva

The next step was to compare recovered levels between recombinant PCT spiked PBS-T and three spiked saliva samples chosen from the healthy non-smoker subjects who had provided saliva samples for these experiments (Table 2.14, Page 121) using the previously discussed (Section 2.4.3, Page 112) VIDAS B.R.A.H.M.S PCT (bioMérieux, France) immunoassay designed for analysis with the mini-VIDAS (bioMérieux, France), which has a lower limit of PCT quantification at 0.05ng/ml for neat serum samples. The saliva samples were spiked with 0.625, 1.25, 2.5, 5, 10 and 20ng/ml of recombinant PCT. The method for this is described in Table 2.19B, Page 136. This experiment enabled a cross-comparison to be performed between each spiked healthy saliva sample and spiked PBS-T samples to ascertain the difference in actual PCT levels between the two spiked samples and a comparative recovery percentage at each PCT concentration (Table 2.21). The observed spiked saliva recombinant PCT levels have a linear relationship in all three healthy subject to the fixed concentration of recombinant PCT (20, 10, 5, 2.5, 1.25 and 0.625ng/ml) used to spike the saliva (Figure 2.15A-C). The r^2 value indicates that the level of observed PCT concentrations can be calculated with at least 96% accuracy when the spiked concentration is known.

Table 2.22: Recombinant PCT levels and recovery in spiked saliva.

		Subject 1		**Subject 2**		**Subject 3**	
*Fixed Concentration Added Recombinant PCT (ng/ml)	Observed PBS-T PCT Level (ng/ml)	Observed Salivary PCT Level (ng/ml)	**Recovery (%)	Observed Salivary PCT Level (ng/ml)	**Recovery (%)	Observed Salivary PCT Level (ng/ml)	**Recovery (%)
20	4.23	2.02	48%	3.29	78%	0.86	20%
10	1.96	0.65	33%	1.11	57%	0.35	18%
5	0.82	0.32	39%	0.48	59%	0.18	22%
2.5	0.35	0.15	43%	0.19	55%	0.10	29%
1.25	0.13	0.08	62%	0.11	87%	0.07	54%
0.625	<0.05	<0.05	n/a	<0.05	n/a	<0.05	n/a
mean ± SD			**45 ± 11%**		**67 ± 14%**		**29 ± 15%**

*Added to both PBS-T and the three subjects' saliva samples, **Compared to the observed PCT level in spiked PBS-T.

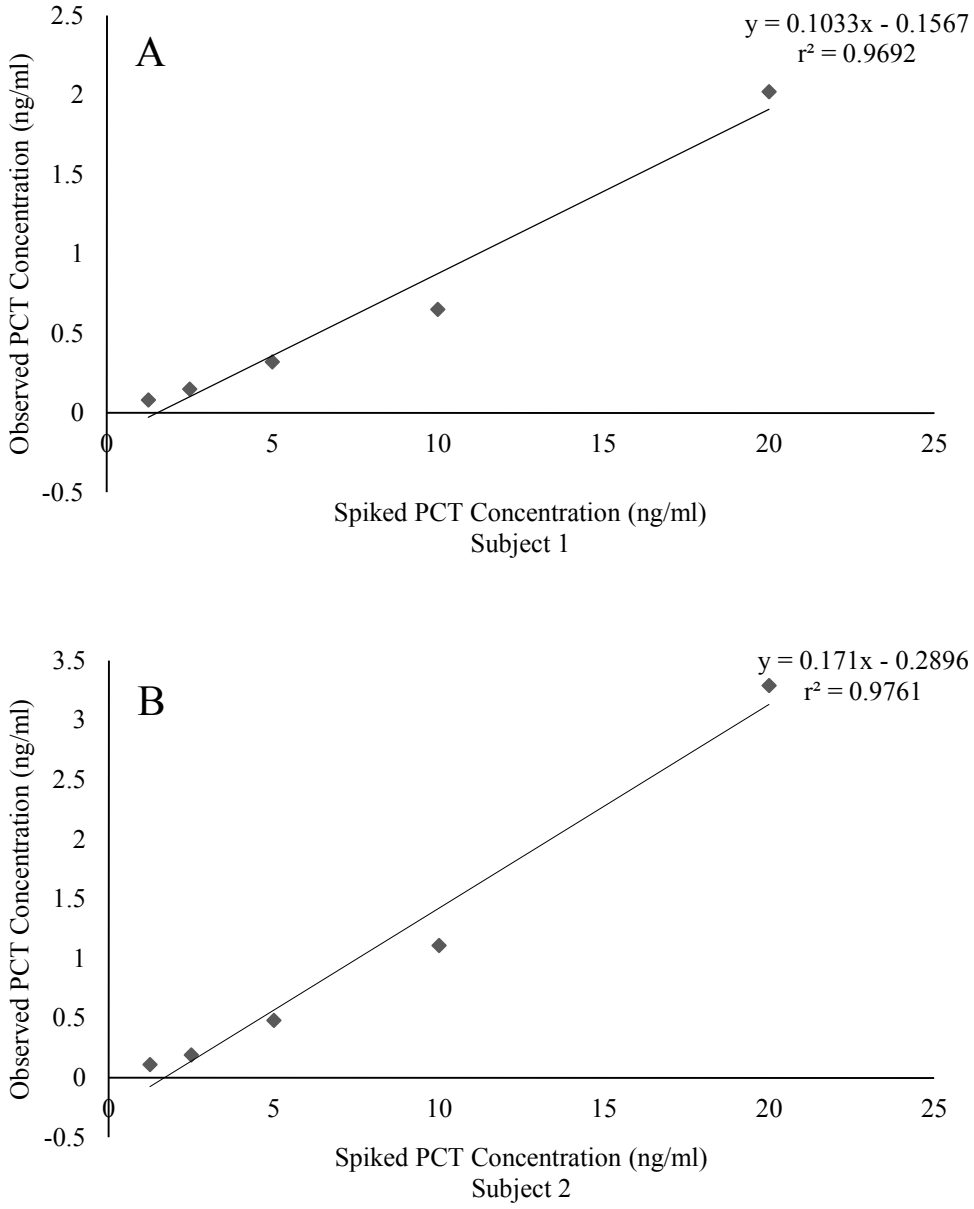


Figure 2.15A-B: Recombinant PCT levels in healthy non-smoker saliva.

Scatter plot (n = 2) with line-of-best-fit for three separate samples of healthy non-smoker saliva spiked with recombinant PCT at six different concentrations: 20, 10, 5, 2.5, 1.25, 0.625ng/ml. 200ul of neat spiked saliva samples were aliquoted onto a VIDAS B.R.A.H.M.S PCT test strip (bioMérieux, France) and analysed on the mini-VIDAS instrument. The r^2 values are greater than 0.98 for both saliva samples (Subject 1: $r^2 = 0.9692$, Subject 2: $r^2 = 0.9761$).

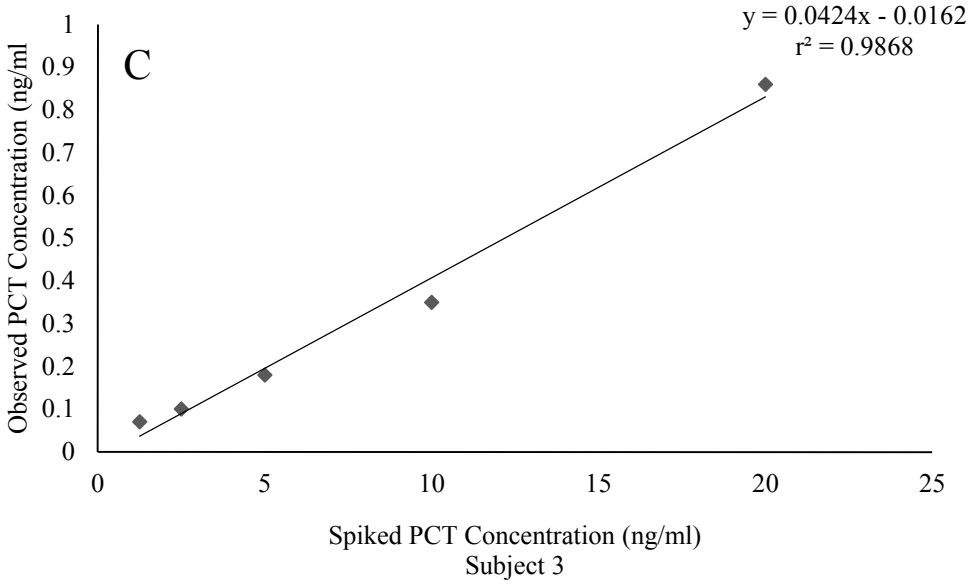


Figure 2.15C: Recombinant PCT levels in healthy non-smoker saliva.

Scatter plot (n = 1) with line-of-best-fit for three separate samples of healthy non-smoker saliva spiked with recombinant PCT at six different concentrations: 20, 10, 5, 2.5, 1.25, 0.625ng/ml. 200ul of neat spiked saliva samples were aliquoted onto a VIDAS B.R.A.H.M.S PCT test strip (bioMérieux, France) and analysed on the mini-VIDAS instrument. The r^2 values = 0.9868.

2.4.3.7.4.3 Conclusion

The recovery of recombinant PCT is lower than the low and high range manufacturer provided PCT controls which were used in the first set of spiking experiments above (Section 2.4.3.5.4, Page 122). It is important to note however that recovered PCT levels display a linear relationship to the expected spiked recombinant PCT concentrations for healthy non-smoker saliva. Although the levels may not be a genuine reflection of the actual concentrations of PCT in saliva this linearity adds reassurance that the salivary PCT levels will be consistently reported across the concentration ranges.

2.4.3.8. Spectral analysis experiment

Based on the results of the PCT spiking and dilution experiments a further experiment was conducted to understand why there was a difference between expected and observed levels of recombinant PCT and the VIDAS BRAHMS PCT kit (bioMérieux, France) manufacturer supplied PCT controls (low and high range) which were both used to spike PBS-T and healthy saliva. Thus there may be compositional difference between the two and this was investigated utilising Fourier transform infrared (FTIR) spectroscopy. FTIR identifies and measures chemical bond vibrations within functional groups in complex biological mixtures and produces infrared absorption spectra "fingerprint" for the particular sample.

2.4.3.8.1. Aim

1. To ascertain the compositional difference if any between recombinant PCT and manufacturer-supplied PCT controls.

2.4.3.8.2. Methods

FTIR spectroscopy was performed on recombinant PCT (Prospec, Israel), two low and high range PCT controls from the VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France) and two matched serum-saliva samples from our archived saliva bio-bank. Spectroscopy was conducted with the Alpha-T spectrometer (Bruker, UK) using a transmission mode in 400 to 900cm^{-1} regions with a 4cm^{-1} resolution. The first step involved calibration of the spectrometer to accommodate for the spectral profile of air. Subsequently a sample was placed on a clear calcium fluoride disc and dried. This is necessary as water can produce background-characteristic spectral interference (eliminated by drying), which can potentially disguise underlying spectra of interest. The calcium fluoride disc is then placed inside the spectrometer and analysed with the results provided after 30 seconds.

2.4.3.8.3. Results

Spectral profiles for serum, saliva, recombinant PCT and both low and high range PCT controls are shown in Figure 2.16A-D. Serum has a variety of peaks reflecting the complexity of its composition. Saliva also has a variety of peaks although the profile as expected is different from serum. The recombinant PCT demonstrates two peaks (the second from left to right) would be consistent with a pure protein band; however both low and high range PCT controls provided in the VIDAS B.R.A.H.M.S PCT assay (bioMérieux, France) displayed a characteristic spectrum, which has similar features to human serum. This spectral profile is markedly different to that of the recombinant PCT indicating that there are likely additional proteins inherent in the VIDAS B.R.A.H.M.S PCT assay (bioMérieux, France) manufacturer-supplied PCT controls (both low and high range) that enhances the recovery of PCT when analysed on the min-VIDAS (bioMérieux, France).

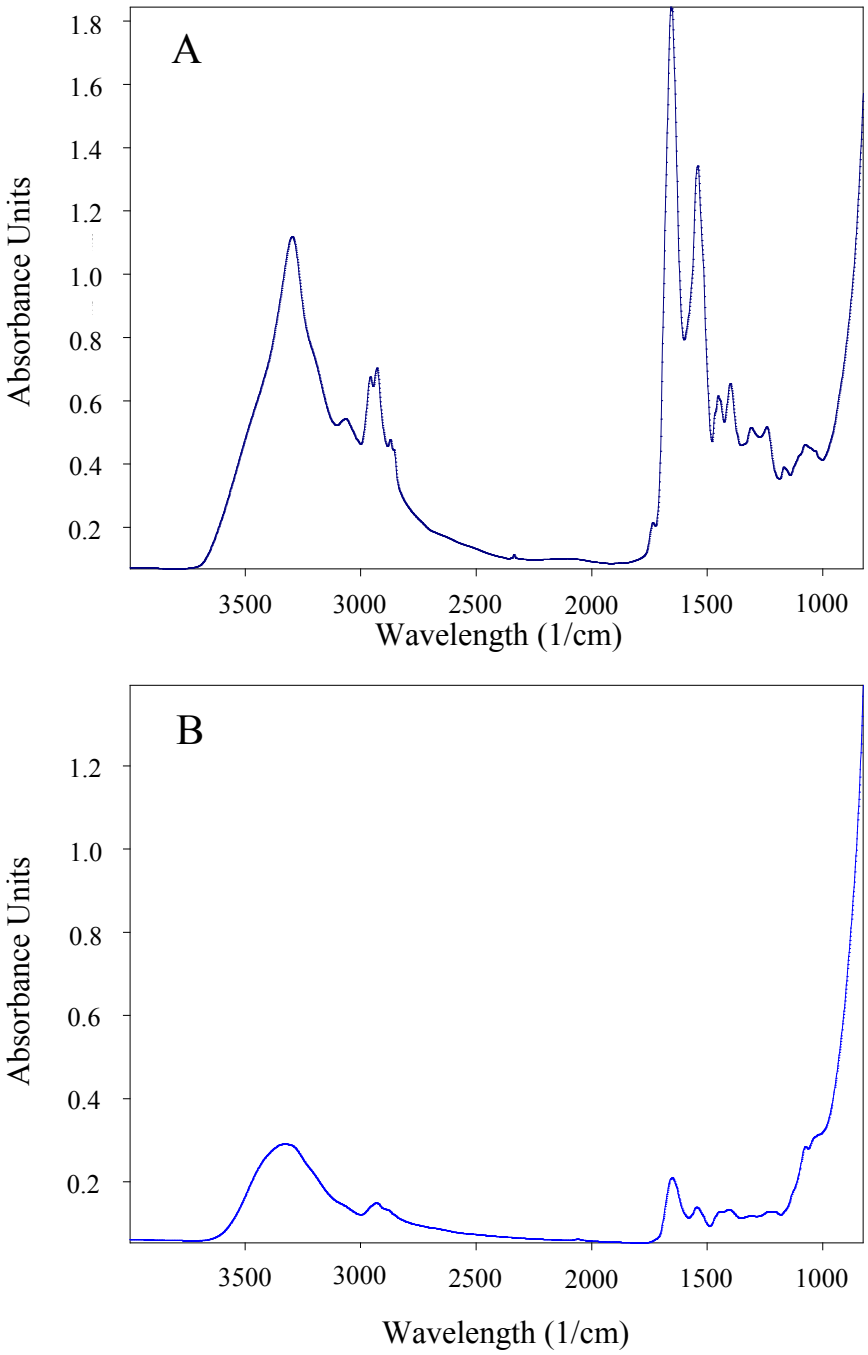


Figure 2.16A-B: FTIR Spectra.

Spectral profiles of A = serum; B = saliva.

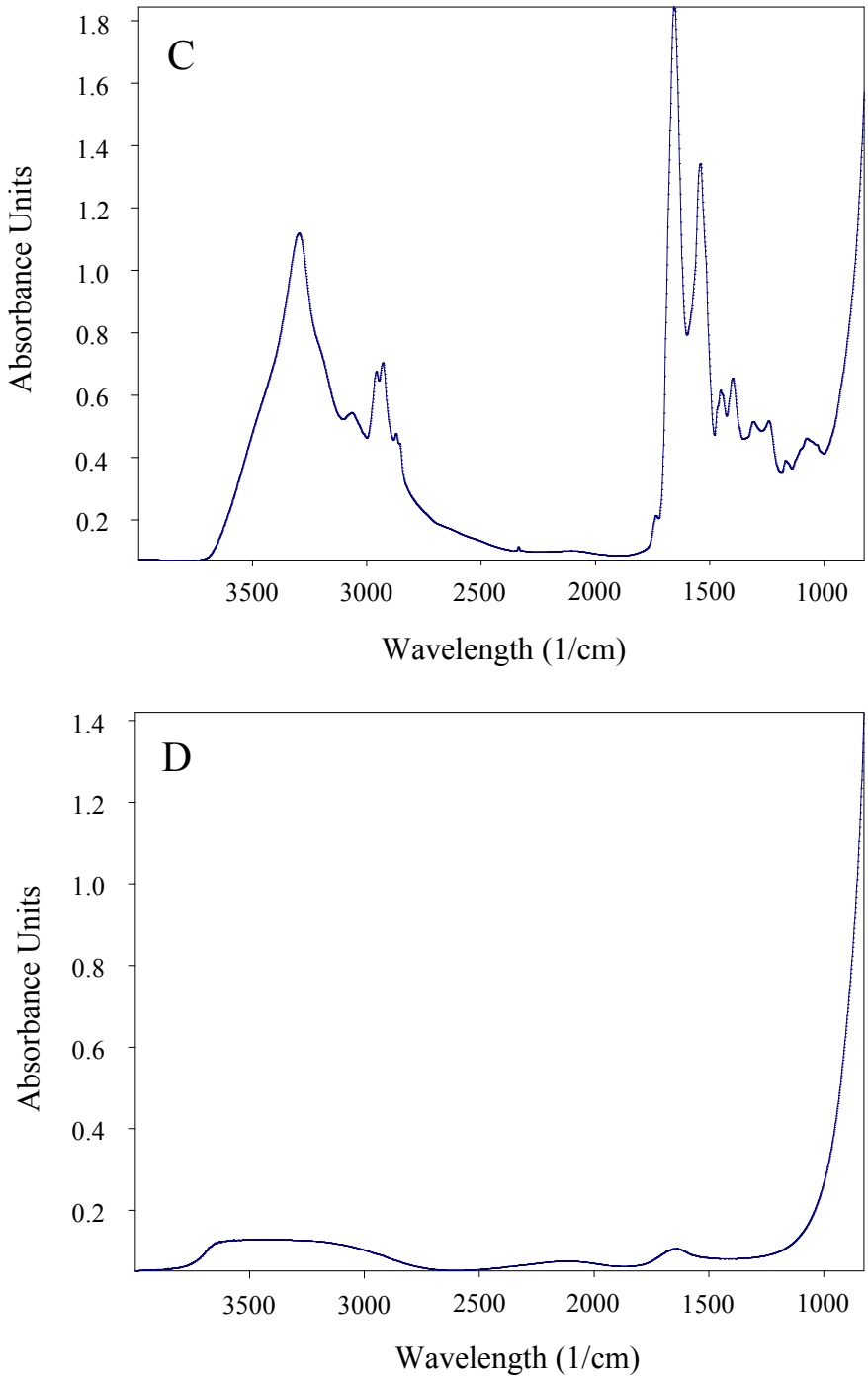


Figure 2.16C-D: FTIR Spectra.

Spectral profiles of C = low and high range PCT control provided in the VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France). As the spectra for both these PCT controls were identical, only one representative spectrum is displayed. D = recombinant PCT.

2.4.3.9. Discussion

PBS-T appears to be a suitable saliva diluent and does not affect the recovery of either low or high range PCT control provided in the VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France). The recombinant PCT experiment demonstrated reduced PCT recovery compared to the low and high range controls at similar PCT concentrations. Spectral analysis of both recombinant PCT and the low and high range controls appears to offer an explanation with the spectra suggesting that both the low and high range controls may contain serum constituents which affect PCT recovery.

The difference in spectra between recombinant PCT and both low and high range PCT controls could be explained by considering that the assay itself is designed to be tested on human serum. Thus the controls may be combined with a human serum substitute or a combination of proteins that form the major components of human serum to enable the analyser to detect them more easily. Fluorescence assays using serum have to overcome limitations associated with fluorescent labeling of the serum constituents, non-specific signals generated by the fluorescence and diffusion properties of the serum (Martin and Jett, 1986). These factors to a lesser or greater extent are accounted for by pre-sets on the machine upon calibration of a new PCT kit. The observed reduced recovery of recombinant PCT may be attributable to the VIDAS calibration. When the analyser is calibrated according to manufacturer's instructions, the MLE card provides information on the expected fluorescence of serum and serum-PCT. Thus as saliva was our target test sample, this pre-set calibration and the consequent intrinsic serum-fluoresce value would be incorrectly applied. This would result in an altered result and could also explain why the recovery was better at higher concentrations as a higher proportion of the fluorescence will be generated by PCT. In considering the results from Section **2.4.3**.7.4, Page 137 one could postulate that the combination of recombinant PCT with serum instead of PBS-

T could have led to enhanced PCT recovery. The recovery of PCT improves as the linear dilution of saliva increases. However, it is important to note that as the dilution of the saliva sample increases the lower limit of PCT quantification for the mini VIDAS also increases: neat = 0.05, 1:2 = 0.10, 1:4 = 0.20 and 1:8 = 0.40. This has important implications for saliva PCT quantification, as meaningful "low" levels will not be quantifiable above dilutions of 1:4. A 1:2 dilution of saliva with PBS-T is optimal for improving PCT recovery and low concentrations, without compromising the working assay range of the mini-VIDAS (bioMérieux, France). Optimal PCT recovery is obtained when diluting saliva 1:2; this is not affected by the concentration of PCT. Interestingly the saliva of smokers does not exhibit an enhanced matrix effect on PCT when compared to the saliva of healthy non-smokers.

2.4.3.10. Overall conclusion

Based on the above experimental results for future PCT analysis, the same pre-analysis saliva preparation as for CRP (Section 2.4.2.1, Page 92) will be adopted. The intra- and inter-assay precision (CV) of 8.2% and 10.1% respectively was below the maximum (10% and 15%) accepted error percentage that had been selected. This will continue to be calculated for all for the VIDAS B.R.A.H.M.S PCT immunoassays (bioMérieux, France) throughout this thesis to ensure that both the intra- and inter-assay precision (CV's) remain below the accepted and clinical thresholds. All saliva samples will be tested in duplicate. Samples will be diluted 1:2 in PBS-T (manufacturer recommendation is neat for serum) and each test strip will continue to receive 200ul of sample (this is in concordance with the manufacturers documented sample amount). The test procedure that occurs internally within the mini VIDAS (bioMérieux, France) does not need to be altered. Due to the 1:2 sample dilution the minimum limit of detection PCT for the mini VIDAS (bioMérieux, France) instrument will be 0.10ng/ml

2.4.4. Optimisation of a serum-based Neutrophil Elastase (NE) assay for use with saliva

The PMN Elastase ELISA kit (Immundiagnostik, Germany) is a validated "for diagnostic use" immunoassay to quantify NE in stool, seminal plasma and serum. There is no published work with respects to whether this kit has been validated in saliva. Jaedicke et al 2012., have described how ELISAs can be modified to quantify target analytes in saliva; if the assay of choice quantifies target analytes in numerous body fluids then the serum-based methodology could be utilised.

Each supplied PMN Elastase ELISA kit (Immundiagnostik, Germany) consists of a 96-well microtitre plate coated with sheep anti-elastase, ELISA wash buffer (diluted by 1:10 with dH_2O), five lyophilised standards (10, 3.3, 1.1, 0.37 and 0ng/ml) and two lyophilised controls (0.65 and 1.60ng/ml respectively). Both standards and controls are reconstituted with 500ul of dH_2O. A lyophilised detection antibody (reconstituted with 600ul of dH_20 and diluted 1:20 in the ELISA wash buffer), Conjugate, TMB substrate and sulfuric acid stop solution (Figure 2.17).

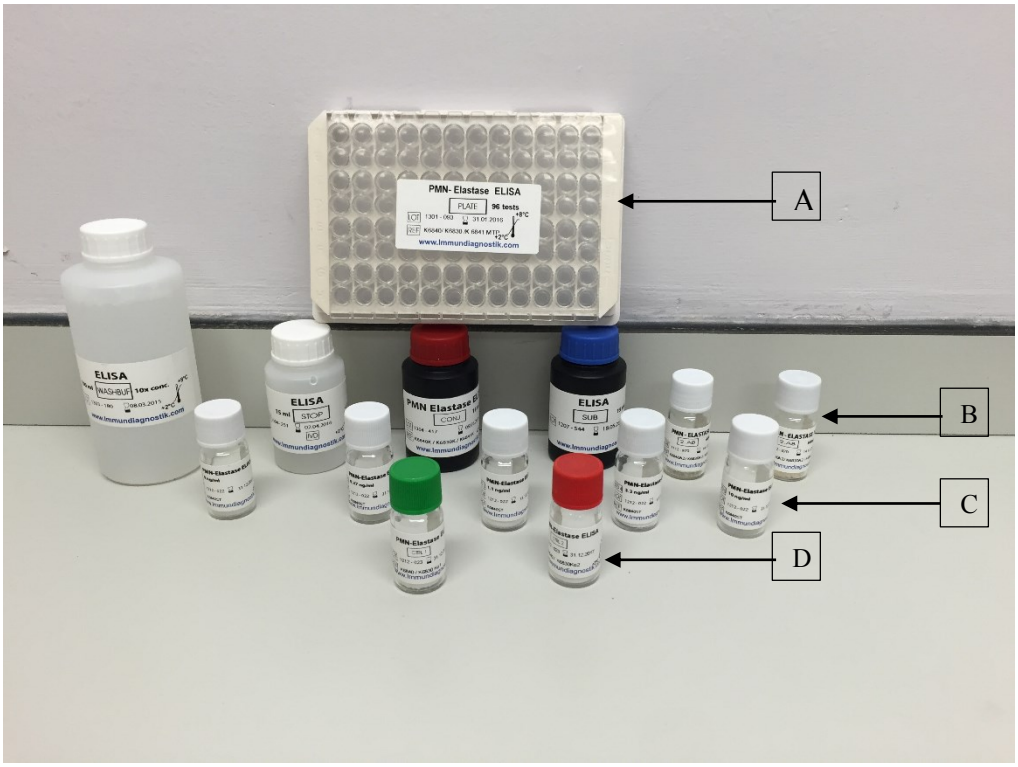


Figure 2.17: PMN Elastase ELISA kit (Immundiagnostik, Germany).

This figure displays the contents of the ELISA kit. A = Microtitre plate; Row B from left to right: ELISA wash buffer, stop solution, conjugate, substrate, antibody; Row C from left to right: 5 sets of standards (0, 0.37, 1.1, 3.3. 10ng/ml); Row D from left to right: low and high control vials.

The above ELISA requires the target sample to be diluted prior to analysis to reduce the concentration of NE into the working assay range: 0.011 to 10ng/ml. The method for serum NE for example involves a minimum sample dilution of 1:500 in the ELISA Wash Buffer. Thus at a 1:500 dilution the quantifiable range of serum NE is 5.5 to 5000ng/ml. For any sample above the working range of PMN Elastase ELISA kit (Immundiagnostik, Germany), the manufacturer recommends a further dilution; this theoretically means that the kit has no upper limit of quantification for NE if the appropriate dilution is incorporated. Subsequently to perform the immunoassay 100ul of target sample, standards and controls are added in duplicate to the wells; thus 40 serum samples can be tested in duplicate per microtitre plate (Table 2.22).

The microtitre plate is then incubated for one hour at room temperature (15 to 30ºC) whilst on a horizontal plate mixer at 500rpm. After a wash step 100ul of detection antibody (mouse monoclonal antibody) is added to the wells. The microtitre plate is then left to incubate for a further hour at room temperature on a horizontal mixer. During this second incubation, a "sandwich" of anti-elastase-elastase-anti-elastase is formed. After a second wash-step, 100ul of conjugate (peroxide labeled goat-anti-mouse-POD) is added, and the microtitre plate is incubated for a further hour at room temperature on a horizontal plate mixer. After a third wash-step, 100ul of TMB is added into each well; this is acted upon by the conjugated enzyme bound in the well and causes a blue discolouration. The microtitre plate is then incubated in the dark (achieved by enveloping the microtitre plate in aluminum foil (Wrap Film Systems, UK)) for 20 minutes. Finally, 100ul of ELISA stop solution (sulfuric acid) is added turning the TMB yellow. The microtitre plate needs to be immediately read on the BioTek Synergy 2 ELISA plate reader (BioTek, USA) at 450nm and 620nm; the later being used as a reference. Total assay time including sample preparation is approximately five hours.

Table 2.22: **Generic NE microtitre plate layout**.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10 ng/ml	10ng/ml	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	3.3 ng/ml	1500 pg/ml	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	1.1 ng/ml	750 pg/ml	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	0.37 ng/ml	375 pg/ml	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	0 ng/ml	187.5 pg/ml	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	High Control	High Control	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	Low Control	Low Control	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	Blank	Blank	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

To calculate the levels of sample NE, a four-parameter logistic regression model is used. This utilizes the known standards and generated optical densities at each standard concentration to generate a "curve-of-best-fit" (Figure 2.18). This "curve-of-best-fit" is generated via the equation "$y = d + \frac{a-d}{1+\left(\frac{x}{c}\right)^b}$" where "y" is the optical density, "x" is the $\log_{10}$ salivary NE level; "a" is the optical density at 0ng/ml standard, "d" is the optical density at 10ng/ml standard, "c" is the point of inflection for the curve and "b" is the steepness of the curve at point "c". Thus a sample of saliva processed on this assay generating an optical density of "y" would have a $\log_{10}$ salivary NE concentration of "$x = c(\frac{-a+y}{d-y})^{\frac{1}{b}}$". The value for "$x$" would then need to be transformed to a number and multiplied by the dilution factor of the saliva sample.

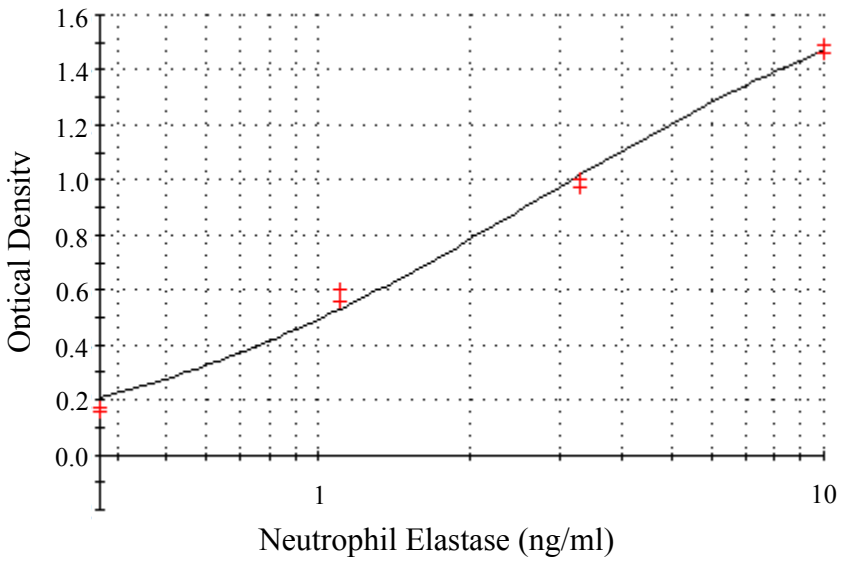


Figure 2.18: Typical four-parameter logistic regression curve

A "curve-of-best-fit" with the "+" in red representing the optical densities at each standard. As the "x" axis scale is logarithmic a zero point cannot be plotted. This "curve-of-best-fit" is generated via the equation "$y = d + \frac{a-d}{1+\left(\frac{x}{c}\right)^b}$" where "y" is the optical density, "x" is the $\log_{10}$ salivary NE level; "a" is the optical density at 0ng/ml standard, "d" is the optical density at 10ng/ml standard, "c" is the point of inflection for the curve and "b" is the steepness of the curve at point "c". The r^2 value for this curve indicates that the "curve-of-best-fit" can calculate with 99.5% accuracy the level of NE.

2.4.4.1. Salivary NE ELISA calibration experiments

In order to calibrate this ELISA for saliva I would need to assess precision, recovery and linear dilution and the minimum limit of detection based on the ideal sample dilution factor (Section 2.4.2, Page 90). I conducted a series of experiments to understand recovery of salivary NE at both "low" and "high" concentrations to determine the effects of saliva sample linear dilution.

2.4.4.1.1. Aims

1. To determine the intra- and inter- assay precision.
2. To ascertain the matrix effect of saliva on the recovery of a fixed concentration of NE provided as part of the PMN Elastase ELISA kit control, and determine whether there is a difference in NE recovery in saliva at low and high NE ranges.
3. To evaluate this matrix effect in four different (linear) dilutions of human saliva.
4. To assign a limit of detection based on a selected saliva dilution.

2.4.4.1.2. Methods

Five healthy non-smokers and five COPD patients (Table 2.23) were randomly selected from the Directorate of Respiratory Medicine's research and outpatient clinic database (Section 2.3, Page 88); gave informed written consent and provided 2mls of unstimulated whole saliva by passive drool (Figure 2.21, Page 176) into ice-cooled containers. Saliva samples were then transported on ice and stored in the Guy Hilton Research Centre Freezer Room (Keele University, UK) at -80^{o}C. Prior to analysis all saliva samples were thawed at room temperature. Saliva samples were stored for no more than 1 week prior to biomarker analysis.

The PMN Elastase ELISA kit (Immundiagnostik, Germany) is validated for the quantification of NE in stool, seminal plasma and serum; thus no protocol exists for pre-analysis saliva sample

preparation. The same pre-analysis saliva preparation as for salivary CRP quantification (Section 2.4.2.1, Page 92) was utilised. Prior to analysis each saliva sample was vortexed and then centrifuged at 3000rpm for 15 minutes to remove debris. Each sample was analysed unadulterated at four different dilutions (1:100, 1:200, 1:400 and 1:800) to determine endogenous levels of NE (Table 2.26). Based on the results from this experiment five samples were chosen and then respectively spiked with "low" NE (1.09ng/ml) and "high" NE (7.98ng/ml) concentrations using the standards provided in the kit. These spiked samples were then analysed at four varying linear dilutions of 1:100, 1:200, 1:400 and 1:800 (Table 2.27 and 2.28). There was no adjustment made to the manufacturer's methodology of the kit and each sample was analysed in duplicate. Two microtitre plates were used to conduct these experiments (Table 2.24 and 2.25).

Table 2.23: Group demographics

Demographics	Healthy Non-Smokers (n = 5)	COPD (n = 5)
Age, [a] years	27.5 ± 1.0	65.7 ± 9.0
Gender, Male, (Female), n	2 (3)	3 (2)
Smoking Status, Current (Ex), n	0 (0)	0 (5)
Duration of COPD, [a] years	n/a	6.7 ± 2.9
FEV_1, [a] % predicted	n/a	53.0 ± 13.6
FVC, [a] % predicted	n/a	73.6 ± 7.0
BMI, [a] (kg/m^2)	23.2 ± 2.6	25.2 ± 2.6
Exacerbations in the last 6 weeks, [a]n	0	0
Exacerbations in the last 1 year, [a]n	0	4 ± 1
Co–morbidities, n		
None	5	0
Cardiovascular Disease	0	2
Type 2 Diabetes Mellitus	0	0
Hypertension	0	4
Gum Disease	0	0
Other	0	2
COPD Treatment, n		
Inhaled β_2 agonists, Short Acting, (Long Acting)	0, (0)	5, (5)
Nebulised β_2 agonists (Short Acting)	0	0
Inhaled Anti-cholinergic, Short Acting, (Long Acting)	0, (0)	1, (4)
Nebulised Anti-cholinergic (Short Acting)	0	0
Inhaled Steroid	0	4
Oral Theophylline, [a]n	0	2

Data are presented as: a = mean ± SD.

Table 2.24: Dilution saliva NE experiment microtitre plate layout.

Healthy Subject (1 to 5)					**COPD Patients (6 to 10)**				
Patient 1 Sample 1	**Patient 2 Sample 2**	**Patient 3 Sample 3**	**Patient 4 Sample 4**	**Patient 5 Sample 5**	**Patient 6 Sample 6**	**Patient 7 Sample 7**	**Patient 8 Sample 8**	**Patient 9 Sample 9**	**Patient 10 Sample 10**
Sample 1 1:100 dilution	Sample 2 1:100 dilution	Sample 3 1:100 dilution	Sample 4 1:100 dilution	Sample 5 1:100 dilution	Sample 6 1:100 dilution	Sample 7 1:100 dilution	Sample 8 1:100 dilution	Sample 9 1:100 dilution	Sample 10 1:100 dilution
Sample 1 1:200 dilution	Sample 2 1:200 dilution	Sample 3 1:200 dilution	Sample 4 1:200 dilution	Sample 5 1:200 dilution	Sample 6 1:200 dilution	Sample 7 1:200 dilution	Sample 8 1:200 dilution	Sample 9 1:200 dilution	Sample 10 1:200 dilution
Sample 1 1:400 dilution	Sample 2 1:400 dilution	Sample 3 1:400 dilution	Sample 4 1:400 dilution	Sample 5 1:400 dilution	Sample 6 1:400 dilution	Sample 7 1:400 dilution	Sample 8 1:400 dilution	Sample 9 1:400 dilution	Sample 10 1:400 dilution
Sample 1 1:800 dilution	Sample 2 1:800 dilution	Sample 3 1:800 dilution	Sample 4 1:800 dilution	Sample 5 1:800 dilution	Sample 6 1:800 dilution	Sample 7 1:800 dilution	Sample 8 1:800 dilution	Sample 9 1:800 dilution	Sample 10 1:800 dilution

Table 2.25: Spiked saliva NE experiment microtitre plate layout.

Sample 1		**Sample 7**		**Sample 8**		**Sample 9**		**Sample 10**	
Low Spike	**High Spike**	**Low Spike**	**High Spike**	**Low Spike**	**High Spike**	**Low Spike**	**High Spike**	**Low Spike**	**High Spike**
Sample 1 1:100 dilution	Sample 1 1:100 dilution	Sample 7 1:100 dilution	Sample 7 1:100 dilution	Sample 8 1:100 dilution	Sample 8 1:100 dilution	Sample 9 1:100 dilution	Sample 9 1:100 dilution	Sample 10 1:100 dilution	Sample 10 1:100 dilution
Sample 1 1:200 dilution	Sample 1 1:200 dilution	Sample 7 1:200 dilution	Sample 7 1:200 dilution	Sample 8 1:200 dilution	Sample 8 1:200 dilution	Sample 9 1:200 dilution	Sample 9 1:200 dilution	Sample 10 1:200 dilution	Sample 10 1:200 dilution
Sample 1 1:400 dilution	Sample 1 1:400 dilution	Sample 7 1:400 dilution	Sample 7 1:400 dilution	Sample 8 1:400 dilution	Sample 8 1:400 dilution	Sample 9 1:400 dilution	Sample 9 1:400 dilution	Sample 10 1:400 dilution	Sample 10 1:400 dilution
Sample 1 1:800 dilution	Sample 1 1:800 dilution	Sample 7 1:800 dilution	Sample 7 1:800 dilution	Sample 8 1:800 dilution	Sample 8 1:800 dilution	Sample 9 1:800 dilution	Sample 9 1:800 dilution	Sample 10 1:800 dilution	Sample 10 1:800 dilution

Table 2.26: Unadulterated saliva dilution experimental protocol.

	Reagent/ Sample Volume (ul)					
Target Saliva dilution	**Neat Saliva**	**ELISA Wash Buffer**	**1:100 diluted neat saliva**	**1:200 diluted neat saliva**	**1:400 diluted neat saliva**	**Total Volume**
1:100 diluted	10ul	990ul				1000ul
1:200 diluted		500ul	500ul			1000ul
1:400 diluted		500ul		500ul		1000ul
1:800 diluted		500ul			500ul	1000ul

Table 2.27: Low range NE spiking experimental protocol.

	Regent/ Sample Volume						Total Volume
Target Low Range (1.09ng/ml) Spiked Saliva Solution	**Neat Saliva**	**1.1ng/ml Standard**	**ELISA Wash Buffer**	**1:100 diluted low range spiked saliva**	**1:200 diluted low range spiked saliva**	**1:400 diluted low range spiked saliva**	
1:100 diluted	10ul	990ul					1000ul
1:200 diluted			500ul	500ul			1000ul
1:400 diluted			500ul		500ul		1000ul
1:800 diluted			500ul			500ul	1000ul

Table 2.28: High range NE spiking experimental protocol.

	Reagent/ Sample Volume						Total Volume
Target High Range (7.92ng/ml) Spiked Saliva Solution	**Neat Saliva**	**8ng/ml Standard A**	**ELISA Wash Buffer**	**1:100 diluted low range spiked saliva**	**1:200 diluted low range spiked saliva**	**1:400 diluted low range spiked saliva**	
1:100 diluted	10ul	990ul					1000ul
1:200 diluted			500ul	500ul			1000ul
1:400 diluted			500ul		500ul		1000ul
1:800 diluted			500ul			500ul	1000ul

* Standard A = 10ng/ml manufacturer provided lyophilised standard 800ul combined with 200ul of ELISA wash buffer.

2.4.4.1.3. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, for the recovery experiment a recovery percentage was calculated using the formula:

"$recovery\ percentage = 100(observed\ NE\ level \div expected\ NE\ level)$"

The expected level was calculated using the formula:

"$expected\ NE\ level = (endogenous\ NE\ level + NE\ spiked\ concentration)$"

Samples above the higher limit of quantification were not included in the analysis. Salivary NE units are given as ng/ml.

2.4.4.1.4. Results

2.4.4.1.4.1. Precision

The intra-assay CV for both microtitre plates was 4.8% and an inter-assay CV was 8.8%.

2.4.4.1.4.2. Endogenous salivary NE levels

Firstly, the ranges of endogenous NE in all saliva samples across 4 different dilutions (Table 2.29) was investigated.

Table 2.29: Endogenous salivary NE levels across varying dilutions in all subjects.

Salivary NE (ng/ml)		**Saliva Sample Dilution**			
Subject Sample		**1:100**	**1:200**	**1:400**	**1:800**
Sample 1	Healthy	93	103	93	115
Sample 2	Healthy	436	305	324	305
Sample 3	Healthy	*Outside Ref	1108	878	769
Sample 4	Healthy	*Outside Ref	*Outside Ref	3918	3258
Sample 5	Healthy	435	400	377	444
Sample 6	COPD	620	469	421	389
Sample 7	COPD	27	22	28	44
Sample 8	COPD	48	51	40	77
Sample 9	COPD	28	32	39	38
Sample 10	COPD	38	57	57	68
Salivary NE (ng/ml) Median, IQR		**71, 402**	**103, 354**	**209, 370**	**210, 362**

* Result above the higher limit of the assay working range (10ng/ml) for the ELISA. Salivary NE levels were multiplied by the dilution factor to standardise the results. Ref = reference range.

Across the four dilutions there was no significant difference in salivary NE levels (p=0.224 by Friedman's test). Thus the levels of NE in saliva across the dilutions appear to be reproducible and not significantly affected by sample dilution. Saliva samples 1 and 7 to 10 were subsequently chosen to perform the spiking experiments below due to low levels of endogenous NE.

2.4.4.1.4.2. Low spike NE saliva recovery and linear dilution experiments

Salivary NE recovery was variable but improved from 1:100 and 1:200 dilutions compared to 1:400 and 1:800 (Table 2.30, Figure 2.19). There was however no significant difference in salivary NE recovery across the 4 dilutions (p=0.120 by repeated measures ANOVA).

Table 2.30: Low spike salivary NE levels across varying dilutions in both COPD and healthy subjects (n = 5).

***Salivary NE (ng/ml)**	**Saliva Sample Dilution**			
Subject Sample	**1:100**	**1:200**	**1:400**	**1:800**
Sample 1	160 (54%)	158 (52%)	256 (152%)	280 (150%)
Sample 7	114 (76%)	102 (65%)	132 (89%)	130 (79%)
Sample 8	108 (49%)	99 (41%)	116 (51%)	116 (45%)
Sample 9	119 (77%)	107 (66%)	131 (83%)	114 (61%)
Sample 10	146 (83%)	124 (63%)	166 (99%)	161 (83%)
Recovery mean ± SD	**68% ± 15%**	**57% ± 11%**	**95% ± 37%**	**83% ± 40%**

*Salivary NE levels were multiplied by the dilution factor to allow for standardisation of the results.

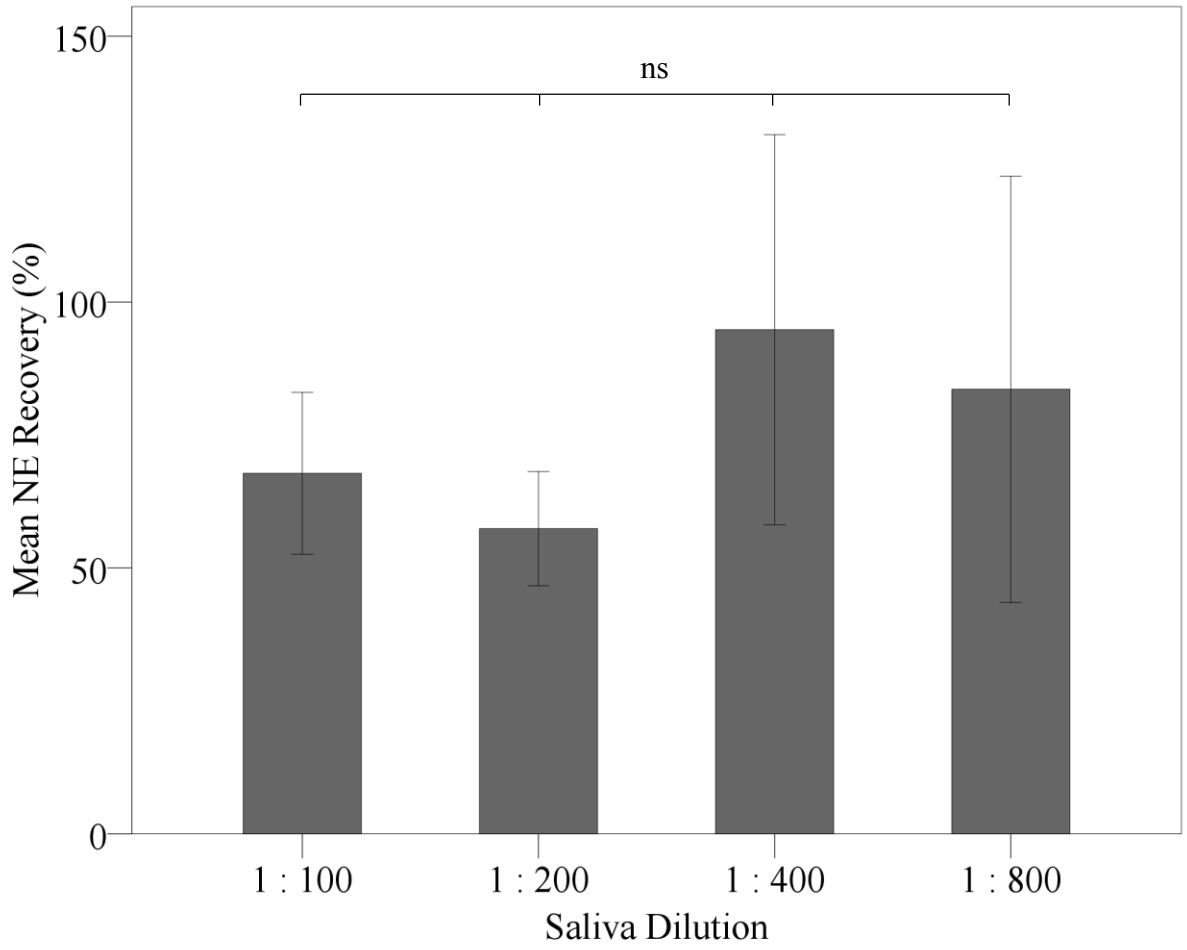


Figure 2.19: Low range spiked saliva NE recovery.

Bar chart with SD error bar of mean salivary NE recovery in samples spiked with low concentration NE at an increasing dilution factor (n = 5). There was no significant difference across dilutions (ns: p=0.120 by repeated measures ANOVA); thus intra-dilution analysis cannot be conducted. There is a large variation in the SD error bars as the dilution factor increased; this may be due to an increased error for the immunoassay as the lower limit of the assay working range (0 to 10ng/ml) is approached; similar to the salivary CRP ELISA as discussed above (Section 2.4.2.7, Page 108).

2.4.4.1.4.3. High spike NE saliva recovery and linear dilution experiments

Overall there was an improvement in recovery percentage with an increased salivary dilution however this was not statistically significant (p=0.681) (Table 2.31, Figure 2.20). Subject samples 7, 8 and 9 demonstrated an improvement in salivary NE recovery as the dilution increased. Sample 9 was unique as recovery decreased; however, this was still above 80% in the 1:800 dilution. This occurred despite selecting samples with "low" endogenous NE concentrations and spiking with 7.92ng/ml which should have ensured that the spiked sample would be in the assay working range (0-10ng/ml). All five samples analysed at a dilution of 1:100 were above the higher limit of quantification for the immunoassay.

Table 2.31: High spike salivary NE levels across varying dilutions in both COPD and healthy subjects (n = 5).

Salivary NE (ng/ml)	**Saliva Sample Dilution**			
Subject Sample	**1:100**	**1:200**	**1:400**	**1:800**
Sample 1	*Outside Ref	*Outside Ref	*Outside Ref	*Outside Ref
Sample 7	*Outside Ref	691 (83%)	767 (92%)	743 (89%)
Sample 8	*Outside Ref	587 (67%)	718 (83%)	799 (93%)
Sample 9	*Outside Ref	799 (96%)	771 (92%)	712 (85%)
Sample 10	*Outside Ref	963 (113%)	901 (106%)	958 (113%)
Recovery mean ± SD	**n/a**	**90% ± 20%**	**93% ± 10%**	**95% ± 12%**

* Result above the 10ng/ml higher limit of quantification for the ELISA.

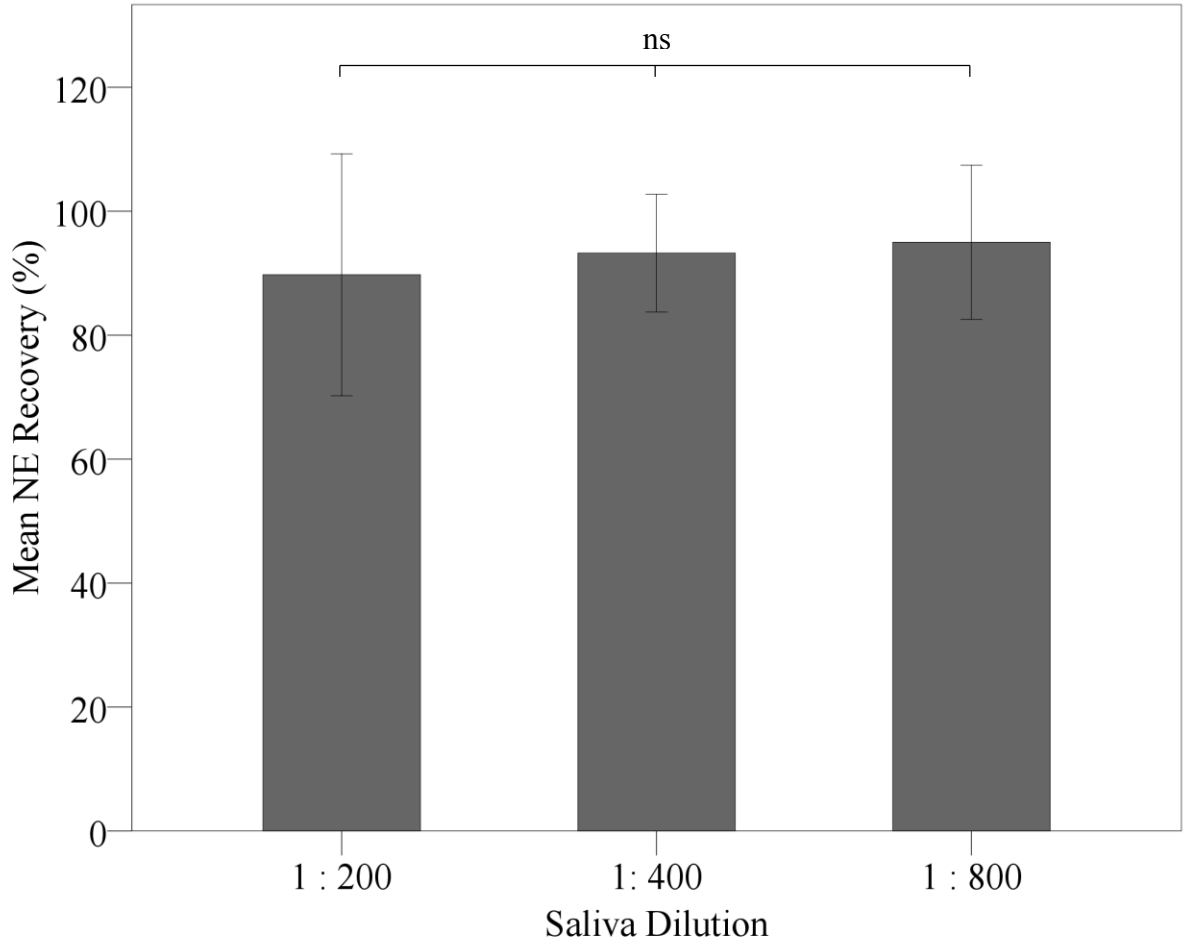


Figure 2.20: High range spiked saliva NE recovery.

Bar chart with SD error bar of salivary NE recovery percentage in samples spiked with high concentration NE at an increasing dilution factor (n = 5). There was no statistically significant difference across the 3 dilutions (ns: p=0.681 by repeated measures ANOVA) and thus intra-dilution analysis could not be performed.

Sub-analysis between the low and high range NE spiked saliva sample recovery results demonstrated no significant difference in recovery of salivary NE between the two concentration ranges (p=0.251 by two-way ANOVA).

2.4.4.1.5. Discussion

The recovery of salivary NE improves with an increasing linear dilution and this effect is more apparent across low range NE spiking experiments. The high range NE recovery experiments demonstrated a consistent recovery of salivary NE (greater than 90%) across all 3 dilutions. Of interest despite selecting saliva samples with known low endogenous salivary NE levels for the spiking experiments, the high range spike of saliva plus dilution at 1:100 yielded salivary NE results above the working range for the immunoassay (0 to 10ng/ml). This suggests that the immunoassay loses effectiveness at concentrations that approach the higher limit of quantification. Interestingly within the low range spike experiments the recovery is approximately 30% lower in dilutions below 1:400; this effect is not seen in the high range spike experiment where recovery of NE is above 90% for 1:200, 1:400 and 1:800 salivary dilutions. Although a 1:400 dilution looks preferable to 1:200 as a minimum saliva sample dilution it is important to note the sample preparation time. For saliva samples that require 1:200 dilution this would involve 7.5ul of saliva and 1.5mls of ELISA wash buffer. A further dilution to 1:400 would require a serial dilution or combining the wash buffer (which is used to dilute saliva) with a smaller volume of saliva. This is impractical due to the inherent viscosity of some saliva samples which does not allow for accurate pipetting of saliva volumes less than 5ul. In view of this my preferred method would be to serially dilute a 1:200 saliva sample to achieve a 1:400 dilution. However, this will increase sample preparation time. A prolonged saliva sample preparation time would result in saliva being exposed at room temperature for longer where further degradation of NE may occur (Chapter 1, Page 50). Thus equilibrium needs to be reached between adequate sample dilution and maximum sample preparation time.

2.4.4.1.6. Overall conclusion

Based on the above experimental results for future NE analysis, the same pre-analysis saliva preparation as for CRP (Section 2.4.2.1, Page 92) will be adopted. The intra- and inter-assay precision (CV) of 4.8% and 8.8% respectively was below the maximum (10% and 15%) accepted error percentage that had been selected. This will continue to be calculated for all PMN Elastase ELISA kit (Immundiagnostik, Germany) throughout this thesis to ensure that both the intra- and inter-assay precision (CV's) remain below the accepted and clinical thresholds. All saliva samples will be tested in duplicate. The recovery of salivary NE appears to be consistent across increasing dilution for both low and high concentration NE samples, although a non-significant improvement is observed as the dilution factor increases. A saliva dilution of 1:200 was selected and the same test procedure as for serum was maintained. The quantifiable ELISA range for salivary NE with a sample dilution of 1:200 is 2.2 to 2000ng/ml. Overall, allowing for the above caveats and the small sample size, it can be concluded that modification of this serum-based assay can be used reliably on saliva.

For saliva to be an effective diagnostic biological fluid, a standardised sampling protocol for saliva collection, processing and storage needs to be established (Chapter 1, Page 50); to deliver the most accurate and meaningful results (Mohamed et al., 2012). A review of the literature discussed later in this chapter (Section 2.5, Page 169) focusing on factors that can affect passive collection of unstimulated whole saliva and the saliva sampling methodology used in studies for the target biomarkers in this thesis. This review enabled the generation a pilot sampling protocol (Figure 2.21, Page 176).

2.5. Optimisation of protocols for saliva collection and processing

The preferred method for saliva sampling in this thesis is unstimulated whole and unfractionated saliva collected via passive drool (Chapter, Page 53). A standardised pre-sampling protocol needs to be provided for individuals prior to saliva sampling to ensure homogeneity of the collected samples. To accomplish this, it was important to understand the underlying physiology of unstimulated whole saliva production, the factors that could affect this complex production and *in situ* release of the target analytes under study, and potential influences on their variable levels from bloodstream diffusion and stimuli such as diurnal release cycles, stress, food intake and medication.

2.5.1. Factors influencing unstimulated salivary flow and analyte levels

2.5.1.1. Un-modifiable factors

There are several un-modifiable factors that contribute to reduced unstimulated whole saliva flow rate. Flow is lower in women compared to men (Inoue et al., 2006) and in individuals of 60 years of age and above (Affoo et al., 2015). The total protein composition of unstimulated whole saliva has not been found to be differ between genders (Agha-Hosseini et al., 2006).

2.5.1.2. Circadian rhythm effects

The flow rate of unstimulated whole saliva collected via passive drool has been demonstrated to show significant circadian rhythms with the highest flow observed in the afternoon, but interestingly total protein content in samples was not found to be variable (Dawes, 1975). Unstimulated whole saliva flow rate for samples collected in the morning appears to be significantly higher when collected at mid-morning (11:30) as opposed to early morning (07:30) (Flink et al., 2005). Circadian rhythms have been observed in salivary glucose (Atwood et al., 1991), IgA (Dimitriou et al., 2002) and cortisol (Powell et al., 2013). Salivary CRP levels

have been observed as being higher in the morning (07:00) on awakening than in the afternoon and evening (Izawa et al., 2013). There are no observations for diurnal effects on PCT and NE levels.

2.5.1.3. Fasting and diet effects

The flow rate of unstimulated whole saliva is decreased after fasting (Rahim and Yaacob, 1991, Johansson et al., 1984). Protein secretion however is not significantly altered; it has been demonstrated that prolonged fasting does not affect the level of total protein in unstimulated whole saliva of healthy individuals (Crosley et al., 2009). Acute dehydration has also been shown to decrease salivary flow and also increase total protein content (Walsh et al., 2004). Salivary flow can be affected by severe caloric restrictions (Humphrey and Williamson). Food or drink high in sugar content or caffeine can stimulate saliva flow rate and lower mouth pH levels; both leading to compromised antibody–antigen binding and enzyme activity in immunoassays (Granger et al., 2004). Thus avoiding food and fluid (except for water) or chewing gum for at least 30 minutes prior to saliva sampling is recommended (Chiappin et al., 2007). Water consumption has been demonstrated to have no effect on the flow of unstimulated whole saliva (Davies et al., 2009) and rinsing the mouth with water prior to saliva sampling helps to eliminate residues that may hamper subsequent immunoassay analyses (Nunes et al., 2011). Acute alcohol consumption has been demonstrated to reduce the total protein content of stimulated whole saliva (Enberg et al., 2001), the effects on composition of unstimulated whole saliva as yet have not been documented.

2.5.1.4. Medication effects

The effects on saliva of commonly used medications have been extensively investigated; these are particularly important to recognise as many COPD patients also present with one or more active co-morbidities for which they are receiving specific treatments.

Furosemide and bendroflumethiazide, which are both diuretics, are commonly used in the treatment of hypertension and cardiac failure. In healthy individuals they have not been shown to affect the salivary flow rate (Nederfors et al., 1989), or total protein content in unstimulated whole saliva (Nederfors et al., 2004). Captopril, an angiotensin converting enzyme inhibitor used in hypertension does not affect unstimulated saliva flow rates or composition in healthy individuals (Nederfors et al., 1995). Additionally, a β_1-adrenergic antagonist (metoprolol) used in the treatment of ischaemic cardiac disease and hypertension, showed no effect on unstimulated salivary total protein concentration or composition (Johnson and Cortez, 1988). However, a study involving only males demonstrated that non-selective and β_1-adrenergic antagonists significantly reduced total protein concentration but did not affect salivary flow in both stimulated and unstimulated whole saliva. A large observational study involving over 1200 participants demonstrated that individuals on diuretics, anti-hypertensive and/or analgesics have a reduced unstimulated salivary flow (Narhi et al., 1992) athough the precise medication details were not published. Overall, the current working consensus is that these types of drugs have the potential to reduce salivary flow.

2.5.1.5. Effects of commonly prescribed COPD medications

COPD patients are invariably prescribed an array of medications to manage their disease. These include: β_2-agonists, anti-muscarinic bronchodilators, inhaled and oral steroids, theophyllines and mucolytics. From this list, the medications that are particularly noted to be xerogenic are

anti-muscarinic bronchodilators (Committee, 2015). One study involving rats receiving chronic treatment with β_2-adrenergic agonists demonstrated no effects on parotid saliva volume, protein concentration or composition (Johnson & Cortez, 1988). However, another study exploring chronic β_2-adrenoreceptor agonist therapy in childhood asthmatics demonstrated decreased flow of stimulated whole and parotid saliva (Ryberg et al., 1991). Inhaled anti-muscarinic medications are associated with xerostomia and/or low salivary flow (Loesche et al., 1995). A cross-sectional study involving forty asthmatic subjects on inhaled corticosteroids and forty non-asthmatic adolescents (median age 13 years) demonstrated no difference in unstimulated salivary flow (Santos et al., 2012). Interestingly, it has been suggested that asthmatics have a significantly reduced rate of salivary flow which is independent of the disease process itself, and likely related to the prescribed inhaled β_2-adrenergic agonists and corticosteroids (Alavaikko et al., 2011). There are at present no documented observations on whether the flow of saliva is affected by theophyllines or mucolytics. Although it is difficult to draw direct conclusions from studies undertaken predominantly in children, it seems appropriate to hypothesise that the medications prescribed for COPD are likely to reduce salivary flow.

2.5.1.6. Effects from blood contamination

Blood and its components can leak into the oral mucosa as a direct result of micro-injury, for example: burns, abrasions from tooth brushing or cuts to the cheek tongue or gums. In addition to this, poor oral health and related intra-oral pathology such as periodontal disease; gingivitis, are likely to increase the chance of blood leakage (Granger et al., 2007a). Smoking can also contribute to poor oral health; although increased blood contamination in unstimulated whole saliva of smokers compared to non-smoker controls has not been demonstrated (Kim et al., 2010). Tooth brushing and related micro-trauma leads to a significant effect on protein

composition of saliva due to contamination with serum constituents. Thus in saliva sampling protocols this should be considered by including a minimum brush to sampling time of 45 minutes (Hoek et al., 2002). Accurate quantification of salivary blood contamination requires assessment of transferrin levels as urine dipstick testing can produce false positives due to endogenous salivary peroxidase (Schwartz and Granger, 2004a). Schwartz et al., measured and found that testosterone levels in saliva were increased when salivary transferrin was greater than or equal to 5000ng/ml. Dehydroepiandrosterone (DHEA) and cortisol were increased at transferrin levels greater than 10000ng/ml. In children blood contamination is rare and its effects on the measurement of salivary hormones (DHEA, cortisol and testosterone) is small (Granger et al., 2007a). For progesterone and estradiol the confounding effect of blood can be controlled by visual inspection of sample discolouration. This study also suggests that sampling of saliva should be avoided in the first 15 minutes post micro-injury and samples visibly contaminated with blood should be discarded (Kivlighan et al., 2005). Recent work has also revealed that blood contamination of saliva at 1%, affected the levels of four oxidative stress markers (advanced oxidation protein products, glycation end products, free reducing antioxidant power and total antioxidant capacity). However, 1% blood contaminated of saliva samples are visibly dis-coloured and thus it is possible to exclude these saliva samples from analysis by visual/macroscopic inspection (Kamodyova et al., 2015). Overall blood contamination appears to only exhibit an affect at visually discoloured or transferrin levels above 5000ng/ml. Presently, there are no target studies investigating the effects of blood contamination in saliva on salivary CRP, PCT and NE levels. Visual inspection appears to offer a crude method for blood contamination screening of saliva sample screen; as urine dipstick testing appears to generate false positives, the transferrin assay may be deemed to be more reliable.

2.5.2. Published saliva sampling protocols for target biomarkers under study

Current review of the literature shows a varied methodology for sample collection when testing unstimulated whole saliva via drool for salivary CRP levels. Several studies do not mention whether the participants are fasted or not prior to saliva collection (Azar and Richard, 2011, Pederson et al., 1995, Punyadeera et al., 2011, Mohamed et al., 2012). Several studies quote (Navazesh, 1993) which states for unstimulated whole saliva collected via passive drool:

Sit in an upright posture, head slightly tilted forward and subjects should refrain from smoking, eating or drinking for 1 to 2 hours prior to saliva sampling, rinse their mouths with water and then let saliva drip off the lower lip into a collection device (Miller et al., 2014).

The commonest pre-sampling fasting protocol is for participants to provide a sample at 1 to 2 hours after any food intake (Dillon et al., 2010, Topkas et al., 2012, Rao et al., 2010, Rao et al., 2011). One study went a step further with pre-sampling fasting protocol by asking participants to refrain from drinking coffee, acidic/sweet liquids and smoking for 30 minutes prior to the visit (Ouellet-Morin et al., 2011). Another recent approach was to collect unstimulated whole saliva upon awakening in the morning (Pace et al., 2013). The only studies to have investigated salivary PCT levels do not mention whether there is any restriction on the participants' habits prior to saliva sampling (Yousefimanesh et al., 2015, Bassim et al., 2008). Similarly, studies exploring NE levels in unstimulated whole saliva do not distinguish restrictions prior to saliva sampling (Pederson et al., 1995, Cox et al., 2006).

In the two studies that have utilised unstimulated whole saliva in COPD patients albeit not for CRP, PCT and NE quantification the protocols have stated fasting for 90 minutes (Yigla et al., 2007, Ji et al., 2014). Ji et al., also documented that subjects were not allowed to brush their

teeth, eat, drink (except water) or smoke for at least 90 minutes before the visit. Subjects were also asked to watch a basket of lemons.

Overall the review above provided the knowledge to generate a pilot saliva sampling protocol (Figure 2.21, Page 176).

Pre-Saliva Sample Collection

- Please avoid alcohol for 12 hours before providing a sample.
- During the hour prior to sampling do not eat, brush or floss teeth or use mouthwash.
- Rinse your mouth with cold water and wait 5 minutes before saliva collection.

Saliva Sample Collection

- Sit in an upright position with your held tilted forward.
- Yawning can also increase saliva.
- When you are ready, hold the container in from of the mouth and simply drool or dribble saliva into the container.
- The container has a marker level to help you know when a sufficient amount of saliva is collected.
- Please close the container with the lid provided.

Do NOT cough or clear your throat when producing a sample, there must not be any sputum or mucus in the sample.

Figure 2.21: Pilot saliva sampling protocol.

2.5.3. Refinement of the pilot sampling protocol

The pilot protocol (Figure 2.21, Page 176) was supplanted to make it more end-user specific. Within this pilot protocol several of the pre-sample saliva collection components cannot be altered. These include minimum time from alcohol consumption, tooth brushing, flossing and mouth wash gargle to saliva sampling. However, one potentially modifiable factor is fast time.

The method of choice for saliva collection in this thesis is passive unstimulated whole saliva via drool the benefits of which have been discussed (Chapter 1, Page 53). One limitation of this method is a reduced saliva flow compared to other sampling modalities for example: stimulated whole saliva. The factors that further limit salivary flow have been discussed earlier in this chapter (Section 2.5.1, Page 169). Briefly, COPD patients represent an older age range, are on a range of xerostermic medications and certain co-morbidities for example: diabetes mellitus all reduce saliva flow. These saliva-flow reduction factors are un-modifiable. Medication burden cannot be manipulated for fear of exacerbating the disease process. Importantly, COPD patients with concurrent type 2 diabetes mellitus may not be able to fast for prolonged periods of time due to problems with glycaemic control. Finally, COPD causes patients to suffer with breathlessness, although this does not affect salivary flow directly, it will affect patients' perceptions of the saliva sampling process if the time to produce an adequate volume of saliva is prolonged due to an excessive fast induced saliva flow reduction.

In view of this and the lack of studies investigating the flow of saliva in COPD patients a series of experiments was conducted to understand the effects of fasting on unstimulated whole saliva flow in COPD patients and its effects on the target analytes in this thesis. These experiments would have important implications on refinement of the pilot saliva sampling protocol (Figure 2.21, Page 176).

2.5.4. Exploration of salivary flow in COPD patients and the optimal fast period prior to sampling

Experiments were designed and conducted to address the following outstanding questions not addressed by the wider literature.

2.5.4.1. Aims

1. Is there a difference in the time taken to produce a set volume (2mls) of unstimulated whole saliva between healthy controls and COPD patients?
2. What specific conditions provide for an optimal sample?
    a. Does the duration of fasting affect the rate at which a predetermined volume of saliva (2mls) is produced in COPD patients?
    b. Does this fasting period alter the level of biomarkers in saliva retrieved from COPD patients?

2.5.4.2. Methods

Six healthy non-smoker subjects and six COPD patients (Table 2.32) in the stable phase (acute exacerbation free for a minimum of six weeks) of their disease were recruited from the Directorate of Respiratory Medicine's research and outpatient clinic database (Section 2.3, Page 88). All participants gave informed written consent and had adhered to the pilot saliva sampling protocol (Figure 2.21, Page 176) prior to the start of the experiment. All saliva collections occurred between 09:00 to 12:00 and were conducted in the home environment of each participant. Prior to collection of saliva, the participants were asked to consume a snack of their choosing for example: toast, biscuits. Once they had finished eating they rinsed their mouths with water. Saliva was then drooled into provided ice-cool 15ml pre-marked centrifuge tubes (Nunc, Denmark), up to a marked set volume of 2mls; the time taken to produce this

volume was timed using the stop-watch function on an iPhone (Apple, USA). Samples were placed on ice and stored in the Guy Hilton Research Centre Freezer Room (Keele University, UK) at -80ºC within 4 hours of collection.

Healthy subjects only provided this initial saliva sample at baseline. COPD patients continued the experiment and were asked to provide repeat 2ml saliva samples every 30 minutes up to two hours, thus providing five samples in total. These samples were vortex and centrifuged at 3000rpm for 15 minutes (Section 2.4.2.1, Page 92) prior to salivary CRP, PCT and NE analysis. The assay procedures were previously described for CRP (Section 2.4.2.1, Page 90), PCT (Section 2.4.3, Page 112) and NE (Section 2.4.4, Page 149). Biomarker analysis for CRP and NE was conducted on one microtitre ELISA plate whilst PCT required one VIDAS B.R.A.H.M.S PCT kit (bioMérieux, France).

Table 2.32: Demographic details for participating healthy and COPD patients.

Demographics	Healthy (n = 6)	COPD (n = 6)
Age, [a] years	57.3 ± 11.5	55.0 ± 8.2
Gender, Male, (Female), n	3 (3)	1 (5)
Smoking Status, Current (Ex), n	0 (2)	1 (5)
Duration of COPD, [a] years	n/a	7.7 ± 3.7
FEV_1, [a] % predicted	97.4 ± 8.8	45.5 ± 23.9
FVC, [a] % predicted	97.4 ± 7.3	75.0 ± 14.4
BMI, [a](kg/m^2)	26.2 ± 4.7	23.1 ± 1.4
Exacerbations in the last 1 year, [a]n	0	4.8 ± 3.5
Co–morbidities, n		
None	4	1
Heart Disease	0	1
Type 2 Diabetes Mellitus	1	2
Hypertension	0	4
Gum Disease	0	2
Other	1	1
Treatment, n		
Inhaled β_2 agonists, Short Acting, (Long Acting)	0, (0)	6, (3)
Nebulised β_2 agonists (Short Acting)	0	3
Inhaled Anticholinergic, Short, (Long Acting)	0, (0)	0, (4)
Inhaled Steroid	0	6
Oral Theophylline, n	0	1
Total Number of Oral Medications, [a]n	0.3 ± 0.5	4.5 ± 2.4

Data are presented as: a = mean ± SD.

2.5.4.3. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, for these experiments to standardise the interpretation of these data, the overall time taken to produce a fixed volume of saliva was divided by this volume (2mls) to determine a rate of flow: ml/min. Salivary NE levels above the higher limit of quantification for the ELISA (2000ng/ml) were assigned as 2001ng/ml. No CRP samples were outside the limits of quantification (0.10 to 30ng/ml) for the immunoassay.

2.5.4.4. Results

Overall the healthy subjects were slightly older: 57.3 ± 11.5 years with a range of 47 to 78 years compared to the COPD patients: 55.0 ± 8.2 years with a range of 45 - 65 years. Within the healthy cohort, subjects 2 and 4 were the only two to have chronic co-morbidities: type 2 diabetes mellitus and osteoarthritis respectively and requiring regular medication. All COPD patients were receiving inhaled β_2-short and long-acting agonists and inhaled steroids. In addition, COPD patients 7 and 9 received inhaled anticholinergic therapy. Patients 7, 8 and 11 were also receiving nebulised β_2 agonists. Within the COPD cohort, total oral medication was 4.5 ± 2.4 tablets, only one COPD patient 8 was on an oral theophylline. COPD severity (FEV_1 % predicted) varied between mild to very severe (FEV_1: 45.5 ± 23.9%, range: 16 to 83% predicted). COPD patient 8 had very severe disease with an FEV_1 of 16% and accordingly was on continuous supplemental oxygen therapy. COPD patients 7 and 10 had stable chronic gum disease, which required no regular treatment. For the whole COPD cohort, the number of COPD exacerbations in the past 12 months was 4.8 ± 3.5 episodes (Table 2.32). The respective intra-assay CV for the CRP immunoassay was 7.8%; PCT immunoassay 4.8%; NE immunoassay 5.0%.

2.5.4.4.1. Time to produce 2mls of saliva

2.5.4.4.1.1. Healthy subjects compared to COPD patients

The results demonstrate that mean salivary flow rate was slightly faster in COPD patients (0.52ml/min) compared to healthy subjects (0.48mls/min); however this result was not statistically significant (p=0.25 by independent t-test). Interestingly the healthy subjects appear to have a higher degree of saliva flow rate variability compared to the COPD patients (Table 2.33).

Table 2.33: A comparison of the time taken by all healthy subjects and COPD patients to produce a set volume of saliva (2mls), with associated saliva flow rates.

Healthy Subjects	*Time to 2mls (mins) Saliva Flow Rate (ml/min)	COPD Patients	*Time to 2mls (mins) Saliva Flow Rate (ml/min)
1	5.37 mins 0.37 ml/min	**7**	4.50 mins 0.44 ml/min
2	6.93 mins 0.29 ml/min	**8**	40.13 mins 0.05 ml/min
3	7.93 mins 0.25 ml/min	**9**	2.47 mins 0.81 ml/min
4	6.40 mins 0.31 ml/min	**10**	3.40 mins 0.59 ml/min
5	8.82 mins 0.23 ml/min	**11**	3.93 mins 0.51 ml/min
6	1.43 mins 1.40 ml/min	**12**	2.85 mins 0.70 ml/min
Mean Flow ± SD	**0.47 ± 0.46**	**Mean Flow ± SD**	**0.52 ± 0.26**

*saliva sampling flow rates immediately post eating.

2.5.4.4.1.2. Saliva sampling flow rates in COPD patients over an increasing fast period

Across the two hour fast time the mean salivary flow rate is variable within a narrow range (0.50 to 0.53ml/min) up to 90 minutes fast time (Table 2.34; Figure. 2.22). At two hour's post eating, a further increase (0.59mls/min) is observed in the COPD patients' cohort. Overall there was no statistically significant change in salivary flow rates across the sub-population (n = 5) with increasing fixed fast time (p=0.854 by repeated measures ANOVA). This result does not allow for further sub-analysis to understand the intra-fast time differences of salivary flow between COPD patients.

Table 2.34: A comparison of the time taken to produce a set volume of saliva (2mls) at different fast times after eating and associated saliva flow rates in COPD subjects.

COPD Patients		Fast Time (mins)				
		0	**30**	**60**	**90**	**120**
7	**Time to 2mls (mins)**	4.50	6.15	6.50	6.27	3.03
	Saliva Flow (ml/min)	0.44	0.33	0.31	0.32	0.66
***8**	**Time to 2mls (mins)**	40.13	n/a	n/a	n/a	n/a
	Saliva Flow (ml/min)	0.05				
9	**Time to 2mls (mins)**	2.47	5.53	3.35	6.28	4.73
	Saliva Flow (ml/min)	0.81	0.36	0.60	0.32	0.42
10	**Time to 2mls (mins)**	3.40	1.73	2.50	2.40	1.83
	Saliva Flow (ml/min)	0.59	1.15	0.80	0.83	1.09
11	**Time to 2mls (mins)**	3.93	14.15	11.98	12.22	12.50
	Saliva Flow (ml/min)	0.51	0.14	0.17	0.16	0.16
12	**Time to 2mls (mins)**	2.85	2.92	2.95	2.28	3.17
	Saliva Flow (ml/min)	0.70	0.69	0.68	0.88	0.63
	Mean Flow ± SD	**0.52 ± 0.26**	**0.53 ± 0.40**	**0.51 ± 0.26**	**0.50 ± 0.33**	**0.59 ± 0.34**

*COPD patient 8 took over 30 minutes to produce the first sample hence there is no time recorded for 30, 60, 90 and 120 minutes. Each subsequent sample was approximately 500ul to ensure biomarker analysis could be performed for each time point.

There are interesting observations when examining the data for individual COPD patients, highlighting the inter- and intra- variability in salivary flow rates that can exist within a study cohort, especially when small (n = 6). COPD patient 7 demonstrated a decrease in salivary flow until the two hour fast time (0.44ml/min to 0.32ml/min), after which the flow rate increased to 0.66ml/min. COPD patient 9's trajectory appears to be consistent with patients 7, 11 and 12 at the 30 minute interval. However at the subsequent fast times COPD patient 9 had another sharp rise in salivary flow rate at 60 minutes followed by a decline, then a rise again at 120 minutes. In contrast, COPD patient 10 had a rapid rise in saliva flow rate from 0 to 30 minute fast time, which subsequently falls from 60 to 90 minutes before rapidly rising by approximately 25% at the two hour fast time. COPD patient 11 demonstrates a rapid reduction in flow after 30 minutes of fasting from 0.51ml/min down to 0.14ml/min, which remained suppressed (0.14 to 0.17ml/min) for the subsequent fast times. Interestingly, COPD patient 12 demonstrates a gradual reduction in flow rate as fast time increases up to one hour, followed by a substantial rise in salivary flow rate to 0.88ml/min, which decreases again by approximately 30% at the two hour mark to 0.63ml/min.

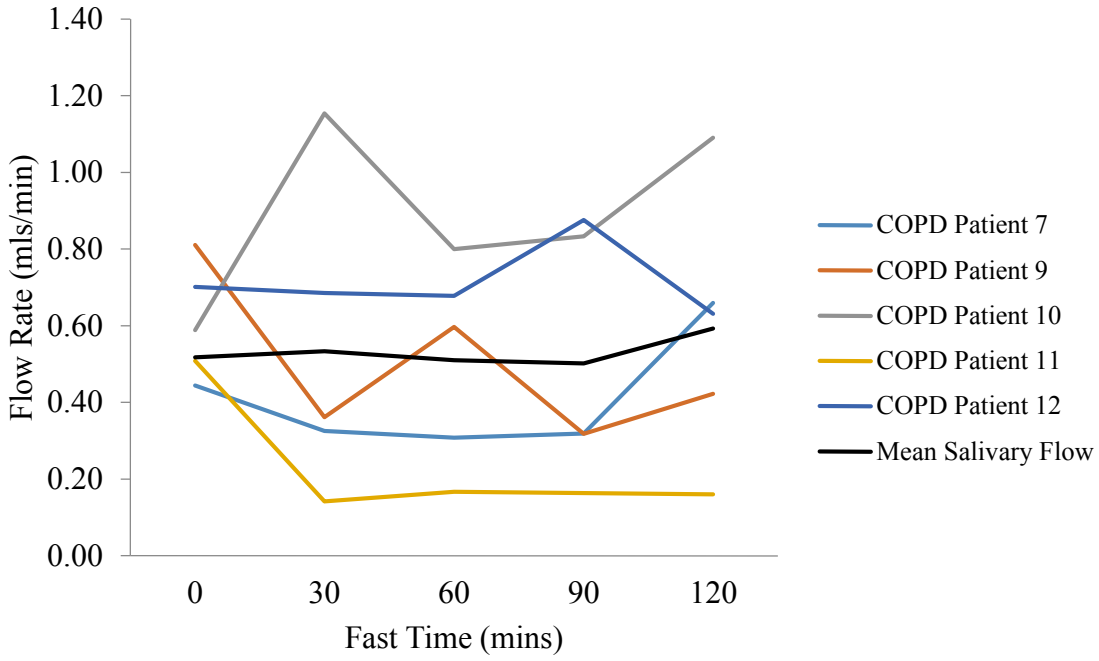


Figure 2.22: Saliva flow time in COPD patients at fixed increasing fast times.

Line chart of saliva flow time across increasing fixed fast times in COPD patients (n = 5). Overall there was no statistically significant change in salivary flow rates across this sub-population with an increasing fixed fast time (p=0.854 by repeated measures ANOVA).

2.5.4.4.1.3. Discussion

COPD patients compared to healthy subjects appear to take a slightly shorter time to produce a pre-set volume of 2mls of saliva immediately after eating. This is an unexpected finding, although it is not statistically significant as all six COPD patients were on both systemic and inhaled therapies that are also known to cause xerostomia (Table 2.32). One possible explanation is that the age ranges for the healthy cohort was higher than the COPD patients (Affoo et al., 2015).

COPD patients 7, 8 and 11 exhibited the slowest salivary flow within their cohort; they were all receiving treatment with nebulised β_2-agonists and aerosolised agonists via meter-dose inhalers. COPD patient 8 had the most severe (FEV_1 = 17%) disease of the entire cohort and correspondingly his salivary flow rate was the lowest. This patient's total medication burden was the highest for the entire COPD cohort with eight oral medications and four inhaled medications in total. An overall explanation for an apparent "healthy-equivalent" saliva flow rate in this cohort of COPD patients remains elusive. It could be concluded that salivary flow in COPD patients is at least equivalent to healthy subjects although the number of subjects studied is small. The variability of salivary flow in healthy subjects is established (Neyraud et al., 2012); however this degree of variability is not as apparent in the COPD subjects and may be related to medication effect. Interestingly the flow rates for COPD patients' saliva are equivalent to published values in healthy subjects (Skopouli et al., 1989, Fenoll-Palomares et al., 2004).

Analysis of the overall COPD cohort data appears to show no significant change in the time taken to produce 2mls of saliva as the fast time increased. The data however indicate that for the majority of COPD patients the fastest time to produce a fixed volume of saliva was

immediately after eating; except for patient 10 whose time for producing set volume of saliva increased with duration of fasting. An interesting observation was COPD patients 7, 9, 10 apparent increase in salivary flow as they approached the two hour fast time. This might be explained as a "mouth-watering" phenomenon. These patients had their saliva sampling performed at 10:00am; so as the two hour time-point would be approaching 12:00, it is possible that the thought of lunch may enhance saliva production. The evidence for this phenomenon occurring in humans, and whether it can be triggered by either visual or olfactory cues is conflicting. The current consensus is that there is no true olfactory-parotid salivary reflex in humans unlike canines; however atmospheric acidic stimuli, both oral and nasal, can cause irritation with an ensuing increase in salivary flow (Lee and Linden, 1991). A separate study demonstrated that an olfactory-submandibular reflex does exist (Lee and Linden, 1992). Interestingly it has also been shown that handling of food elicits an increase in the total volume of saliva in the mouth (Ilangakoon and Carpenter, 2011) perhaps in support of Lee et al. Visual cues on the other hand have not been established to increase the flow of parotid saliva (Drummond, 1995). Thus it could be concluded that an olfactory stimulus or handling of food may have led to an increase in the flow of whole saliva. This provides a potential explanation for COPD patient 7 who had begun to prepare a meal. The handling and olfactory stimulation of the food may have resulted in the 48% increase in salivary flow.

2.5.4.4.1.4. Conclusion

Overall 2mls is an acceptable volume for COPD patients to produce within a timeframe of approximately five minutes and the overall flow rate in this small group is no different to healthy subjects. The flow of saliva is accelerated after eating which subsequently slows and then accelerates towards the two hour fast time. The flow of saliva remains above 0.50mls/min

(range: 0.50 to 0.59mls/min), which would result in a minimum sampling time of 4 minutes to produce 2mls of saliva.

2.5.4.4.2. Salivary biomarker levels at different fast times

2.5.4.4.2.1. Salivary CRP

The median level of salivary CRP in all COPD patients increased significantly as fast time was extended ($p<0.006$ by Friedman's test) (Table 2.35, Figure. 2.23). The post-hoc analysis however does not demonstrate a particular fast time as having a significant influence on salivary CRP levels perhaps due to the small sample size (Table 2.36).

Table 2.35: Salivary CRP levels at increasing fast time in COPD patients.

Salivary CRP (ng/ml)	**Fast Time (mins)**				
COPD Patients	**0**	**30**	**60**	**90**	**120**
7	2.13	2.44	2.81	3.70	2.98
8	45.63	36.85	28.10	23.16	25.13
9	0.71	1.10	1.13	1.38	1.54
10	2.94	12.26	5.99	13.56	5.73
11	0.71	0.90	0.83	1.01	1.28
12	0.26	0.26	0.38	0.65	0.39
Median, IQR	**1.42, 2.03**	**1.77, 8.86**	**1.97, 4.29**	**2.54, 9.99**	**2.26, 3.69**

Table 2.36: Salivary CRP significance levels at increasing fast time in COPD patients.

Salivary CRP (ng/ml)	**Fast Time (mins)**				
Fast Time (mins)	**0**	**30**	**60**	**90**	**120**
0	n/a	p=0.271	p=0.343	p=0.344	p=0.344
30	p=0.271	n/a	p=0.892	p=0.171	p=0.499
60	p=0.343	p=0.892	n/a	p=0.248	p=0.752
90	p=0.344	p=0.171	p=0.248	n/a	p=0.752
120	p=0.344	p=0.499	p=0.752	p=0.752	n/a

Post-hoc analysis to determine significant differences in intra-fast time by Wilcoxon Signed Rank test. n/a = not applicable.

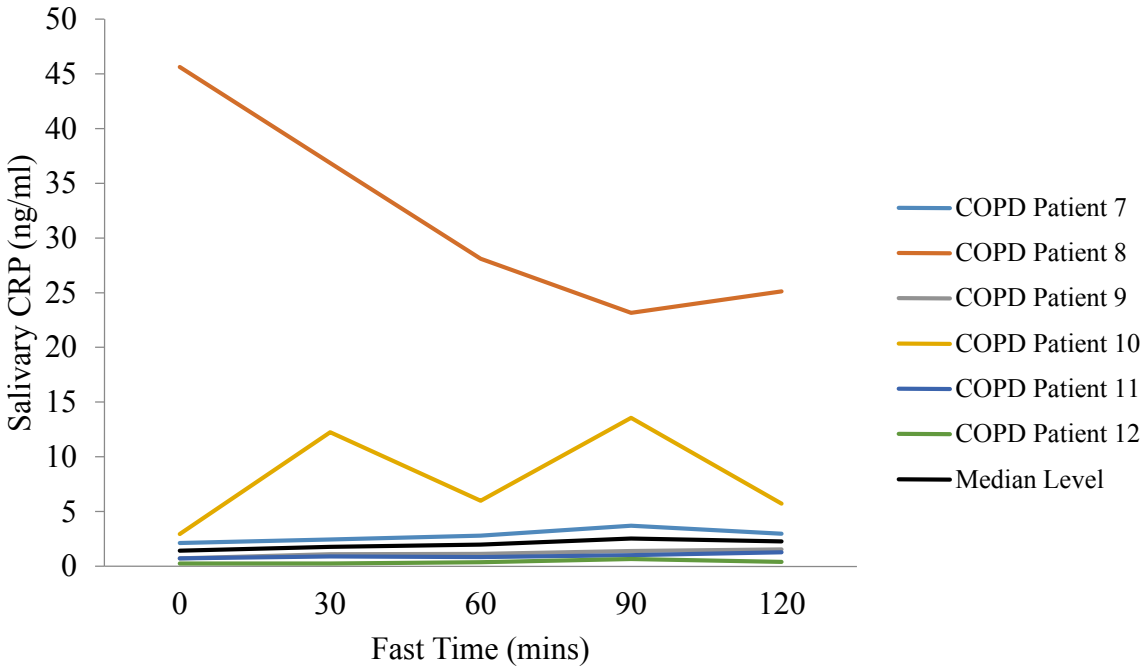


Figure 2.23: Salivary CRP levels at increasing fast time in COPD patients.

Line chart representing salivary CRP levels in a COPD cohort at increasing fast time (n = 6). There was a significant increase in salivary CRP ($p<0.006$ by Friedman's Test) as fast time increased (n = 6). Intra-fast-time analysis does not demonstrate any significant differences between the individual fast-time points.

All COPD patients, except for patient 8, demonstrated higher levels of salivary CRP after two hours of fasting compared to baseline; although there are subtle individual variations. Interestingly COPD patient 8's baseline salivary CRP level was well above the others and dramatically declined as fasting continued, with a small CRP rise between the 90 minutes and two hour fasted samples. In COPD patients 7 and 10 baseline levels of salivary CRP were also higher than their peers with the exception of patient 8. COPD patients 7 and 12 demonstrated a gradual increase in salivary CRP levels until the 90 minute fast time and fell thereafter. In patients 9 and 11, salivary CRP levels gradually rose as fast time increased, although patient 11 does demonstrate a small rise and fall between 30 and 60 minutes fast times. Of note,

salivary CRP levels in COPD patient 10 were highly variable demonstrating a sine wave pattern as fast time increased.

The median levels of CRP in the COPD cohort increased significantly as fast time increased with a reduction in levels between sampling at 90 and 120 minutes. Interrogation of the results revealed that only patient 8's salivary CRP level decreased as fast time increased with a small increase between 90 to 120 minutes. This patient's salivary CRP levels at all fast times were approximately 10 to 20 times higher than the other COPD patients. This could be explained by the patient having very severe COPD (FEV_1 16%) and thus the elevated levels actually may reflect a systemic footprint of the advanced disease status. In the rest of the cohort, COPD patient 7 had higher levels of salivary CRP compared to patients 9, 11 and 12. Although patient 7 had mild COPD (FEV_1 83%), in the prior 12 months the patient has experienced 10 acute exacerbations requiring antibiotics and steroids. The higher CRP levels seen in patient 10 likely reflect a severe disease state (FEV_1 28%) as well as a background history of gum disease; with both also likely contributing to sine wave fluctuations in levels.

2.5.4.4.2.2. Salivary PCT

The median levels of salivary PCT in the six COPD patients demonstrated no change over the 2-hour fast time. As expected this result was not statistically significant (p=0.828 by Friedman's test) (Table 2.37; Figure. 2.24).

Table 2.37: Saliva PCT levels at increasing fast time in all COPD patients.

Salivary PCT (ng/ml)	**Fast Time (mins)**				
COPD Patients	**0**	**30**	**60**	**90**	**120**
7	0.10	<0.10	<0.10	<0.10	0.27
8	0.20	0.21	0.22	0.29	0.21
9	<0.10	<0.10	<0.10	<0.10	<0.10
10	<0.10	<0.10	<0.10	<0.10	<0.10
11	<0.10	<0.10	<0.10	<0.10	<0.10
12	<0.10	<0.10	<0.10	<0.10	<0.10
Median, IQR	**0.09, 0.01**	**0.09, 0.004**	**0.09, 0.004**	**0.09, 0.004**	**0.09, 0.09**

*salivary PCT below the lower limit of assay quantification 0.10ng/ml assigned as 0.09ng/ml.

The median levels of salivary PCT across patient cohort remained the same for the entire 120 minutes post eating. COPD patients 9 10, 11 and 12 had salivary PCT levels below the lower limit of assay quantification (0.10 ng/ml) throughout the fast period. The reason for COPD patient 7's sudden rise in PCT at the 2 hour fast time is unclear; it is possible it could be due to existent gum disease or the onset of an inflammatory response. This PCT rise at 2 hours is however not reflected in concomitant salivary CRP levels for this patient: CRP did rise markedly from baseline (Table 2.35) at the 90 minute fast point. COPD patient 8's baseline salivary PCT level was higher than the others and was mirrored concomitant in his salivary CRP levels; again this likely reflects the underlying severe disease state and frequent exacerbation status.

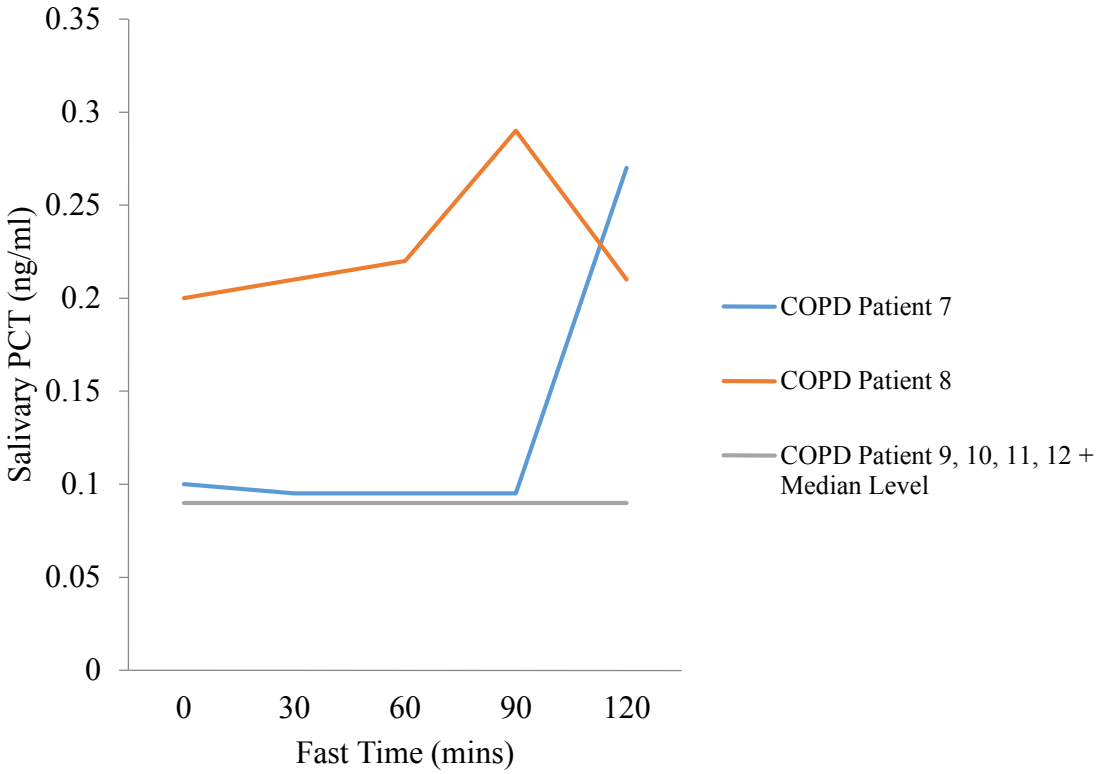


Fig. 2.24: Salivary PCT levels at increasing fast time in COPD patients.

Line chart representing COPD patients salivary PCT levels at increasing fast time (n = 6). There was a no significant change in salivary PCT levels (p=0.828 by Friedman's Test) as fast time increased.

2.5.4.4.2.3. Salivary NE

The median levels of salivary NE in COPD saliva appeared to rise as the fast time increased but not statistically significant ($p<0.119$ by Friedman's Test) (Table 2.38; Figure. 2.25), although as 2 patients had levels above the higher limit of quantification for the entire experiment it is difficult to fully interpret these results.

Table 2.38: Salivary NE levels at increasing fast time in all COPD patients.

Salivary NE (ng/ml)	**Fast Time (minutes)**				
COPD Patients	**0**	**30**	**60**	**90**	**120**
7	>2000	>2000	>2000	>2000	>2000
8	>2000	>2000	>2000	>2000	>2000
9	185	>2000	>2000	>2000	>2000
10	42	433	112	1273	>2000
11	278	282	394	316	518
12	4	4	3	34	4
Median, IQR	**232, 1493**	**1199, 1172**	**1180, 1810**	**1619, 1437**	**1983, 1121**

*salivary NE above the higher limit of assay quantification 2000ng/ml assigned as 2001ng/ml.

COPD patients 7 and 8 demonstrated stable salivary NE levels above the higher limit of quantification (2000ng/ml) for the immunoassay throughout the two hour test period. COPD patient 7 had higher levels of CRP and PCT throughout the fast time although there was more variability when compared to peers. The high NE levels in patient 8 reflect concomitant high salivary CRP and PCT levels. In contrast COPD patient 9's salivary NE levels increased from 185ng/ml at baseline to 2001ng/ml at 30 minutes and then remained high thereafter; in this patient concomitant salivary CRP levels showed gradual increase over the fast time (although levels are low in comparison to peers) whilst PCT did not change.

COPD patient 10's trajectory for salivary NE levels mirrored changes in CRP levels; there was an immediate large progressive rise in NE levels as fast time increased from 42ng/ml at start time to greater than 2000ng/ml at two hours. COPD patient 11 demonstrated a much steadier

increase in salivary NE with fast time; also reflected in salivary CRP levels. Interestingly COPD patient 12 demonstrated very low levels of salivary NE throughout the two hour fasting period; consistent with this patient's salivary CRP levels (the lowest of all COPD patients in this experiment) and negative salivary PCT levels.

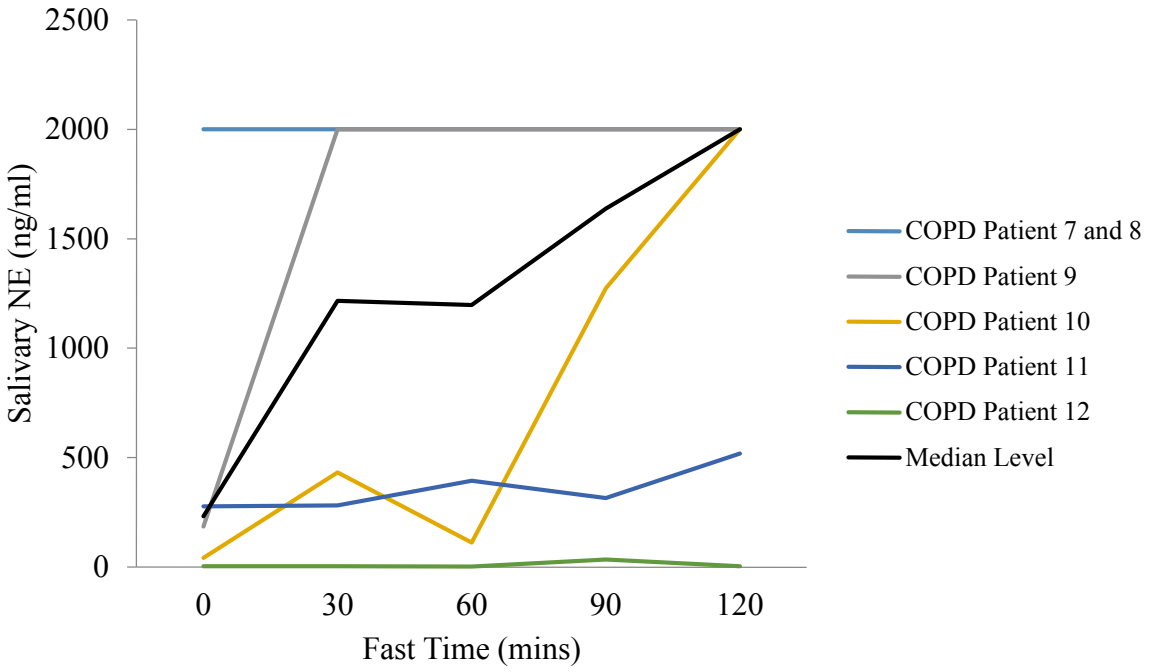


Figure 2.25: Salivary NE levels at increasing fast time in COPD patients.

Line chart representing COPD patients salivary NE levels at increasing fast time (n = 6). The line for COPD Patients 7 and 8 shares the line for COPD patient 9 as the salivary NE levels are the same from the 30 minute fast time towards 2 hours. There was a no significant change in salivary NE levels (p=0.118 by Friedman's Test) as fast time increased.

2.5.4.4.2.4. Discussion

Overall salivary CRP in COPD patients' significantly increases up to 90 minutes post fasting with levels then reducing at the 2-hour time-point. Interestingly for patient 8 who had the most severe disease, CRP levels were the highest post food consumption and steadily declined over the two fast period. This observation for COPD patients over two hours may be consistent with an initial micro trauma caused by eating which results in higher levels of salivary CRP due to blood contamination. Subsequently as the effects of this trauma subside levels fall and perhaps if the experimental observations had continued beyond the 2 hour period, levels might have reduced further. This would then be consistent with a diurnal effect that has previously been observed demonstrating lower salivary CRP levels in the afternoon compared to the morning (Izawa et al., 2013). Salivary PCT is stable across 2 hours, with negligible levels for 4 out of the 6 COPD patients. This is consistent with the literature, in that PCT levels are normally undetectable during "stable health". Salivary NE levels are difficult to interpret due to 3 subjects providing samples that were above the higher limit of quantification for the assay for the entire experiment. A significant change over the 2 hours is not demonstrate although median levels increase, which like CRP may possibly be related to oral microtrauma. There are no published studies for the diurnal variation of NE in saliva; however it is possible that contrary to CRP salivary NE levels could be elevated in the afternoon compared to the morning.

2.5.4.5. Overall conclusion

These preliminary experiments appear to show that most COPD patients can effortlessly produce 2mls of saliva within an acceptable timeframe. The effect of xerostermic medications in this small COPD group do not appear to significantly influence the flow of saliva when compared to healthy subjects and that this saliva volume in clinical studies would not cause a problem with patient compliance. Overall salivary biomarker levels do fluctuate post fasting:

for PCT. The diurnal changes in salivary CRP warrant attention to time-of-day of testing in sample collection protocols whilst the NE results are difficult to interpret but perhaps in a few subjects demonstrate the effects of oral microtrauma.

2.5.5. Blood contamination in saliva experiments

Blood contamination appears to have a small effect on salivary hormone levels (Chapter 2.5.1.6, Page 173); however the potential effects on salivary CRP, PCT and NE have not previously been explored. To quantify and compare the levels of blood contamination in saliva a commercially available transferrin assay (Biocare Diagnostics, China) and an 8-parameter urine reagent stick (Siemens, Germany) were utilised. A transferrin assay was selected as a review of the literature highlighted several studies that had quantified salivary transferrin as a surrogate for salivary blood contamination (Granger et al., 2007a & Kivlighan et al., 2005). If blood contamination is shown to significantly affect the salivary levels of CRP, PCT and NE this will have important implications for saliva sampling protocols and pre-analysis saliva sample preparation (Section 2.4.2.1, Page 92).

2.5.5.1. Aims

Accordingly, experiments were carried out to:

1. Determine effects of blood contamination on levels of CRP, PCT and NE in human saliva.
2. Explore whether urine reagent sticks can provide meaningful information on blood contamination in human saliva samples.

2.5.5.2. Methods

Firstly, both the transferrin assay and urine reagent test stick assay were modified for saliva-based testing.

2.5.5.2.1. Transferrin assay

The transferrin assay used in this experiment is a lateral flow test-strip (Figure 2.26) designed for use with stool samples. The test procedure involves adding 100ul of substrate to 1.5mls of

assay buffer, which is provided in a purpose-built container: Three drops of the mixture are then aliquoted onto the test-strip (S). This is performed by removing a detachable head (which reveals a stoma) on the assay solution container, inverting the bottle and pressing. To understand the volume per drop and thus standardise the amount of substrate-assay buffer mixture being dispensed onto the test-strip, a volume per drop was caluculated. This was achieved by aliquoting a series of drops into a 1.5ml eppendorf up to a volume of 500ul. In total 17 drops were required to reach the target volume which equates to a volume per drop of 29.41ul. A volume of 30ul was used for ease of titration and thus 90ul substrate-assay buffer solution needs to be dispensed onto the test strip. The assay can then be read after 15 minutes. A line is required to form at point (C) for the test to be valid. If transferrin is present above the detection threshold, a line appears at point (T). The assay has a lower limit of quantification of 10ng/ml.

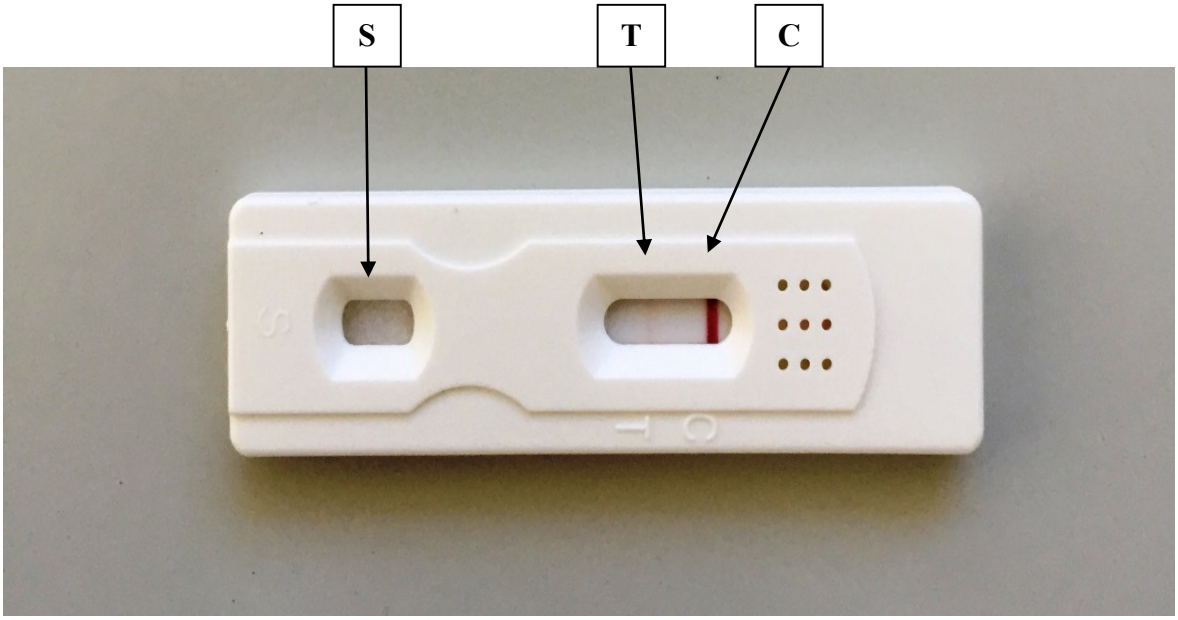


Figure 2.26: Transferrin assay test strip.

Samples are deposited at the point (S). A control line must appear at the point (C); if transferrin is present in the sample above the lower limit of quantification (10ng/ml) a line will also appear at point (T). The colour intensity of the line at either point (C) or (T) is not important.

A review of the literature does not identify transferrin levels that can contaminate and affect the biomarkers to be investigated in this thesis; yet there is suggestion that certain proteins can be affected at levels greater than or equal to 5mg/l, with possible effects observed at levels greater than 10mg/l (Section 2.5.1.6, page 172). This assay's lower limit of quantification is 10ng/ml. Baseline transferrin levels in unstimulated whole saliva via drool in healthy subjects have been shown to be less than 4mg/L (Schwartz and Granger, 2004); thus using this assay in its unmodified form would have likely resulted in all saliva samples testing positive for blood. The assay was modified to reduce the lower limit of quantification from 10ng/ml to 4mg/l. This was achieved by reducing the volume of saliva combined with assay buffer from 90ul to 0.25ul.

2.5.5.2.2. Urine reagent stick

The 8-parameter urine reagent stick (Siemens, Germany) (Figure 2.27) used to assess urinary blood contamination contains a series of eight reagent squares which change colour when immersed in urine. The colour change for the blood reagent square (A) is compared to a reference chart after approximately 60 seconds to quantify the level of blood. The chemistry for the blood reagent square is based on haemoglobin's pseudo-peroxidase activity to catalyse a reaction between hydrogen peroxide and TMB. This produces a colour change (Figure 2.27) from green to dark blue depending on the quantity of haemoglobin. A review of the literature (Section 2.5.1.6, Page 173) demonstrates that this method can be unreliable as saliva peroxidases can interfere with this reaction, thereby resulting in an increased rate of false positives. It may be hypothesised that a high false positive rate generated by the urine blood reagent square due to saliva peroxidases may be overcome by reading the reagent square after a shorter time frame than recommended for urine.

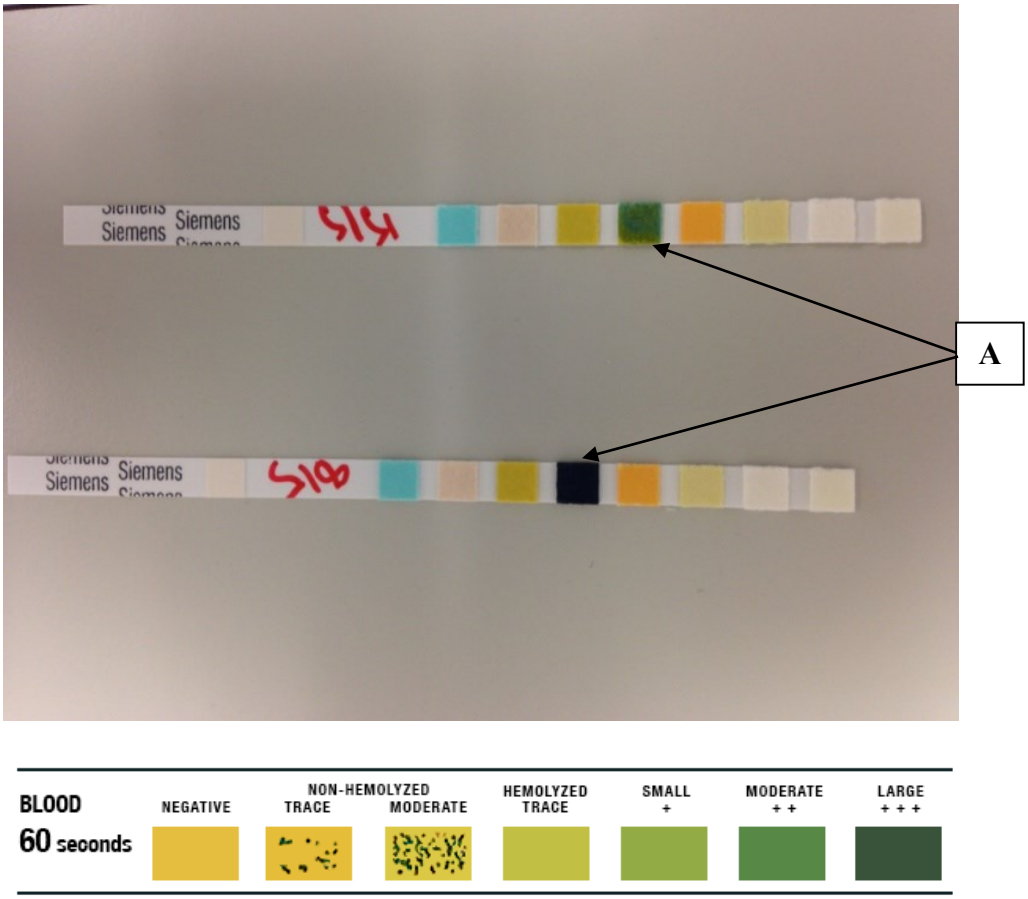


Figure 2.27: Urine reagent stick and colour chart.

A = blood reagent square, Blood = colour reference chart

The test procedure for the blood reagent square on the 8-parameter urine stick was modified. To avoid having to immerse the entire urine reagent stick in saliva 10ul of saliva was aliquot onto the reagent square (this volume covered the whole square in saliva) and the colour change was subsequently read after 5 seconds; timed with the stopwatch function on an iPhone (Apple, USA)

Forty-eight randomly selected saliva samples from 8 different COPD patients' (6 samples per patient) (Table 2.39) who were previously recruited from the Directorate of Respiratory Medicine's research and outpatient clinic database (Section 2.3, Page 88) tested for blood contamination. Each COPD patient had previously given informed written consent and had

provided multiple unstimulated whole saliva samples via passive drool across a varying but short time-frame (2 month) All COPD patients had adhered to the pilot sampling protocol (Figure 2.21, Page 176) and all samples were collected in ice-cooled collectors and stored in the Guy Hilton Research Freezer Room (Keele University, UK) at -80^{o}C as part of an established saliva bank until analysis. Analysis of said saliva samples occurred no later than 6 months after collection. Each sample had previously tested for salivary CRP, PCT and NE using the methods described earlier in this chapter for CRP (Section 2.4.2.1, Page 90), PCT (Section 2.4.3, Page 112) and NE (Section 2.4.4, Page 149). The purpose of testing multiple samples in the same individual was to understand whether there was any inter-sample blood contamination variability. Blood contamination was tested for using the modified transferrin assay (Biocare Diagnostics, China) and modified 8-parameter urine reagent stick (Siemens, Germany) as described above. Pre-analysis sample preparation involved sample thawing at room temperature; vortexed and centrifuged at 3000rpm for 15 minutes (Section 2.4.2.1, Page 92) as per salivary biomarker analysis. Each sample was tested in duplicate with the modified transferrin assay and 8-parameter urine reagent stick.

Table 2.39: Demographic details of COPD patients.

Demographics	COPD Patients (n = 8)
Age, [a] years	67.3 ± 8.7
Gender, Male, (Female), n	5 (3)
Smoking Status, Current (Ex), n	0 (8)
Duration of COPD, [a] years	6.8 ± 4.7
FEV_1, [a] % predicted	52.4 ± 17.6
FVC, [a] % predicted	78.4 ± 10.9
BMI, [a](kg/m^2)	24.7 ± 2.8
Exacerbations in the last 1 year, [a]n	5.8 ± 2.8
Co–morbidities, n	
None	1
Heart Disease	2
Type 2 Diabetes Mellitus	0
Hypertension	5
Gum Disease	2
Other	2
Treatment, n	
Inhaled β_2 agonists, Short Acting, (Long Acting)	8, (7)
Nebulised β_2 agonists (Short Acting)	0
Inhaled Anticholinergic, Short, (Long Acting)	1, (7)
Inhaled Steroid	6
Oral Theophylline, n	2

Data are presented as: a = mean ± SD.

2.5.5.3. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, salivary biomarker data were logarithmically transformed to allow univariate analysis and determination of covariate effect: age, gender, BMI, time of day of sampling, percentage positive blood contamination and total co-morbidities on salivary biomarker levels. For the transferrin assay saliva samples were assigned a score: 0 = no contamination and 1 = positive contamination to allow statistical testing. For the 8-parameter urine stick saliva samples were assigned a score: 0 = no contamination and 1 = positive contamination. This allowed correlation with transferrin values. The 8-parameter urine stick results were also divided according to the respective colour changes using the heamolysed colour scale (Figure 2.27): 0 = negative, 1 = trace, 2 = small, 3 = moderate, 4 = large. Spearman's Rank test was used to assess correlations

between the 2 modified assays. Only salivary PCT levels were below the lower limit of assay quantification (0.10ng/ml) in this experiment; these samples were assigned as 0.09ng/ml for calculation purposes.

2.5.5.4. Results

In total 13 out of 48 saliva samples tested positive for transferrin and 20 out of 48 samples tested positive for blood using the modified testing procedure described above (Table 2.40). The level of blood contamination in this group of COPD patients (n = 8) was: transferrin assay (27 ± 23%) and 8-parameter urine stick (42 ± 39%). All saliva samples that were positive for blood using the transferrin assays were also tested positive for blood when using the 8-parameter urine stick.

Salivary CRP levels were not significantly different between samples that were classified as contaminated with blood either by urine dipstick (p=0.205 by Univariate analysis; p=0.118 unadjusted for covariates) or transferrin assay (p=0.672; p=0.228 unadjusted for covariates) when adjusted for the covariates of age, BMI, gender, COPD disease severity and total co-morbidities. Sub-analysis of the salivary biomarker levels across the 5 different colour scales for the 8-parameter urine sticks revealed no significant difference for: CRP (p=0.170 by Kruskall Wallis test), PCT (p=0.562) and NE (p=0.08). There was no significant correlation (p=0.677 by Spearman's Rank test) between the transferrin assay and 8-parameter urine testing.

Table 2.40: Demographic breakdown of all saliva samples tested for blood contamination.

Demographics	Blood Contamination	
	Negative	Positive
Transferrin Assay, n	35	13
Urine Reagent Stick, n	28	20
Salivary Biomarkers (Transferrin assay),		
CRP, ng/ml	3.16, 3.68	5.13, 3.50
PCT, ng/ml	0.09, 0.03	0.11, 0.06
NE, ng/ml	204, 586	1076, 1133
Salivary Biomarkers (8-parameter urine test stick),		
CRP, ng/ml	3.59, 3.22	4.25, 5.24
PCT, ng/ml	0.09, 0.04	0.09, 0.05
NE, ng/ml	278, 535	721, 1301

Salivary biomarker data is presented as median, IQR.

2.5.5.5. Discussion

Approximately 27% of the samples tested positive for transferrin, however there was no effect on salivary biomarker levels when adjusted for covariates. This result implies that the routine testing for blood in saliva samples is unnecessary as significant levels of contamination which I defined as greater than 4000ng/ml do not appear to affect salivary CRP, PCT and NE levels. Whilst urine reagent sticks appeared to generate comparatively higher false positive results, there was 100% agreement with a positive transferrin test. Thus modifications in colour change timing for reading the reagent stick may provide useful information. Urine reagent sticks therefore may have a role in identifying saliva samples that are not contaminated with blood.

2.5.5.6. Conclusion

Blood contamination in saliva does not appear to significantly affect levels of CRP, PCT and NE. Urine reagent sticks can provide an accurate and easy assessment on blood contamination in collected saliva samples with the developed modified protocol. Thus saliva samples will be tested for blood contamination by aliquoting 10ul of saliva onto the blood reagent square of the urine reagent stick and reading the colour change after five seconds. Although salivary levels of CRP, PCT and NE are not affected, blood contamination testing will provide a

possible insight into pre-sampling protocol adherence, for example: tooth brushing may lead to oral microtrauma and leeching of serum proteins into saliva if the time between sampling and brushing is not adhered to. Testing for blood contamination will be conducted for randomly chosen saliva samples throughout this thesis. It will occur at the pre-biomarker analysis saliva sample preparation stage (Section 2.4.2.1, Page 92) post vortex and centrifugation.

2.5.6. Refined saliva sampling and collection protocol

Taking all above experimental data together, duration of fasting does not have an impact on the production of a pre-set volume of saliva. Regarding biomarker levels in the collected saliva, PCT and NE levels are not significantly altered as the fast time increases; this is not the case for CRP. This has important implications as the pilot protocol (Figure 2.21, Page 176) is to have participants abstain from food for at least 2 hours prior to their saliva collection. The experiments on salivary analyte levels across increased fast time (Section 2.5.4.4.2, Page 188) demonstrates that it is not necessary to have such a prolonged fast time, as there is probably a minimal effect on the eventual levels of the biomarkers, at least for PCT and NE. With respects to CRP, as the fast time does appear to affect the level of CRP, sampling should be carried out at a consistent time of day for each subject. A reduction in fast time from two hours can be justified. Accordingly, the new protocol will stipulate a fast time of 30 minutes prior to saliva collection. The result for CRP further enforces that a consistent time of day for sampling as far as is practically possible needs to be adhered to with the diurnal effect of saliva sampling on CRP, PCT and NE levels being incorporated in analyses throughout this thesis.

The pilot protocol (Figure 2.21, Page 176) was accordingly updated and would be provided to patients for all further saliva sampling in this thesis. With respects to blood contamination samples visually discoloured with blood will be discarded and participants providing a visually discoloured saliva sample will be asked to produce a further sample after approximately 30 minutes. Saliva samples will be randomly tested to quantify the level of blood contamination in the COPD population using an 8-parameter urine test stick (Siemens, Germany) with the modified protocol developed earlier in this chapter (Section 2.5.5.2.2, Page 200).

Pre-Saliva Collection Instructions

- Please avoid alcohol for 12 hours before providing a sample.
- You only need to fast for 30 minutes prior to providing a sample
- Avoid tooth brushing, flossing or mouthwash for at least 1 hour prior to providing a sample.
- Please let us know if you have recently used inhalers.

Saliva Sample Collection

- Rinse your mouth with cold water before saliva collection.
- Sit in an upright position with your held tilted forward.
- Thinking of lemons may help.
- When you are ready, hold the container in front of the mouth and simply drool or dribble saliva into the container.
- The container has a marker level to help you know when a sufficient amount of saliva is collected.
- Please close the container with the lid provided.

Do NOT cough or clear your throat when producing a sample, there must not be any sputum or mucus in the sample.

Figure 2.28: Refined saliva sampling protocol.

2.6. Development of a COPD PRO Score

COPD is a complex disease, the assessment and monitoring of which cannot be accurately encompassed by spirometry, biomarkers or symptom assessment in isolation. As discussed in Chapter 1, Page 34 there remains the need to combine these metrics to produce an accurate model of disease status and to capture the "biological march" of COPD in patients.

Having a standardised procedure for saliva collection and processing and established methods for biomarker analysis, a PRO score was developed to encompass measureable aspects of COPD-related features and incorporation into a patient self-assessment diary. A variety of questionnaires have been previously developed and their pros/cons discussed (Chapter 1, Page 27; Table 1.4, Page 30). Such questionnaires are invariably completed by COPD patients at intermittent intervals with a varied recall period. At present there remains the need to develop a PRO that can used daily which is accurate in monitoring COPD disease state and simple for patients to use. It is now increasingly recognised that a more suitable alternative to periodic symptom assessment would be a COPD health status questionnaire that is capable of capturing patient-driven disease relevant metrics on a frequent regular basis (Leidy et al., 2014). Such a questionnaire within the context of a daily diary would need to be simple and non-intrusive specifically avoiding the degree of complexity often associated with, for example the SGRQ (Jones, 2001) which is in-practical for daily monitoring. Additionally, PRO scores in COPD need to assess some or all of the key symptoms that are important to COPD patients: dyspnoea (breathlessness), cough and sputum production, effects on wellbeing and the ability to perform activities of daily living (ADL) (Walters et al., 2012). Sputum metrics could include colour, volume and texture, the relevance of which have been discussed (Chapter 1, Page 32). Although the PRO questionnaires discussed in Chapter 1, Page 27 provide meaningful measures on symptom burden, there remains a need to shift from a periodic assessment to a daily patient-

driven completed diary. This approach is required to examine the temporal and dynamic nature of symptoms and health status in COPD patients.

The EXACT-RS (respiratory symptoms) has been shown to be a reliable and valid instrument for evaluating respiratory symptoms daily in stable COPD (Leidy et al., 2014). The Shortness of Breath and Daily Activates questionnaire has also been shown to be a reliable, valid and response measure of dyspnoea when assessed daily in randomised control trials for medical product development in COPD patients (Tabberer et al., 2015). The CAT score incorporated into a daily diary has been demonstrated to increase during an acute exacerbation of COPD when measured daily (Alahmari et al., 2014).

2.6.1. Wellbeing and Self-Assessment diary

Working from this knowledge base, a PRO was designed and called: "COPD Wellbeing Score" questionnaire which enquired about (1) breathlessness, (2) ADL, (3) cough and (4) sputum metrics (Figure 2.29). The responses for breathing score, ADL and cough were specifically configured so that patients could assign a value based on the baseline or "normal" burden (Burge and Wedzicha, 2003). This questionnaire was incorporated in the first instance into a paper-based diary "Wellbeing Self-Assessment Dairy" (Figure 2.29) for use alongside salivary biomarker testing in a community based study on COPD patients (Chapter 3, Page 213).

1. **Breathing Score**

   How was your breathing today?

   Excellent = 1; Good = 2; Fair = 3; Bad = 4; Very Bad = 5

2. **Activity of Daily Living Score**

   Has your breathing affected your ability to perform daily activities such as self-wash/dress, cooking, housework?

   Not at all = 1; A little = 2; A fair amount = 3; Much = 4; Very much = 5.

3. **Do you have a cough?**

   Yes = 1; No = 0

   If Yes,

   Much better than usual = 1, Usual =2, Same = 3, Worse = 4, Much worse = 5

4. **Sputum Amount**

   How much sputum have you produced?

   None = 1; Up to 5mls (1 teaspoon) = 2; Up to 15mls (1 tablespoon) = 3; Up to 30mls (1 egg cup) = 4; Up to or greater than 50mls (1 cup) = 5.

5. **Sputum Texture**

   How would you describe the produced sputum?

   Watery = 1; Sticky Liquid = 2; Semi-solid = 3; Solid = 4.

6. **Sputum Colour (Participants were provided with a colour chart)**

   What is the colour of your sputum?

   Watery, clear, transparent = 1; Watery, cloudy, colourless = 2; Creamy = 3; Light green = 4; Dark green = 5.

Figure 2.29: COPD Wellbeing Score - manual self-assessment diary.

The numerical figure after each response indicate the score attached to that particular item.

Chapter 3:

Exploration of Levels of C-Reactive Protein, Procalcitonin and Neutrophil Elastase in Saliva: Comparison Between Health and COPD.

3.1. Introduction

As discussed in Chapter 1, Page 46 saliva is increasingly used as a non-invasive easily accessible bio-sample for POC diagnostics instead of blood (Denny et al., 2008, Wong, 2008) to inform on infection (Blackbourn et al., 1998, Gallo et al., 1997, Ikuta et al., 2000, Li et al., 1996, Thieme et al., 1992), drugs (Berlin et al., 2011, Mandel, 1993) and disease states (Hanemaaijer et al., 1998, Ji and Choi, 2015, Kaufman and Lamster, 2002, Miller et al., 2010, Raff, 2009, Sugimoto et al., 2010, Tishler et al., 1996, Zhang et al., 2015).

Within the field of respiratory medicine, studies using saliva as a bio-sample have explored isolation of respiratory tract infections (Robinson et al., 2008) and monitoring of smoking status (Berlin et al., 2011, Fagan et al., 2015) and therapeutic drugs (Henkin, 2012).

Salivary eosinophil cationic protein can differentiate between asthmatic and healthy subjects (Schmekel et al., 2001). Increased salivary CRP and haptoglobin levels are demonstrated in childhood allergic asthma (Rao et al., 2011); raised salivary leukotriene levels differentiate aspirin-intolerant asthmatics from tolerant counterparts (Gaber et al., 2008). Recent work has demonstrated that inhaled corticosteroid in patients with asthma results in reduced concentrations of salivary mucin (Navarrete et al., 2015).

Biomarkers in various body fluids have been associated with COPD pathogenesis and clinical outcome (Barnes et al., 2006, Koutsokera et al., 2013). The importance of CRP, PCT and NE in COPD has been clearly demonstrated (Chapter 1, Page 64, 69 and 73). Yet despite the merits that saliva could offer to practical monitoring of COPD and its exacerbations, only two studies have explored its potential clinical role (Ji et al., 2014, Yigla et al., 2007). Ji., et al investigated IL-8 and MMP-9, both markers of inflammatory response, demonstrating an inverse correlation

with lung function measured by FEV_1. They concluded that salivary analysis may be suitable for assessment of disease severity in COPD. Yigla., et al explored markers of COPD pathogenesis (total antioxidant status, uric acid, peroxidase and super oxide dismutase) in both saliva and BAL. They demonstrated that salivary analysis was comparable to BAL.

Presently a gap in knowledge exists for saliva levels of PCT and NE in healthy subjects both non-smokers and smokers and for the role of salivary CRP, PCT and NE in patients with COPD. The relationship between saliva and serum concentrations of PCT and NE has also yet to be clarified. In this community-based cohort study, to address these gaps in the literature CRP, PCT and NE levels were explored in unstimulated whole saliva via passive drool (Chapter 1, Page 53) using commercially-validated and in-house modified immunoassays, (Chapter 2, Page 90). This determined differences between healthy subjects with normal lung function and patients with COPD. The target biomarkers were measured at 3 time points within a 14-day period. As smoking can influence steady-state biomarker levels (Cazzola et al., 2008), the control cohort included life-long never-smokers and current smokers.

Additionally, recognising that biomarkers in isolation may lack sensitivity or specificity for disease monitoring in COPD without symptom assessment (Hurst et al., 2006) a novel PRO instrument: COPD Wellbeing score was designed (Chapter 2, Page 211) and utilised as part of a purposeful paper-based diary "Wellbeing and Self-assessment diary" (Chapter 2, Figure 2.29, Page 212). Diary responses were used to determine whether components of the COPD Wellbeing score correlated with salivary biomarker levels.

In order to relate salivary biomarker levels to COPD status, data in COPD patients were analysed relative to the GOLD stage (percentage predicted FEV_1) (Vestbo et al., 2013);

alongside MRC scores. This provided sufficient information for correlations between target salivary biomarkers and validated COPD-relevant objective and subjective clinical metrics. For further validation of saliva biomarkers, randomly chosen study participants across all cohorts also provided simultaneous blood samples.

The objectives for this chapter:

1. To further determine the precision of the saliva based and modified immunoassays.
2. To understand whether saliva sampling is feasible in a study setting with COPD patients.
3. To understand whether the COPD Wellbeing assessment score can be used as a PRO instrument and the relationship of said scores with salivary biomarkers.
4. To evaluate whether target biomarker levels in saliva can be used to differentiate between health and COPD status (Chapter 1, Page 77).

3.2. Materials and methods

3.2.1. Study design

From January 2010 to March 2012, 143 individuals were recruited consecutively from the Directorate of Respiratory Medicine's research and outpatient clinic databases (Chapter 2, Page 88) to one of 3 cohorts: life-long never-smokers (n = 20); current smokers (greater than 20 pack years) (n = 25); or COPD, confirmed by spirometry (Koko Legend II, nSpire, USA) according to GOLD criteria (n = 98) (Vestbo et al., 2013). Patients with other respiratory disorders were excluded. All never-smokers and smoker subjects had normal lung function. Participants were monitored over 14 days (3 visits, one week apart). At visit 1, demographic details were recorded (Table 3.1); participants with any infection or unstable illness in the preceding 6 weeks were excluded. On each visit, the MRC dyspnoea score was recorded (Fletcher, 1960), spirometry (Koko Legend II, nSpire, USA) performed and unstimulated whole saliva via passive drool collected (2ml). All participants were provided with, and asked to complete, a daily paper (Wellbeing and Self-assessment diary) (Chapter 2, Figure 2.29, Page 212), which incorporated scores on breathing, ADL, cough presence and sputum features (volume, colour and texture. In-between scheduled visits, study participants were asked to contact me on developing any change in symptoms. An exacerbation in COPD patients was defined as an increase in respiratory symptoms for two consecutive days, with at least two major symptoms (dyspnoea, sputum purulence, sputum volume) or a major plus a minor symptom (wheeze, cold, sore throat, cough) (Mackay et al., 2014). Randomly-selected subjects provided simultaneous saliva and blood samples. The study had received prior approval from the South Staffordshire Research Ethics Committee, 09/H1203/77; Informed written consent was obtained from all participants before enrolment into the study.

3.2.1.1. Unstimulated whole saliva collection and processing

Each study participant was provided with verbal instructions and a printed protocol for pre-saliva collection (Chapter 2, Figure 2.28, Page 209). Briefly, participants were asked to abstain from alcohol for at least 12 hours; fast for 30 minutes; refrain from brushing their teeth and smoking for 30 minutes, prior to providing saliva samples. Oral hygiene was checked and mucosal examination performed at each visit. All visit samples were collected at same time of day for each subject where practical. Immediately before collection participants rinsed their mouths with 10mls water; they then sat in an upright position, tilted their heads forward, and allowed saliva to pool in the mouth before passively drooling into an ice-cooled marked centrifuge tubes (Nunc, Denmark) up to a marked set volume of 2mls.

Collected saliva samples were transported on ice and stored in the Guy Hilton Research Centre Freezer Room (Keele University, UK) at -80^{o}C until analysis. Pre-saliva analysis sample preparation involved thawing of stored saliva samples and centrifugation at 3000rpm for 15 minutes (Chapter 2, Page 92). All saliva sample measurements were undertaken within three months of storage; all biomarker assays were performed in duplicate. Thirty randomly selected saliva samples were tested for blood contamination using an 8-parameter urine regent strip (Siemens, Germany) with a modified testing procedure (Chapter 2, Page 200).

3.2.1.2. Analysis of biomarkers in saliva

Saliva biomarker analysis for CRP, PCT and NE was optimised in Chapter 2, Page 90. Briefly, CRP was measured in 15ul of saliva using a Salivary ELISA kit (Salimetrics Europe, UK) (Chapter 2, Page 90), which has a range of quantification of 0.10 to 30ng/ml. Salivary PCT and NE were measured using in-house modified commercial serum based ELISAs. PCT was determined in 100ul of saliva diluted 1:2 in PBS-T using VIDAS® BRAHMS PCT kit

(bioMérieux, France) (Chapter 2, Page 112) which has a range of quantification of 0.10 to 400ng/ml. NE was measured in 7.0ul of saliva diluted 1:200 in ELISA wash buffer using PMN-Elastase ELISA kit (Immundiagnostik, Germany), (Chapter 2, Page 149) which has a range of quantification of 2.2 to 2000ng/ml.

3.2.1.3. Analysis of biomarkers in blood

Peripheral blood was collected in supplement-free tubes and ethylene diaminetetra-acid vacutainer tubes (BD Bioscience, New Jersey, USA). Samples were then centrifuged at 2000rpm for 15 minutes; retrieved serum was stored at -80^{o}C until analysis. Serum CRP was measured using ADVIA 2400 Chemistry System (Siemens, Germany) with a lower limit of detection as 0.3mg/L; concentrations below this limit were assigned as 0.29mg/L. Serum PCT and NE were quantified using same assay kits as for saliva, but following manufacturers' protocols. Serum levels were expressed as ng/ml except for CRP (mg/L). All assays were performed in duplicate.

3.3. Statistical analysis

The statistical tests employed are discussed in Chapter 2, Page 86. Specifically, biomarker data were logarithmically transformed to allow Univariate Analysis and determination of covariate effect. The reproducibility of salivary biomarker levels was explored using Bland-Altman plots expressing the change within a subject. Reproducibility of both the immunoassays and salivary biomarker levels was also assessed using CV.

3.4. Results

In total 143 individuals were recruited all control subjects had normal lung function; and 98 COPD patients (GOLD Stage I, 16; Stage II, 32; Stage III, 39; Stage IV, 11) were all ex-smokers (over 20 pack year history). Thirty-six COPD patients experienced an exacerbation during the course of the community-based cohort study; all controls remained clinically stable. Salivary levels of CRP, PCT and NE were measured in all participants (Table. 3.1), with an intra- and inter-assay co-efficient of variances of less than 7% and less than 12% respectively for all 3 assays. Microscopic blood contamination was tested in 30 randomly selected saliva samples using an 8-parameter urine test strip (Siemens, Germany) according to the methodology described in Chapter 2, Page 200. Overall there was no significant positive contamination detected and no saliva samples were discarded or repeated due to visual discolouration.

During the study all participants provided feedback on the method of saliva collection used. The general consensus revealed that saliva sampling was preferable to blood. However, study participants felt that the aperture of the collector resulted in saliva spillage although interestingly the research nurse and study participants both commented that this improved, as they got familiar with the sampling process. Further refinements to saliva collection methods incorporating patient feedback is considered in Chapter 6, Page 384.

Additionally, patients provided informal feedback on the provided Wellbeing and Self-Assessment diary; in addition to the structured questions they wished to see a “free-text” area to enter their own thoughts related to their health status this is addressed in Chapter 4, Page 251.

Table 3.1: Subject demographics and salivary biomarker profiles for the healthy non-smokers, smokers and stable COPD subjects (n = 107).

	Control Subjects (n = 45)		Stable COPD Subjects (n = 62)					P-value
Demographics	**NES**	**Sm**		***I	***II	***III	***IV	
	(n = 20)	**(n = 25)**	**(n = 62)**	**(n = 12)**	**(n = 19)**	**(n = 25)**	**(n = 6)**	
Age, [a] years	53 ± 17	42 ± 12	67 ± 7	65 ± 10	64 ± 8	68 ± 5	72 ± 4	**<0.001**
Gender, Male, (Female)	7, (13)	17, (8)	34, (28)	4, (8)	12, (7)	13, (12)	5, (1)	=0.525
FEV_1, [a](% predicted)	98.1 ± 3.7	99.7 ± 4.7	55.7 ± 22.0	90.4 ± 9.2	64.4 ± 7.6	44.1 ± 3.7	25.1 ± 4.2	**<0.001**
BMI, [a] (kg/m^2)	29.8 ± 3.6	25.4 ± 3.3	27.3 ± 7.8	28.0 ± 7.0	28.6 ± 2.6	27.3 ± 1.9	19.8 ± 3.6	=0.378
Co-Morbidities								
None	15	20	27	9	10	5	3	
***Cardiovascular**	3	3	31	2	7	19	3	
Type 2 Diabetes	1	2	10	1	2	7	0	
Gum Disease	2	0	1	0	0	1	0	
Treatment								
Inhaled β_2 Agonist Short, (Long) Acting	0, (0)	0, (0)	0, (0)	9, (8)	19, (16)	25, (25)	6, (6)	
Inhaled Anticholinergic Short, (Long) Acting	0, (0)	0, (0)	0, (0)	1, (3)	2, (8)	3, (18)	0, (5)	
Inhaled Steroid	0	0	0	8	17	25	6	
Oral Theophylline	0	0	0	0	1	7	2	
Symptom and sputum metrics, [b]								
MRC Score	1.00, 0.25	1.00, 0.25	4.00, 1.67	3.00, 2.25	4.00, 1.50	5.00, 1.00	5.00, 0.00	**<0.001**
****Breathing Score**	2.00, 1.00	2.00, 0.25	3.00, 0.00	3.00, 1.00	3.00, 0.00	3.00, 0.00	3.00, 0.75	**<0.001**
****ADL Score**	1.00, 0.00	1.00, 0.00	3.00, 2.00	1.00, 2.00	3.00, 2.00	4.00, 2.33	3.00, 1.50	**<0.001**
****Cough**								
****Sputum Amount**	1.00, 0.00	1.00, 1.00	2.00, 2.00	1.50, 1.00	2.00, 1.84	3.00, 1.00	2.50, 2.50	**<0.001**
****Sputum Colour**	3.00, 0.00	3.00, 0.00	3.00, 1.00	3.00, 0.75	3.00, 0.83	3.00, 1.00	3.50, 1.00	**<0.001**
****Sputum Texture**	1.00, 1.00	2.00, 0.00	2.00, 0.00	2.00, 0.50	2.00, 0.00	2.00, 0.00	2.00, 0.00	**<0.001**
Salivary Biomarkers, [b]								
CRP, ng/ml	0.89, 0.35	1.70, 1.07	1.66, 2.30	1.62, 1.36	2.44, 2.63	1.45, 2.34	2.34, 5.94	**<0.002**
PCT, ng/ml	0.09, 0.03	0.13, 0.09	0.09, 0.04	0.10, 0.06	0.09, 0.04	0.09, 0.04	0.11, 0.03	**<0.012**
NE, ng/ml	152, 96	408, 748	189, 508	227, 104	161, 491	189, 687	163, 181	**<0.001**

Data presented as a = mean ± SD and b = median, IQR. P-values represent the difference between control and stable COPD subjects. *Combined with hypertension, **Components of the COPD Wellbeing Score. NES = never-smokers, Sm = smokers. ***GOLD classification has been previously described Chapter 1, Table 1.2, Page 24.

3.4.1. Biomarkers across health status

To reduce the potential bias of stable baseline metrics for the exacerbation cohort within the COPD group only stable COPD participants (n = 62) were included in the between-group analyses.

3.4.1.1. Salivary CRP

Salivary CRP levels differed between the 3 groups ($p<0.002$ by ANOVA), with significant increase in COPD patients (median: 1.66ng/ml, IQR: 2.55ng/ml) compared to never-smokers (0.89, 0.35ng/ml, $p<0.001$($) by Mann Whitney U), but not to smokers (1.70, 1.07ng/ml, $p=0.604$). Smokers had higher salivary CRP levels than never-smokers ($p<0.001$($)) (Figure 3.1). These differences remained statistically significant ($p<0.05$ by Univariate Analysis) following adjustment for age, gender, sampling time and total co-morbidities; but not for BMI ($p=0.401$).

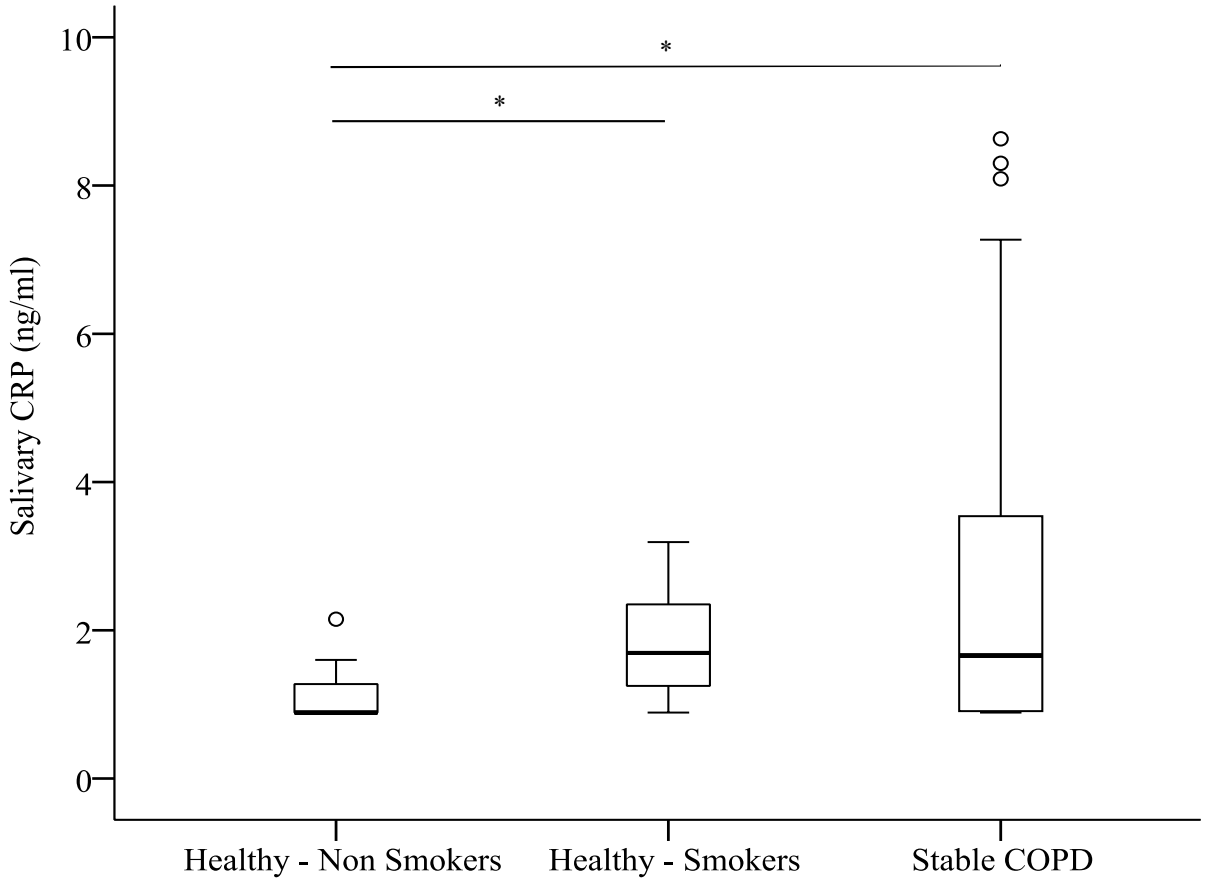


Figure 3.1: Salivary CRP levels from healthy non-smoker, healthy smokers and stable COPD subjects.

A box and whisker plot representing salivary CRP levels in healthy non-smokers and smokers alongside stable COPD patients (n = 107). The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{o} (1.5 x the interquartile range) Salivary CRP levels differed between the 3 groups ($p<0.002$ by ANOVA), with significant increase in stable COPD patients compared to non-Smokers (*$p<0.001$($) by Mann Whitney U), but not to healthy smokers ($p=0.604$). Smokers had significantly higher salivary CRP levels than never-smokers (*$p<0.001$($)). These differences remained statistically significant ($p<0.05$ by Univariate Analysis) following adjustment for age, gender, sampling time and total co-morbidities; but not for BMI ($p=0.401$).

The CV for salivary CRP variability within subjects was 13%, 27% and 15% for never-smokers, smokers and stable COPD respectively. The Bland-Altman plot with upper and lower limits (1.96 SD) combing all 3 groups (n = 107) showed good data consistency (difference between stable baseline values), with only 2 outliers (Figure 3.2).

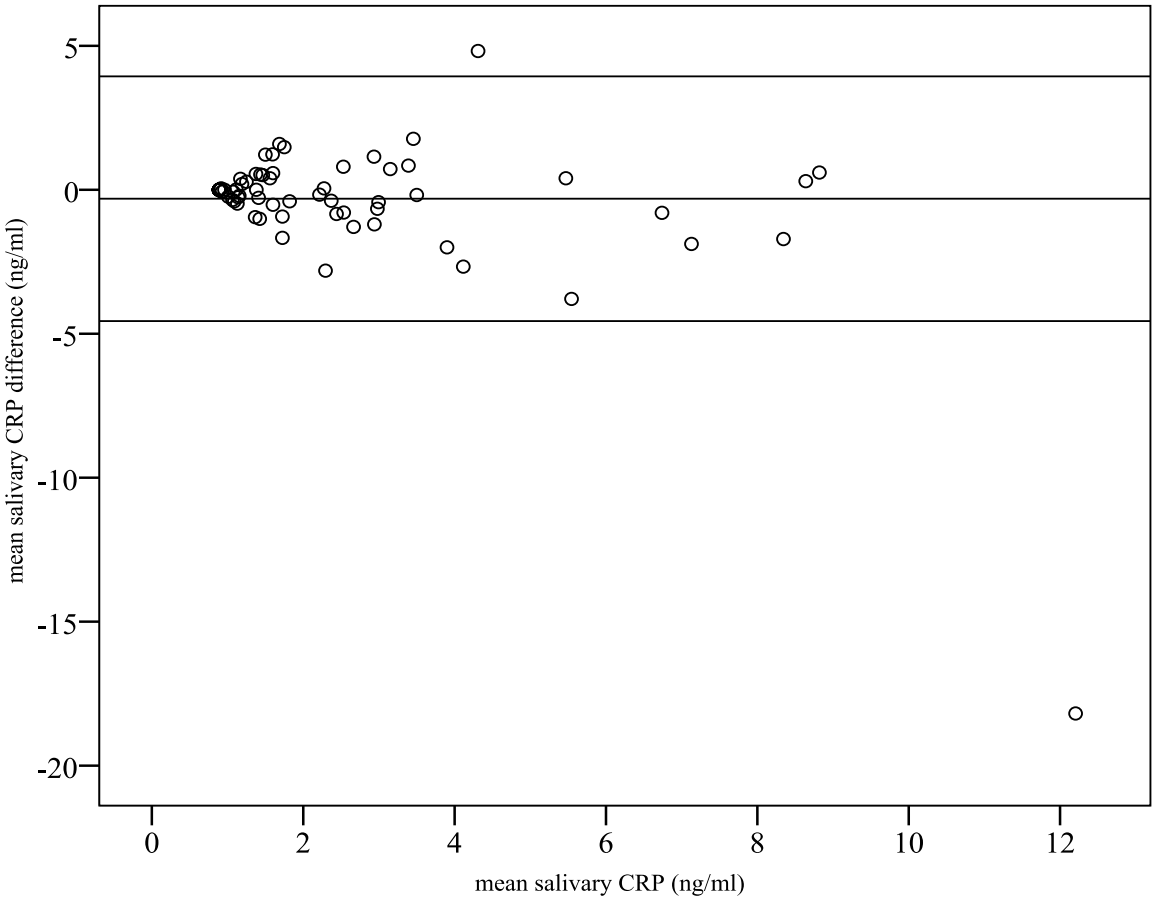


Figure 3.2: Baseline variability of salivary CRP levels across healthy non-smokers, health smokers and stable COPD.

A Bland-Altman plot of baseline salivary CRP variability in healthy non-smokers and smokers alongside stable COPD patients (n = 107). The upper and lower bars represent 1.96 standard deviations from the mean. Almost all replicates bar 2 outliers fell within the 95% limits of agreement (difference between stable baseline values).

3.4.1.2. Salivary PCT

Salivary PCT levels differed between groups ($p<0.012$). Salivary PCT was significantly elevated in healthy smokers (0.13, 0.09ng/ml) compared to never-smokers (0.09, 0.03ng/ml, $p<0.011(\$)$) and COPD patients (0.09, 0.04ng/ml, $p<0.01(\$)$); but not between COPD patients and never-smokers ($p=0.362$**)** (Figure 3.3). Following covariate adjustment (age, BMI, gender, sampling time and total co-morbidities), there was no significant difference ($p=0.563$) between cohorts. Gender adjustment showed salivary PCT levels were generally lower in females, (0.11 compared to 0.14 ng/ml [males]: $p<0.05$).

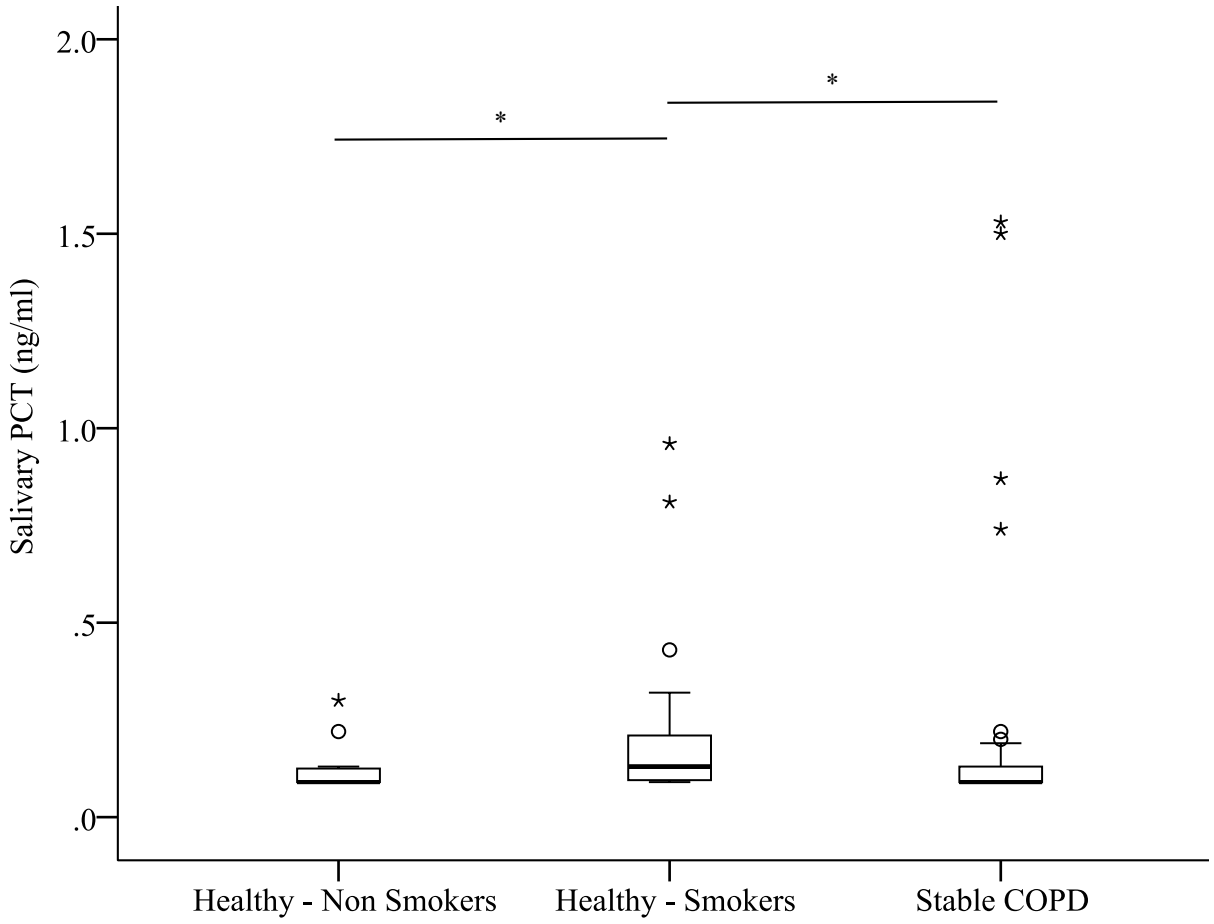


Figure 3.3: Salivary PCT levels from healthy non-smokers, healthy smokers, stable COPD subjects.

A box and whisker plot of salivary PCT levels in healthy non-smokers and smokers alongside stable COPD patients (n = 107). The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{o} (1.5 x the interquartile range) and * (3 x the interquartile range). Salivary PCT levels differed between groups ($p<0.01$). Salivary PCT was significantly elevated in healthy smokers compared to non-smokers (*$p<0.01$($)) and COPD patients (*$p<0.01$($)); but not between COPD patients and never-smokers ($p=0.362$**).** Following co-variate adjustment, there was no significant difference ($p=0.563$) between cohorts. Gender adjustment showed salivary PCT levels were generally lower in females, (0.11 compared to 0.14ng/ml [males]: $p<0.05$).

The CV for PCT variability within subjects was 19%, 15% and 14% for never-smokers, smokers and stable COPD respectively. The Bland-Altman plot with upper and lower limits (1.96 SD) combing all 3 groups (n = 107) showed good data consistency, with only 4 outliers (Figure 3.4).

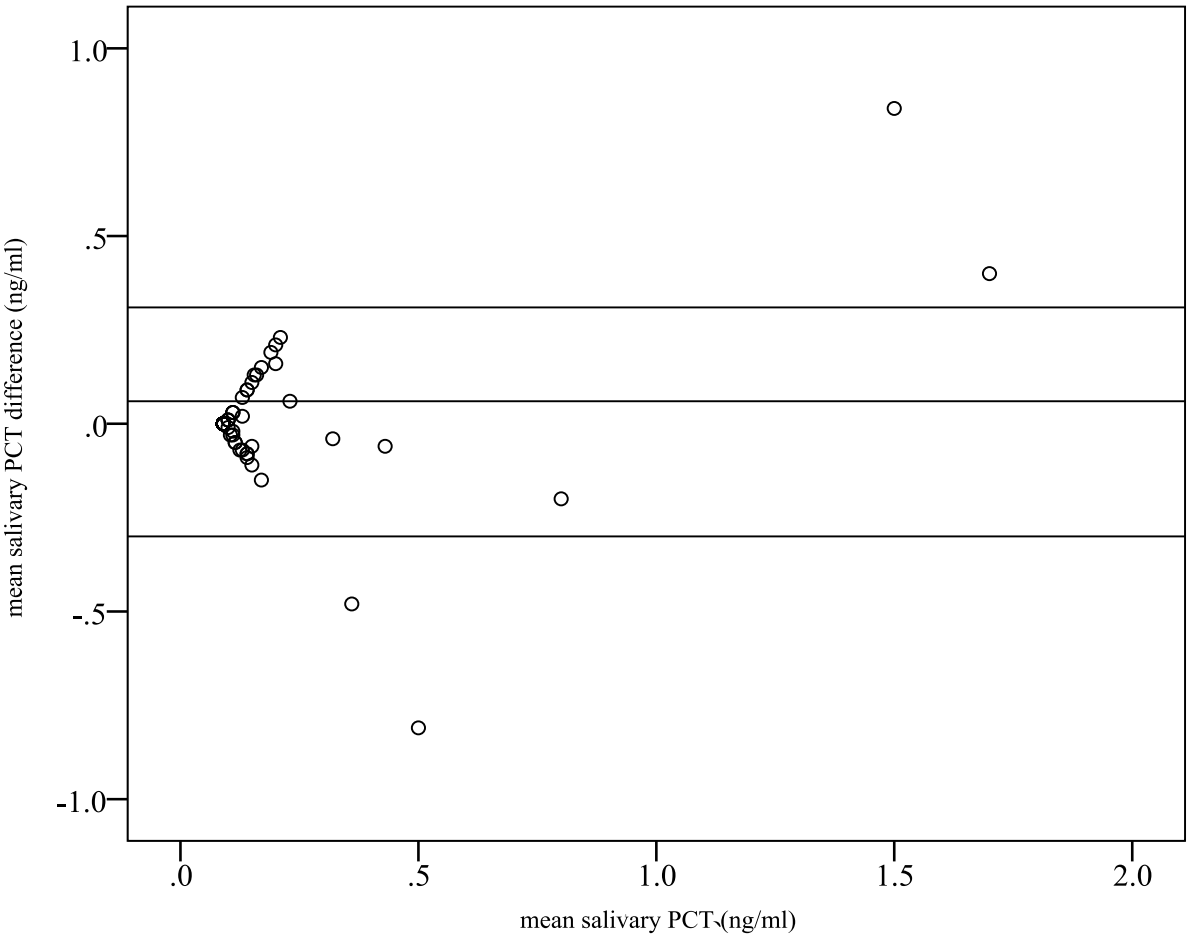


Figure 3.4: Baseline variability of salivary PCT levels across healthy non-smokers, health smokers and stable COPD.

A Bland-Altman plot of baseline salivary PCT variability in healthy non-smokers and smokers alongside stable COPD patients (n = 107). The upper and lower bars represent 1.96 standard deviations from the mean. All replicates bar 4 outliers fell within the 95% limits of agreement (difference between stable baseline values).

3.4.1.3. Salivary NE

Differences in salivary NE levels were observed between cohorts ($p<0.001$), irrespective of covariate adjustment ($p<0.01$). Healthy smokers had significantly raised NE levels (408, 748ng/ml) compared to never-smokers (152ng/ml, 96ng/ml, *$p<0.001$($)), and stable COPD patients (189, 508ng/ml, *$p<0.001$($)); with no significant difference between never-smokers and stable COPD patients ($p=0.234$) (Figure 3.5). Age appeared to affect salivary NE levels ($p<0.04$), with around 60ng/ml decline for every increasing decade in COPD patients, regardless of treatment.

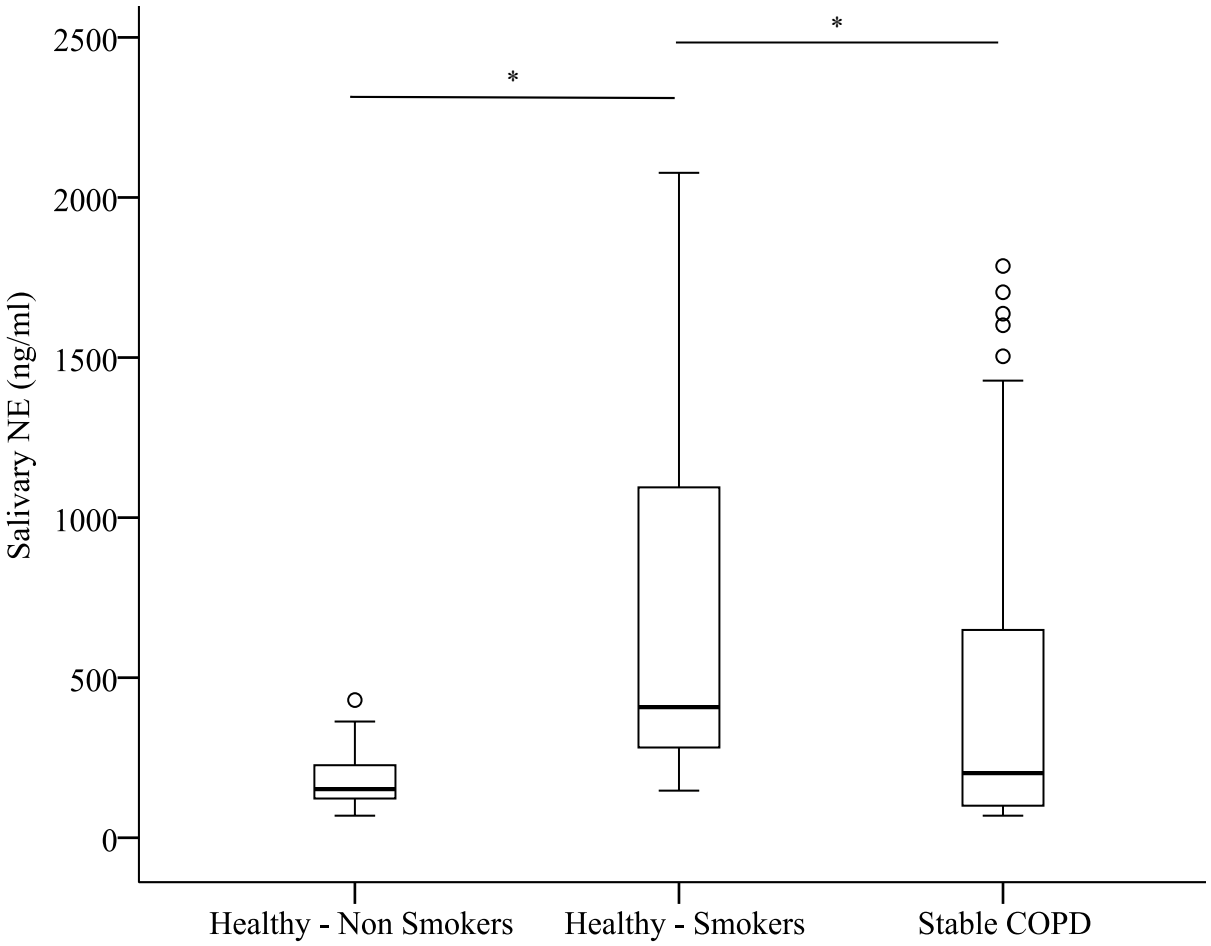


Figure 3.5: Salivary NE from healthy non-smokers, healthy smokers and stable COPD subjects. A box and whisker plot of salivary NE levels in healthy non-smokers and smokers alongside stable COPD patients (n = 107). The horizontal bar represents the median; the box length represents the interquartile range. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{o} (1.5 x the interquartile range). Differences in salivary NE levels were observed between cohorts (*p<0.001), irrespective of covariate adjustment (p<0.011). Healthy smokers had significantly raised NE compared to never-smokers (p<0.001($)), and stable COPD patients (189ng/ml, 508ng/ml, *p<0.001($)); with no significant difference between healthy non-smokers and stable COPD patients (p=0.234).

The CV for NE variability within subjects was 32%, 41% and 37% for never-smokers, smokers and stable COPD respectively. The Bland-Altman plot with upper and lower limits (1.96 SD) combing all 3 groups (n = 107) showed good consistency of data (difference between stable baseline values) with only 7 outliers (Figure 3.6).

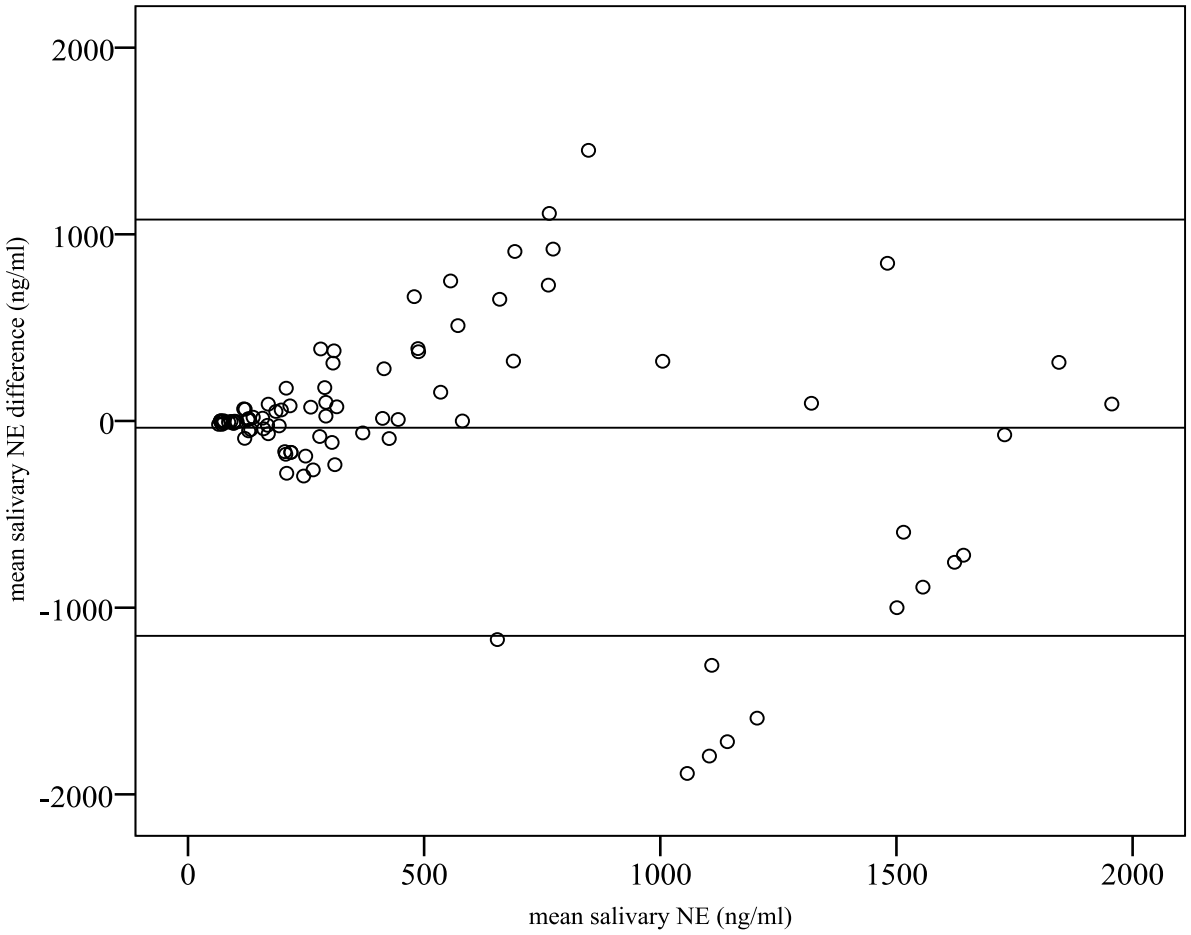


Figure 3.6: Baseline variability of salivary NE levels across healthy non-smokers, health smokers and stable COPD.

A Bland-Altman plot of baseline salivary NE variability in healthy non-smokers and smokers alongside stable COPD patients (n = 107). The upper and lower bars represent 1.96 standard deviations from the mean. All replicates bar 7 outliers fell within the 95% limits of agreement (difference between stable baseline values).

3.4.2. Stratification by disease severity in stable COPD patients

No significant difference was found across COPD severity defined by FEV_1 for: Age, BMI, CRP, PCT, NE, breathing score, sputum colour and sputum texture: ($p=0.378$; $p=0.402$; $p=0.558$; $p=0.945$; $p=0.619$; $p=0.126$; $p=0.823$; $p=0.227$) FEV_1 significantly decreased as COPD patients' severity increased ($p<0.001$), whilst MRC, ADL and Sputum Amount significantly increased: ($p<0.001$; $p<0.002$; $p<0.011$ respectively)

3.4.3. Subject-completed PRO scores

All study participants completed a paper-based Wellbeing and Self-Assessment diary (Chapter 2, Figure 2.29, Page 212) and MRC score. MRC scores significantly correlated with two components of the COPD Wellbeing score (Figure 2.29, Page 212) Breathing score ($r = 0.55$; 95% Confidence Interval (CI): 0.34 to 0.70) and ADL score ($r = 0.47$; 95% CI: 0.25 to 0.64; $p<0.001$ by Spearman's Rank Correlation Co-efficient).

3.4.3.1. PRO scores across health status

Inter-group analysis demonstrated differences in all components of the COPD Wellbeing score between stable COPD, never-smokers and smoker subjects ($p<0.001$) (Table 3.1). There was no significant difference however between never-smokers and smokers for any COPD Wellbeing score components.

3.4.3.2. Correlation of salivary biomarker levels to subject-completed PRO score.

To understand and identify whether a relationship exists between the components of the COPD Wellbeing Score, MRC and salivary biomarkers further sub-analysis was undertaken.

3.4.3.2.1. Whole study population analysis

Analysis between salivary biomarkers, COPD Wellbeing score components and MRC score across all participants (n = 143), (Table 3.2) revealed correlation of salivary CRP with ADL ($r = 0.23$, $p<0.02$); sputum amount ($r = 0.23$, $p<0.02$) and colour ($r = 0.24$, $p<0.02$). Salivary PCT did not significantly correlate with any of the PRO instrument metrics. Salivary NE only correlated with MRC score ($r = 0.29$, $p<0.01$).

Table 3.2: Correlations of whole study population (n = 143) symptom scores and sputum metrics to salivary biomarker levels.

		COPD Wellbeing Score				
Salivary Biomarkers	**MRC Score**	**Breathing Score**	**ADL Score**	**Sputum Amount**	**Sputum Colour**	**Sputum Texture**
CRP	r = 0.08, p=0.216	r = 0.05, p=0.448	r = 0.23, **p<0.02**	r = 0.23, **p<0.02**	r = 0.24, **p<0.02**	r = 0.08, p=0.219
PCT	r = 0.16, p=0.425	r = 0.05, p = 0.903	r = 0.02, p=0.778	r = 0.08, p=0.219	r = 0.16, p = 0.425	r = 0.07, p=0.278
NE	r = 0.29, **p<0.01**	r = 0.09, p=0.135	r = 0.004, p=0.952	r = 0.05, p=0.386	r = 0.02, p=0.692	r=0.12, p=0.06

3.4.3.2.2. COPD population sub-analysis

Separate sub-analysis on all COPD subjects (n = 98) for the COPD Wellbeing and MRC score (Table 3.3) (note the COPD Wellbeing score had been designed for use in COPD patients), (Chapter 2, Page 211) demonstrated salivary CRP correlated with MRC score (r = 0.16, p<0.01), breathing score (r = 0.14, p<0.02) (Figure 3.7A-B) sputum amount (r = 0.15, p<0.01), colour (r = 0.32, p<0.001) and texture (r = 0.13, p<0.03). Salivary PCT correlated with breathing score (r = 0.13, p<0.04) (Figure 3.7C), sputum amount (r = 0.13, p<0.03) and colour (r = 0.23, p<0.001). Salivary NE did not correlate with any clinical features. Sputum amount and colour correlated with breathing (r = 0.34, p<0.001) and ADL scores (r = 0.34, p<0.001); texture correlated only with ADL (r = 0.24, p<0.001).

Table 3.3: Correlations of all COPD patients (n = 98) symptom scores and sputum metrics to salivary biomarker levels.

		COPD Wellbeing Score				
Salivary Biomarkers	**MRC Score**	**Breathing Score**	**ADL Score**	**Sputum Amount**	**Sputum Colour**	**Sputum Texture**
CRP	r = 0.16, **p<0.006**	r = 0.14, **p<0.02**	r = 0.11, p=0.07	r = 0.15, **p<0.013**	r = 0.32, **p<0.001**	r = 0.13, **p<0.032**
PCT	r = 0.04, p=0.443	r = 0.13, **p<0.04**	r = 0.12, p=0.05	r = 0.13, **p<0.033**	r = 0.23, **p<0.001**	r = 0.11, p=0.077
NE	r = -0.07, p=0.221	r = 0.11, p=0.081	r = 0.03, p=0.646	r = 0.08, p=0.218	r = 0.07, p=0.265	r = -0.12, p=0.15

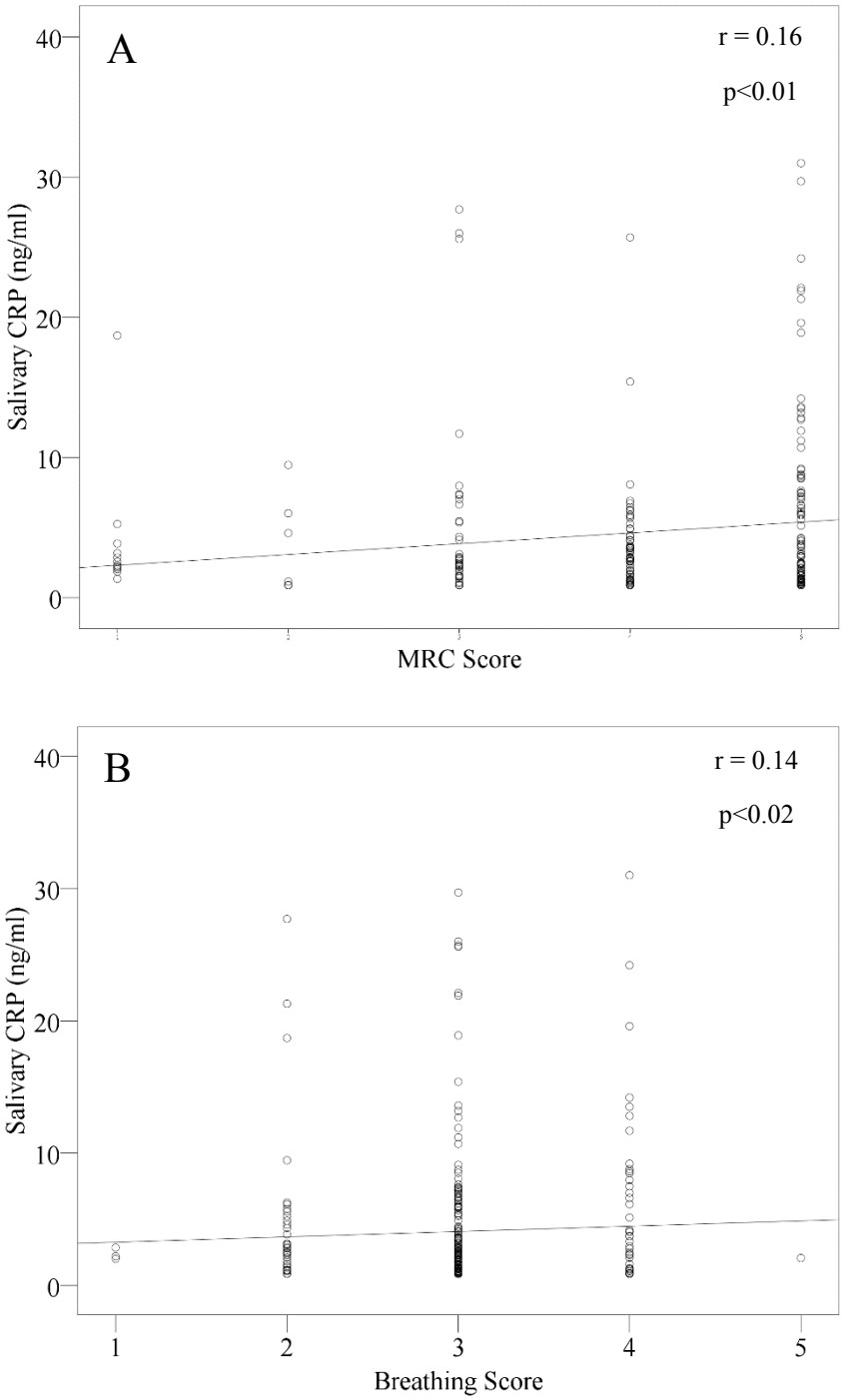


Figure 3.7A-B: Correlations of COPD patients' PRO and salivary biomarkers levels.

Scatter plot with line-of-best-fit between all COPD PRO and salivary biomarkers (n = 98). (A) Salivary CRP correlated to MRC score (r = 0.16, p<0.01); (B) Salivary CRP correlated to Breathing score (r = 0.14, p<0.02) and (C) Salivary PCT correlated to Breathing score (r = 0.13, p<0.04).

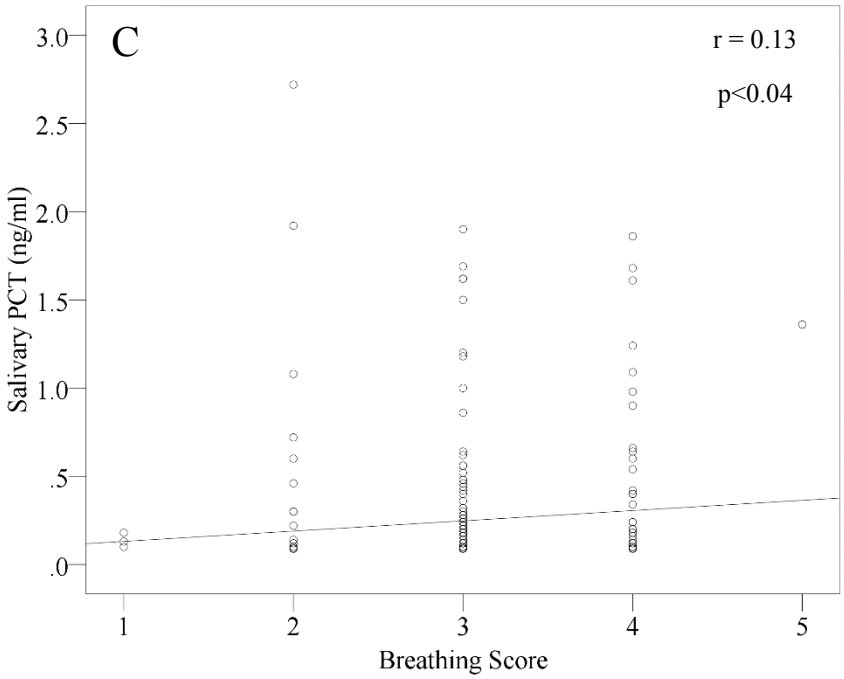


Figure 3.7C: Correlations of COPD patients' PRO and salivary biomarkers levels.

Scatter plot with line-of-best-fit between all COPD PRO and salivary biomarkers (n = 98). (C) Salivary PCT correlated to Breathing score ($r = 0.13$, $p<0.04$).

Overall the MRC score and all components of the COPD Wellbeing Score demonstrated relevant correlations with salivary biomarkers and intra-correlations as well as comparisons with FEV_1. Sputum texture however did not offer any additional information beyond other sputum metrics after assessment of the total number of individual significances for each sputum parameter (Table 3.2, Page 233; Table 3.3, Page 234; Table 3.4 Page 238) and thus further refinement of the COPD Wellbeing score would remove this component, (Chapter 4, Page 251).

3.4.4. COPD Patient exacerbation sub-population

Thirty-six COPD patients experienced an exacerbation at day 11 ± 3 during the study period (Table 3.4). This assessment was performed on either visit 2 or 3 during the study where the COPD patients would provide information on the onset of an exacerbation following the previous visit. There was no difference in the median baseline exacerbation frequency (1 to 3 episodes per year) between these patients and those COPD patients that remained stable throughout the study. During an exacerbation episode as would be expected there was a reduction in FEV_1 ($p<0.001$), breathing and ADL scores ($p<0.006$ and $p<0.0014$); alongside changes in sputum (amount: $p<0.001$, texture: $p<0.05$ and colour: $p<0.001$ respectively).

Table 3.4: Same COPD patients in stable and exacerbation phase (n = 36) demographics, salivary biomarker levels and symptom profiles.

Demographics	Stable	Exacerbation	p-value
Age, [a] years	68 ± 9	***	
Gender, Male (Female)	17 (19)	***	
$FEV_{1,}$ [a](% predicted)	53 ± 23	48 ± 19	**<0.001**
BMI, [a] (kg/m^2)	24.0 ± 6.3	***	
Co-Morbidities			
Nil	5	***	
Cardiovascular	30	***	
Type 2 Diabetes Mellitus	4	***	
Gum Disease	2	***	
Treatment			
Inhaled β_2 agonists Short, (Long) Acting	35, (32)	***	
Inhaled Anticholinergic Short, (Long) Acting	5, (25)	***	
Inhaled Steroid	31	***	
Oral Theophylline	6	***	
Symptom & Sputum Metrics, [b]			
MRC Score	5.00, 1.25	5.00, 1.25	=0.15
****Breathing Score**	3.00, 0.00	4.00, 1.00	**<0.006**
****ADL Score**	3.00, 1.00	4.00, 2.00	**<0.014**
****Increased Cough**	0	10	**<0.001**
****Sputum Amount**	2.00, 2.00	3.00, 2.25	**<0.001**
****Sputum Colour**	3.00, 1.00	4.00, 0.41	**<0.001**
****Sputum Texture**	1.94, 0.33	2.06, 0.41	**<0.05**
Salivary Biomarkers, [b]			
CRP, ng/ml	1.61, 1.10	7.35, 10.04	**<0.001**
PCT, ng/ml	0.09, 0.06	0.50, 0.71	**<0.001**
NE, ng/ml	128, 190	769, 1680	**<0.001**

Data are presented as: a = mean ± SD or b = median, IQR. P-values represent the difference between the two cohorts. *Hypertension included with cardiovascular disease, **COPD Wellbeing Score components. ***Variables remained unchanged during an exacerbation from stable values.

3.4.4.1. Salivary biomarker levels during a COPD exacerbation

Comparison of paired stable and pre-treatment exacerbation saliva samples from those COPD patients who experienced an exacerbation during the community-based cohort study, demonstrated significant elevation in all target salivary biomarkers with exacerbation onset ($p<0.001$), (Figure 3.8). Median salivary CRP concentration increased by 5.74ng/ml (95% CI: 3.72 to 11.47); median salivary PCT concentration increased by 0.38ng/ml, (95% CI: 0.31 to 0.54) and median salivary NE concentration increased by 539ng/ml (95% CI: 169 to 982) from baseline stable levels during an exacerbation of COPD.

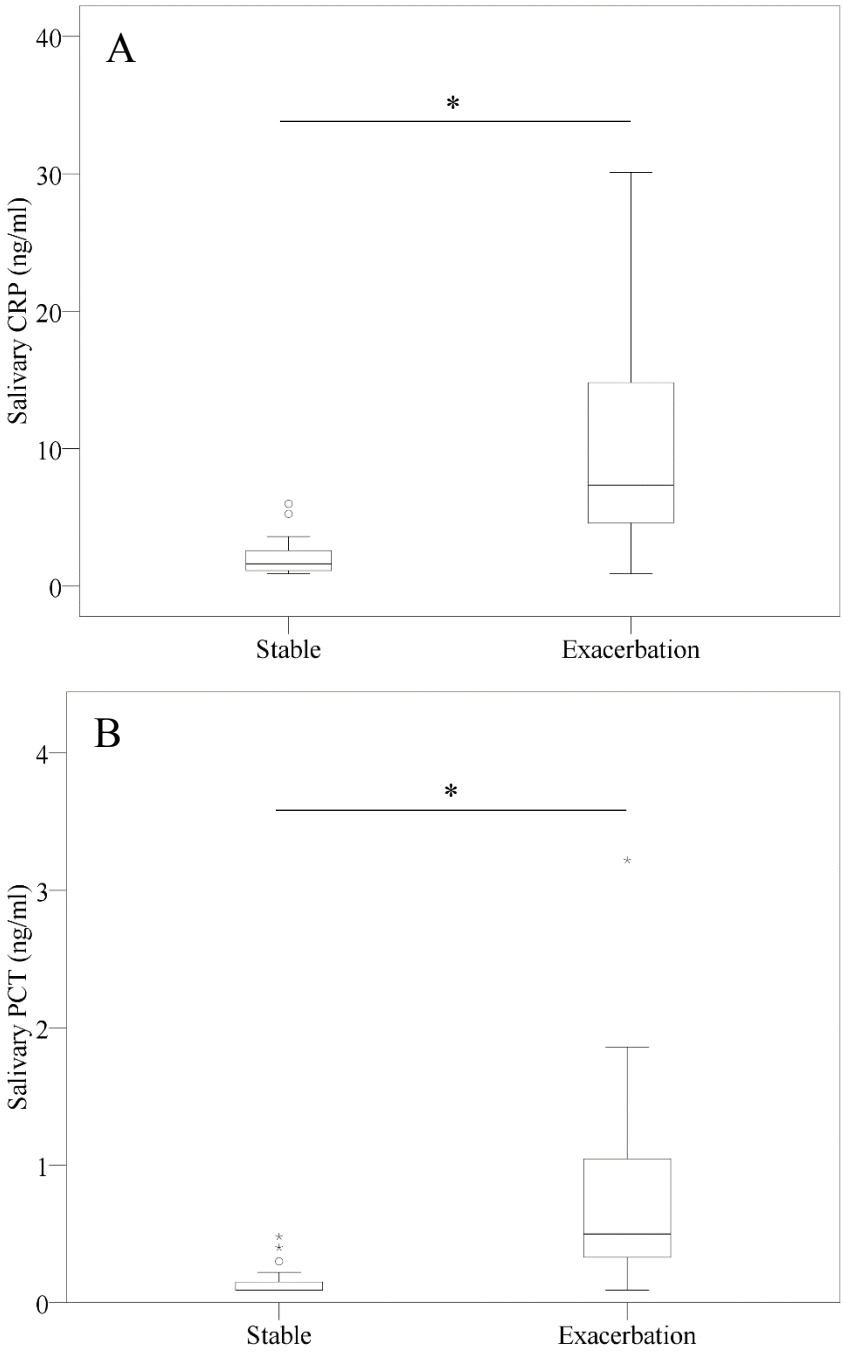


Figure 3.8A-B: Salivary biomarkers in COPD exacerbations.

Box and whisker plots of all 3 salivary biomarker levels in COPD patients in the stable phase and during an acute exacerbation of COPD (n = 36). The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{o} (1.5 x the interquartile range) and * (3 x the interquartile range). A = salivary CRP; B = salivary PCT. A significant elevation in all target salivary biomarkers with exacerbation onset is observed (*$p<0.001$).

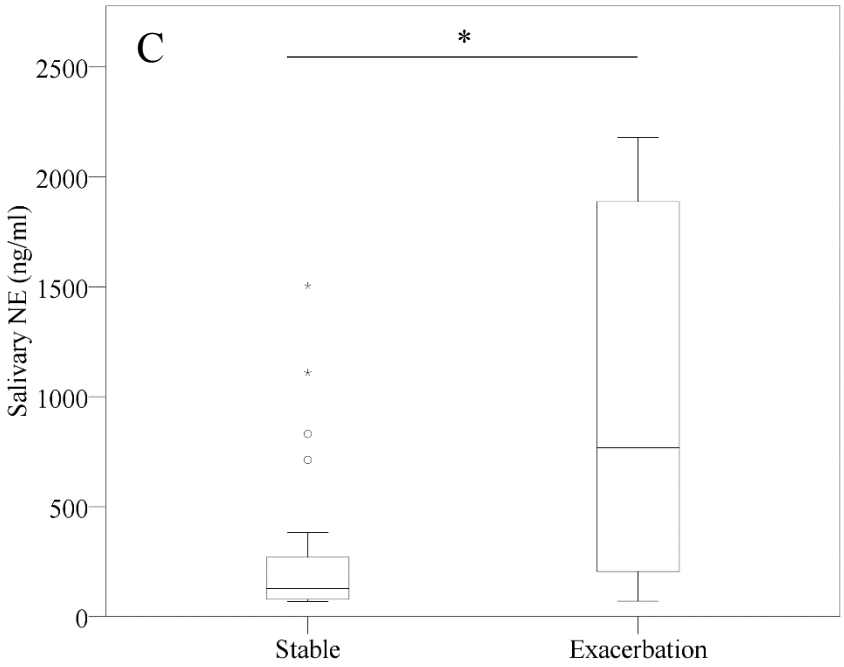


Figure 3.8C: Salivary biomarkers in COPD exacerbations.

Box and whisker plots of all 3 salivary biomarker levels in COPD patients in the stable phase and during an acute exacerbation of COPD (n = 36). The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ^{o} (1.5 x the interquartile range) and * (3 x the interquartile range). C = salivary NE levels in COPD patients during their stable and exacerbation states. A significant elevation in all target salivary biomarkers with exacerbation onset is observed (*$p<0.001$).

-

3.4.5. Comparison of subject-matched saliva and serum biomarker levels

Relationships between saliva and serum biomarkers were studied in 22 randomly-selected subjects, providing a total of 66 paired saliva–serum samples. Salivary CRP levels were approximately 200 times lower than serum; salivary PCT and NE levels were about two-fold higher. Salivary CRP and PCT correlated with serum equivalents, r = 0.82, (95% CI: 0.72 to 0.87), p<0.001 by Spearman's; and r = 0.53, (95% CI: 0.33 to 0.69), p<0.006 respectively (Figure 3.9A-B). Salivary and serum NE did not correlate (r = -0.24, p=0.24).

3.4.5.1. Intra-biomarker correlations

Biomarker cross-analysis demonstrated salivary PCT correlated with serum and salivary CRP, r = 0.53, (95% CI: 0.33 to 0.69), p<0.006; and r = 0.73, (95% CI: 0.59 to 0.83), p<0.001 respectively (Figure 3.9C-D). Salivary NE correlated with both salivary CRP, r = 0.45, (95% CI: 0.23 to 0.63), p<0.001, and salivary PCT, r = 0.58, (95% CI: 0.39 to 0.72), p<0.001 (Figure 3.9E-F).

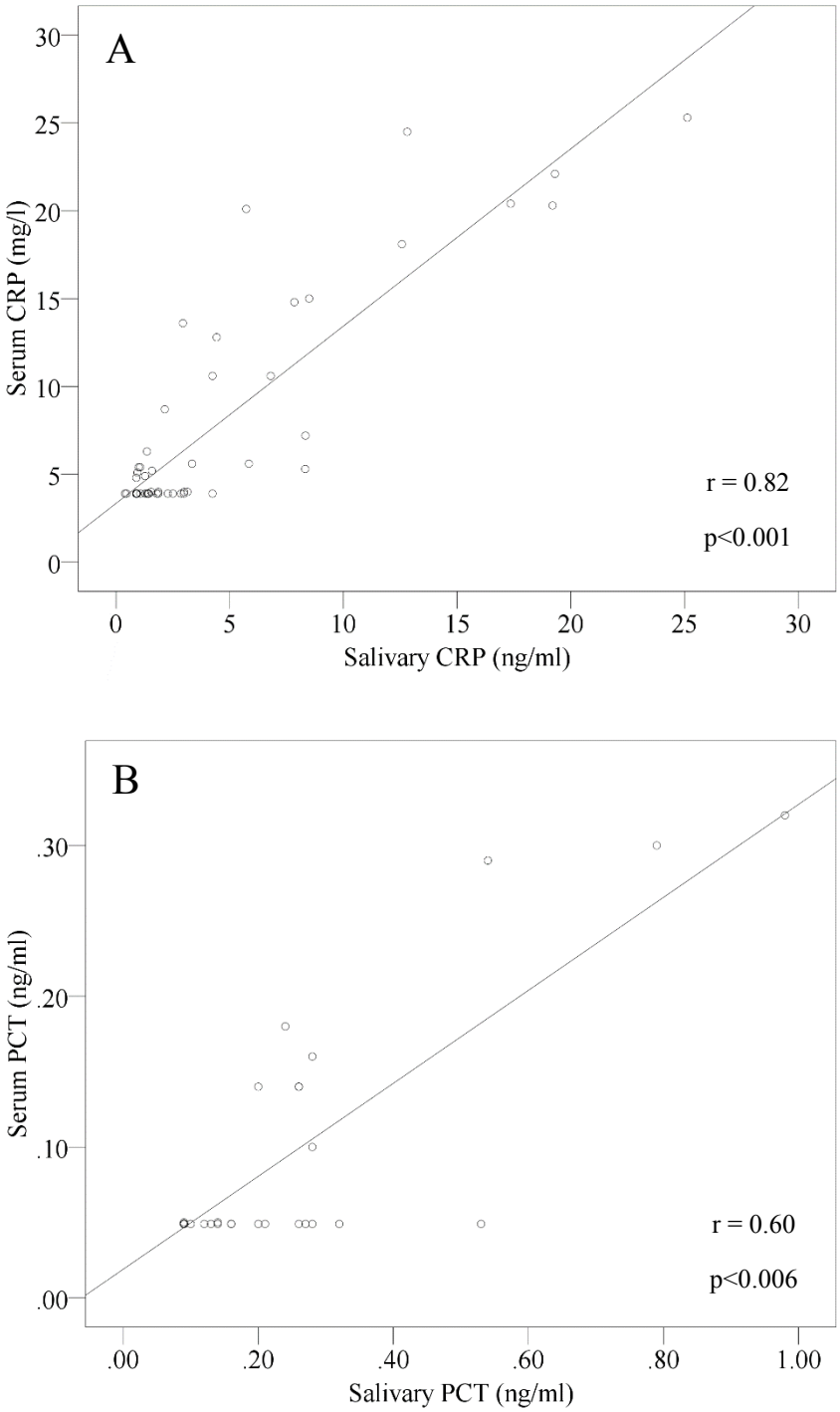


Figure 3.9A-B: Correlations between same subject serum and salivary biomarkers.

Scatter plots with a line-of-best-fit between salivary and serum biomarker levels in the same subject (n = 22). These study participants provided both serum and saliva samples on the same visit. A = Salivary CRP levels correlated to serum CRP ($r = 0.82$, $p<0.001$); B = Salivary PCT levels correlated to serum PCT ($r = 0.60$, $p<0.006$).

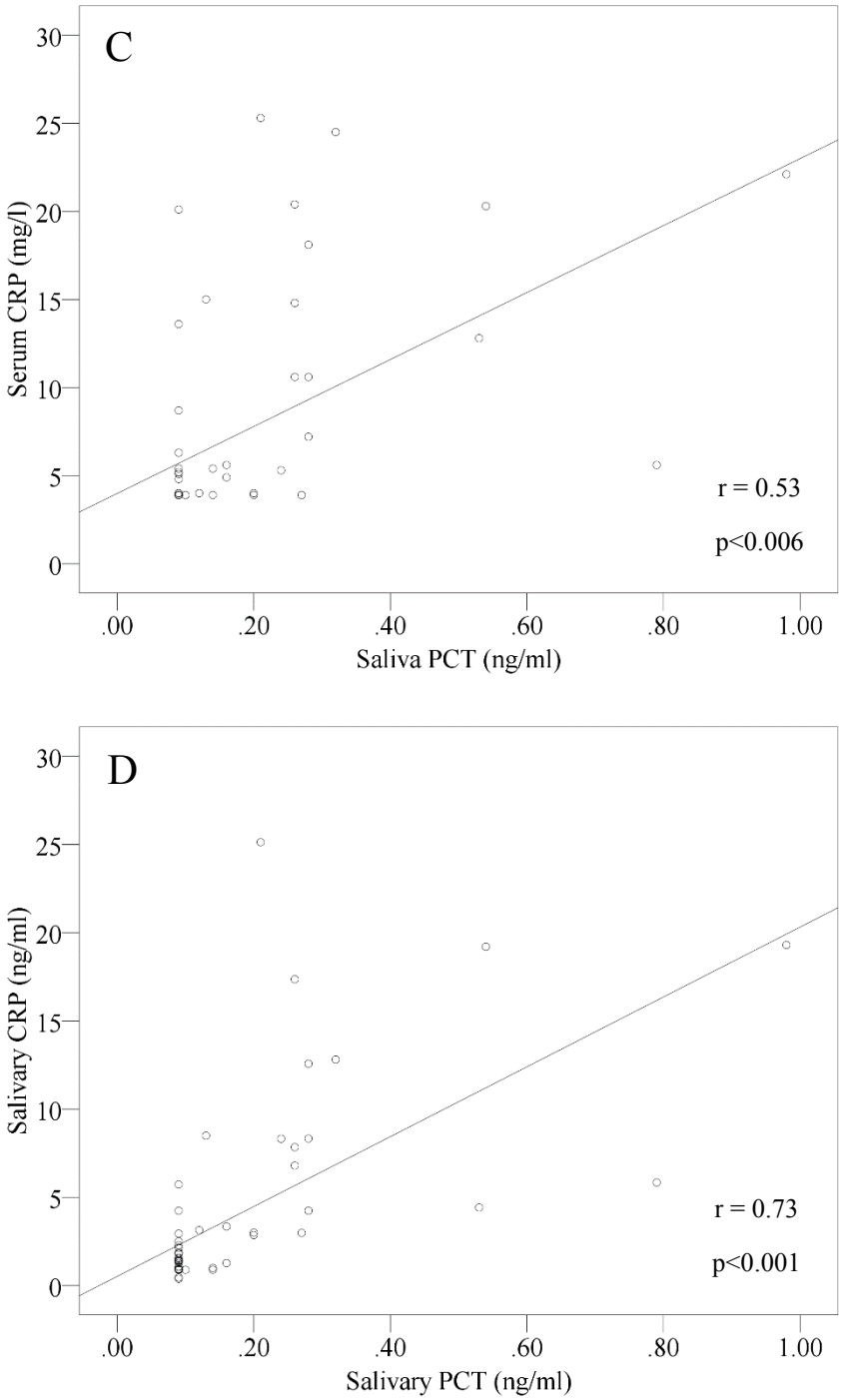


Figure 3.9C-D: Correlations between same subject serum and salivary biomarkers.

Scatter plots with a line-of-best-fit between salivary and serum biomarker levels in the same subject (n = 22). These study participants provided both serum and saliva samples on the same visit. C = Salivary PCT levels correlated to serum CRP (r = 0.53, p<0.006); D = Salivary PCT levels correlated to salivary CRP (r = 0.73, p<0.001);

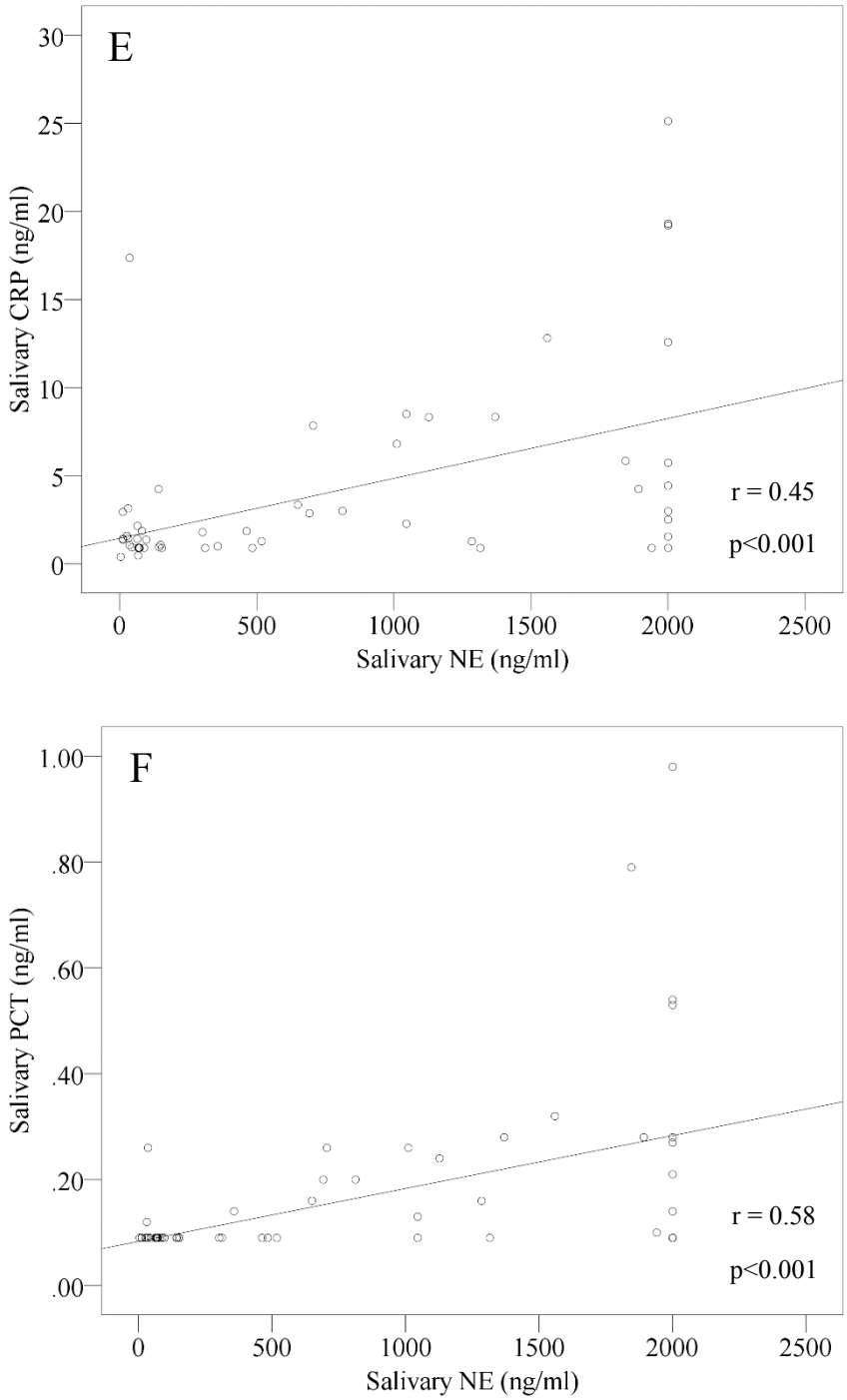


Figure 3.9E-F: Correlations between same subject serum and salivary biomarkers.

Scatter plots with a line-of-best-fit between salivary and serum biomarker levels in the same subject (n = 22). These study participants provided both serum and saliva samples on the same visit. E = Salivary NE levels correlated to salivary CRP ($r = 0.45$, $p<0.001$) and, F = Salivary NE levels correlated to salivary PCT ($r = 0.58$, $p<0.001$).

3.4. Discussion

As disease management shifts increasingly towards POC, there is urgency to develop easier, less stressful sampling methods especially for monitoring chronic conditions. Having established that levels of CRP, PCT and NE can be measured in saliva, this is the first study to explore their potential role of salivary CRP, PCT and NE in COPD.

Salivary biomarker targets in COPD patients of varying severity were compared to controls (never-smokers and smokers) under real world/working conditions; hence study participants with co-morbid conditions were included provided these were clinically stable at time of enrolment. To minimise across-cohort demographic variations and circadian influences, analysed measurements were then adjusted for potential covariate bias (Janes and Pepe, 2008), including sampling times (Izawa et al., 2013). Non-smoker salivary CRP levels at 0.89ng/ml (IQR: 0.35ng/ml) compared favourably to previous observations showing a healthy CRP range of 0.02 to 2.5ng/ml in saliva (Izawa et al., 2013, Topkas et al., 2012). Serum CRP has been shown to distinguish between COPD and controls (Gan et al., 2004), but not healthy smokers from non-smokers (Pinto-Plata et al., 2006). However, no difference in salivary CRP levels was demonstrated between my study cohorts following all-covariate adjustment, possibly because my controls had relatively high BMIs; indeed, significant differences emerged when adjustment excluded BMI. In support, strong correlations between serum CRP and BMI have been previously demonstrated (Choi et al., 2013). Whilst correlations between serum CRP levels and FEV_1 have been reported (Broekhuizen et al., 2006, de Torres et al., 2006), in this study there was no association between salivary CRP and FEV_1 in stable COPD; this possibly reflects the inhaled corticosteroid usage in the COPD patients (Pinto-Plata et al., 2006).

This study is the first to explore the presence of PCT in saliva. There was no difference in salivary PCT levels between stable COPD patients and healthy controls following covariate adjustment. This is not surprising as PCT is normally hardly detectable in blood (below 0.05ng/ml) unless there is presence of bacterial infections and sepsis or following trauma (Uzzan et al., 2006). In agreement with previous observations (Ozcaka et al., 2011, Weitz et al., 1987) salivary NE was found to be higher in smokers, but not in stable COPD patients (Higashimoto et al., 2008); possibly because all study COPD patients were ex-smokers.

The observed increases in salivary CRP, PCT and NE during COPD exacerbations reflect the well-documented elevated CRP and PCT levels in blood (Broekhuizen et al., 2006, Stolz et al., 2007a, Uzzan et al., 2006) and NE levels in sputum (Ilumets et al., 2008, Ozcaka et al., 2011), and have clinical implications. Whilst salivary CRP (or any of the other analytes) may not be sufficiently sensitive for evaluating COPD risk and outcome, it could serve as a potential surrogate for determining exacerbation onset. However, evidence for CRP or any biomarker in isolation to confirm an exacerbation is minimal. On the other hand, these results give support to future development of single-platform immunodiagnostics for near-patient measurement of salivary CRP alongside other readily available biomarkers, for example, PCT, to enable sufficient confidence for exacerbation prediction and stratified intervention.

Alongside such developments, there is still a need to improve understanding of the association between biomarker/physiological measurements and PRO in COPD (Paladini et al., 2010). This is particularly crucial as no one parameter appears to be sufficiently sensitive or specific in monitoring disease status or predicting exacerbation onset. This study reveals significant differences in self-assessed symptom scores and sputum metrics (COPD Wellbeing Score) in COPD patients, similar to studies using SGRQ and CAT (Nishimura et al., 2013). Furthermore,

significant correlations were observed between salivary levels of CRP and PCT and the breathing component of the COPD Wellbeing Score, with simultaneous changes occurring in both target analyte levels and breathing plus the ADL component of the COPD Wellbeing Score during exacerbations of COPD. As other PRO instruments have shown similar correlations (Tu et al., 2014), it is likely that particular COPD symptoms will be shown to be driven by underlying inflammatory events, with those very severe COPD exacerbations requiring hospitalisation possibly exhibiting different clinical and inflammatory profiles (Huerta et al., 2013).

Thus, biomarkers or symptoms in isolation will not be sensitive or specific enough to monitor longitudinal wellbeing in COPD, and combined bio-clinical profiling is essential, particularly if the long-term goal is to enable patient-led prediction of exacerbations and prompt intervention. Indeed, combining serum CRP with one increased major exacerbation symptom (dyspnoea, sputum volume or purulence) was found to be more sensitive than CRP alone in diagnosing exacerbations (Hurst et al., 2006). Of 36 biomarkers analysed, none were sensitive or specific enough to diagnose exacerbations without symptom assessment (Hurst et al., 2006).

Most serum components are present in saliva, although compositional differences show that saliva is not a passive ultra-filtrate of serum (Rehak et al., 2000). Biomarkers can enter saliva by cellular diffusion or active transport, ultra-filtration within salivary glands and/or via the gingival sulcus (Spielmann and Wong, 2011). The precise mechanisms explaining CRP, PCT and NE presence in saliva are unclear. Whilst blood contamination via micro-leakages, crevicular fluid overflow from micro-injuries or poor oral health is plausible, biomarker measurements in my study were not affected by adjustment for gum disease; samples also tested negative for blood.

Both salivary CRP and PCT levels correlated with serum counterparts. Saliva-serum CRP correlations have been previously established (Ouellet-Morin et al., 2011, Punyadeera et al., 2011). Although Ouellet-Morin et al observed a moderate to strong association between saliva and serum CRP, lower correlations were found at serum CRP below 2000 ng/ml compared to higher CRP (greater than or equal to 2000 ng/ml). However, Punyadeera et al., demonstrated saliva to serum CRP correlation at concentrations up to 5mg/L. Whilst these studies suggest that prediction of serum CRP from saliva CRP is more accurate at higher serum concentrations, strong correlations at both low and high CRP levels have been demonstrated. The only study on saliva to serum PCT relationship (Bassim et al., 2008) showed no significant correlation between the two fluids; however, saliva samples were stored at -27^{o}C rather than the recommended -80^{o}C (Slavkin, 1998).

No correlation was found between saliva and serum NE levels. Whilst one possible explanation could be localised NE production not manifesting systemically, this contradicts the observed moderate to strong correlation of salivary NE to both salivary CRP and PCT levels. An alternative explanation could be the rapid inactivation of NE *in-vivo* (Carter et al., 2013), leading to comparatively slower inactivation in saliva than serum.

In acknowledgement that there may be some study limitations which require consideration. Although subjects had three assessments over 14 days, longitudinal studies are required and essential in order to establish steady–state baselines for the target salivary analytes. These would offer precise correlations of biomarker changes to PRO, specifically in the important prodromal period leading to an exacerbation. Furthermore, as BMI-matched cohorts appear to influence salivary CRP between-group differences, BMI status may need consideration in future larger studies. Another possible shortfall is that this study did not specifically exclude

for potential microbial airway colonisation in the COPD group, although participants were excluded in the event of any infection or unstable illness in the preceding six weeks to enrolment. Acknowledging that the presence of lower airway bronchial colonisation can be associated with elevated serum CRP levels in stable COPD patients (Marin et al., 2012), and with increased exacerbation frequency (Patel et al., 2002). In mitigation, separate analysis for the COPD subjects that underwent an exacerbation and for those who remained stable throughout the study; thus minimising bias on target biomarker level results. Furthermore, there was no difference in median exacerbation frequency between the exacerbation group and the stable group, which may indirectly indicate that airway microbial colonisation was not significantly different between the two groups.

In conclusion this first study established that levels of CRP, PCT and NE can be reliably and reproducibly measured in saliva, providing useful clinical information as blood. All three target salivary biomarkers increased during COPD exacerbations, with CRP and PCT correlating with patient-derived metrics. These findings provide the conceptual basis for the further development of salivary biomarkers, alongside PROs, for practical POC monitoring of COPD and prediction of exacerbations. This concept will be further explored in a longitudinal cohort study (Chapter 4, Page 251).

Chapter 4:

Exploration of Longitudinal Monitoring of Salivary C- Reactive Protein, Procalcitonin and Neutrophil Elastase Levels Alongside Patient Reported Outcomes in COPD

4.1. Introduction

The study described in Chapter 3, Page 213 has demonstrated that CRP, PCT and NE can be reliably detected in the saliva of healthy non-smokers, smokers and COPD patients with measurable differences between the three groups (Chapter 3, Page 223). In, addition all three biomarkers demonstrate a significant rise during an acute exacerbation of COPD. A novel PRO (COPD Wellbeing Score) (Chapter 2, Page 210) could also be used in a purposeful paper-based diary (Wellbeing and Self-Assessment diary) (Chapter 2, Figure 2.29, Page 212) with correlations between components of this score, the levels of three salivary biomarkers and conventional MRC score. Additionally, clinically-relevant changes in the COPD Wellbeing score were also observed during exacerbations (Chapter 3, Table 3.4, Page 238).

COPD is a complex disease the severity and progression of which is primarily graded by FEV_1 (% predicted). On the other hand, COPD patients do not uniformly exhibit a monotonous decline in FEV_1 over time (Casanova et al., 2014). Furthermore, FEV_1 only weakly correlates with PROs (Casanova et al., 2011). This heterogeneity and lack of a complete comprehensive COPD assessment for health status monitoring and progression alongside difficulties in early diagnosis support the need for well-designed longitudinal studies that assess the "biological march" from unaffected but at risk individuals to those with very severe end-stage COPD (Bourbeau et al., 2014).

4.1.1. COPD health status monitoring

4.1.1.1. Spirometry

As described above FEV_1 decline in COPD patients is variable. Sub-populations within the general COPD population have been identified, for example frequent exacerbators (defined as more than 2 acute exacerbations per annum) are known to have an accelerated decline in FEV_1

compared to non-frequent exacerbators (Wedzicha et al., 2013). Increased rates of decline in FEV_1 have also been observed in COPD patients with the following characteristics: current smokers, bronchodilator reversibility and emphysema over a 3-year monitoring period (Vestbo et al., 2011).

4.1.1.2. Patient reported outcomes (PRO)

Current longitudinal studies of PROs in COPD patients have focused on PRO assessments over time points several months or years apart. A reduction in FEV_1, 6-minute walk distance and SGRQ between two time points 4 years apart has been reported; interestingly the study also demonstrated a significant reduction in serum CRP (Fu et al., 2014). A decline in exercise capacity at 6 monthly intervals over 5 years has also been observed (Oga et al., 2005). A separate study with the same protocol (6 monthly assessments over 5 years) investigating changes in PROs such as health status, dyspnoea and psychological status also identified a significant deterioration in these outcomes. (Oga et al., 2007). Oga et al., found that the deterioration in PROs correlated weakly to changes in physiological metrics. A recent study assessed changes in CAT Score at baseline and after 1 year in a stable COPD cohort and demonstrated a corollary relationship with MRC score (de Torres et al., 2014).

Daily longitudinal symptom monitoring has been used to identify COPD exacerbations, but there is lack of information on whether such data can provide sufficient advance warning for exacerbation prediction (Johnston et al., 2013). One study utilising a paper-based daily symptom diary demonstrated 2 distinct groups: (1) “gradual” with a change in symptoms 4 days prior to an exacerbation and (2) “sudden” with a change in symptoms on the day of an exacerbation (Aaron et al., 2012).

Issues relating to compliance and faked-entries for paper-based diaries persist. A recent study that utilised paper-based daily diary scores of clinically relevant COPD symptom metrics demonstrated a "sharp" increase in scores 2 days prior to an exacerbation of COPD (Walters et al., 2012). However, Walters et al 2012., reported a total daily diary compliance of only 53%. It can be argued that the validity of this study's observations; specifically, the challenges of validity of unobserved paper diary records as well as, the results and conclusions from paper-based diary studies are well documented (Stone et al., 2002, Kudielka et al., 2003).

Digitalised (electronic-based) daily diaries could help overcome the aforementioned limitations. An electronic-based diary appears to offer obvious advantages over a paper-based platform for example: remote data access and supervision; however, a number of key issues need to be considered:

1. Does electronic information accurately represent information collected by paper?
2. Do participants prefer electronic data capture?
3. Is individual compliance improved?
4. Can the habit of retrospective data entries be reduced?

Data collected electronically has been shown to be valid and of comparable quality to data obtained via paper survey, for the same eventual clinical outcomes. Importantly, patients also appear to prefer electronic data entry over paper (Tiplady et al., 2010, Ring et al., 2008, Bliven et al., 2001, Ryan et al., 2002, Boyer et al., 2002, Jamison et al., 2002).

Several studies have demonstrated reduced compliance with paper diaries compared to electronic entries; with one study showing only 11% compliance (Stone et al., 2003).

Interestingly, paper diaries showed a high rate of "faked" compliance, suggesting retrospective data entry (Meltzer et al., 2008). This implies that electronic diaries with compliance-enhancing features are a more effective and reliable method for the collection of daily diary data than a paper-based diary (Lauritsen et al., 2004). Electronic diaries thus appear to encourage patients to comply with pre-set protocols and prevent retrospective data entry. Electronic diaries that have the ability for participants to provide a "usability evaluation" allow the researchers to pick up on areas that are sub optimal. Additionally, participants are willing to generate ideas on how to improve the diary interface (Stinson et al., 2006). Electronic diaries can also be more easily verified, analysed and summarised than a paper form as data does not need to be manually inputted into a database (Walker et al., 2004).

In conclusion from the literature review an electronic diary would provide a clear advantage over a paper-based diary for data validity in a longitudinal study. For such an electronic diary to be engaging to patients, its design would need to provide a clear, simple and intuitive interface.

4.1.1.3. Biomarkers

It is recognised that serum biomarkers cannot be reliably ascertained on a single measurement and multiple measurements over time are required to give a more reliable and precise estimate of their levels in stable COPD (Aaron et al., 2010). The ECLIPSE study monitored a panel of 6 serum biomarkers: white blood cell count, CRP, IL-6, IL-8, fibrinogen and TNF-α and identified distinct sub-populations observed at baseline and at one year follow up. A cohort of COPD patients (30%) did not show evidence of systemic inflammation whilst 16% had evidence of persistent systemic inflammation with the latter group having a higher co-morbidity and annual exacerbation frequency (Agustí et al., 2012). Presently there are no

studies that have monitored biomarkers or panels of biomarkers in COPD patients at a greater frequency than annually.

4.1.2. Multidimensional monitoring of health status

As discussed in Chapter 1, Page 27, multidimensional scores appear to provide a more comprehensive assessment of long term mortality. A study monitoring BODE scores over 2 years concluded that BODE was superior to FEV_1 in monitoring progression of COPD; however, this study did not attempt to combine the two parameters (Casanova et al., 2014).

Presently there is an incomplete understanding of multidimensional sub-populations in COPD and their relationship to monitoring health status in COPD. The combination of some systemic biomarkers perform better than single biomarkers in identifying the causal aetiology of an acute exacerbation of COPD (Shaw et al., 2014).

The combination of both biomarkers and PROs appears to be a valid strategy as biomarkers in isolation are not sensitive enough for diagnostic precision, without symptoms to determine an exacerbation of COPD (Hurst et al., 2006). Recent work has used baseline study-enrolment characteristics to create sub-population clusters, which were then followed-up longitudinally for 3 years. These clusters had variable baseline demographics and differed regarding outcomes (Rennard et al., 2015). This is one of the first instances where a combination of baseline variables has been used to define sub-population clusters, with the particular cluster being monitored longitudinally over a protracted time period. There is however the potential of missing a possible temporal component of any of these variables, by just taking a snap-shot of data at study enrolment. Overall a well-designed longitudinal study incorporating a holistic dynamic of, for example, spirometric indices, PROs and biomarker levels would allow better

characterisation of the variable sub-population phenotypes known to exist within the whole COPD population. Such an approach enables corollary analysis to determine how all 3 chosen metrics would work best together towards design of a sensitive tool for monitoring an individual's COPD journey.

This chapter describes a community-based longitudinal study for COPD patients designed to understand whether frequent saliva based testing of the three target biomarkers (CRP, PCT and NE) alongside spirometry and the COPD Wellbeing Score could be used to monitor health status in COPD. The objectives for this study were:

1. To further determine the precision of the saliva based and modified immunoassays
2. To understand whether the COPD Wellbeing and Self-Assessment score can be used daily in COPD patients.
3. To explore whether dynamic pattern changes between these 3 chosen metrics could provide an early warning (prediction) of deterioration in health status, for example, during the "prodromal phase" of an exacerbation.
4. To determine the baseline variability in these 3 metrics during stable phases of COPD, and whether a corollary relationship between them could be established.

4.2. Materials & Methods

4.2.1. Development of an electronic Wellbeing & Self-Assessment diary

Acknowledging the potential benefits of electronic diary data capture discussed above (Section 4.1.1.2, Page 253) and the work undertaken in Chapter 3, Page 213 (demonstrating that the COPD Wellbeing Score provided potentially meaningful results), an electronic-based Wellbeing and Self-Assessment diary was developed.

The results from Chapter 3, Page 221 generated ideas for refinement of the paper-based Wellbeing and Self-Assessment diary. The individual components of the COPD Wellbeing Score (Breathing, ADL, cough, sputum colour and volume) remained; sputum texture was removed (Chapter 3, Page 237). In response to informal patient feedback in the community-based cohort study (Chapter 3, Page 221), a free-text section was incorporated for COPD patients to document their thoughts on how they were feeling and to send messages to the research team. The electronic platform was extended to include a separate section for the clinical research team to manage their patients and to record: (1) medical and medication details; (2) physiological and biological metric results; and (3) a free-text section for clinical observations, serving as healthcare reported outcomes (HRO). This latter section would permit future cross-reference between the PROs and HROs.

4.2.1.1. Design process

The initial phase in the construction of the electronic Wellbeing and Self-Assessment diary was the design of a story board with key features for the diary and a working logic algorithm for how each diary "page" self-interacts. This story-board acted as a framework for the software developers (Serious Games International Ltd, Coventry, UK) in the design process. The electronic Wellbeing and Self-Assessment Diary App also required a secure support-website

to "host" the diary, thus acting as a data repository. The website allowed two-way data transfer and crucially remote data observation for entries submitted by patients via the electronic Wellbeing and Self-Assessment diary. Both the App and website were programmed by Serious Games International.

4.2.1.2. Story-board

4.2.1.2.1. Electronic platform

The first step was to choose the software platform for the electronic diary and the hardware platform to host the software. The iOS (Apple, USA) operating system was chosen for the electronic Wellbeing and Self-Assessment diary development and an iPad (Apple, USA) as the platform to install the electronic diary. The advantages of using a tablet over a smart phone included: a larger display with better material presentation and easier handling for the target patient group. Once installed, the iPad (Apple, USA) required mobile internet capability for data transmission to the host website. In the event of poor connectivity, the App functionality allowed storage of daily diary data entry until sufficient connectivity was available to transmit the data.

4.2.1.2.2. Electronic Wellbeing and Self-Assessment diary components

4.2.1.2.2.1. Home screen

The home screen allowed log-in with a unique user name and password (Figure 4.1A).

4.2.1.2.2.2. Logged-in screen

This screen acted as a welcome page for both COPD patients and the clinical research team. This page had two "buttons" for separate access into (1) the Wellbeing and Self-Assessment

Diary for patients; and (2) the HRO section for the clinical research team which had its own password-protection (Figure 4.1B).

4.2.1.2.2.3. COPD Wellbeing and Self-Assessment screen

The paper-based COPD Wellbeing and Self-Assessment diary (Chapter 2, Figure 2.29, Page 212) developed in Chapter 2, Page 210 served as the template for this section. COPD patients could select an appropriate response by touching a particular bullet point next to the set of questions and assigned an appropriate score (Figure 4.2A-B). Once all entries were completed, the assessment was sent by pressing a "submit" button at the bottom of the page. This page also contained the "free-text" section where COPD patients could record any other information or queries. Once submitted, this section was locked out until the next day to prevent changes in entries and/or multiple entries. In the event of missing entries, a message (excluding a blank "free-text" section) would appear when the submission button was pressed informing the patient that some questions still required a response. If the electronic diary was not submitted by 12:00, an alert would be generated to remind individuals. This alert also set off a tone and appeared in the notification centre of the iOS (Apple, USA).

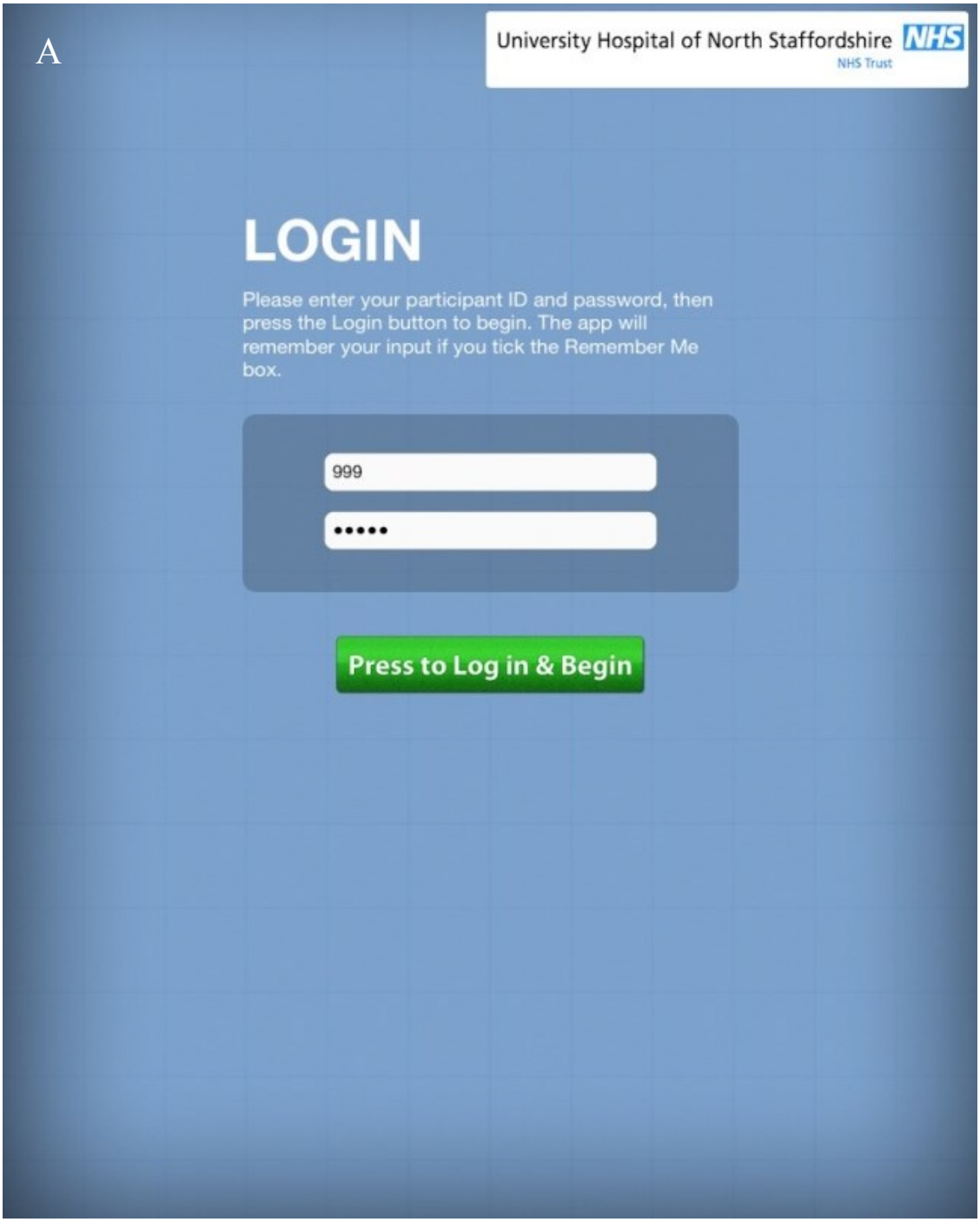


Figure 4.1A: Electronic Wellbeing and Self-Assessment diary log-in screen shots.

These screen shots were captured from the electronic Wellbeing and Self-Assessment diary. A: Home screen where COPD patients or the research team press the green button to log-in the diary which leads to a welcome screen (B).

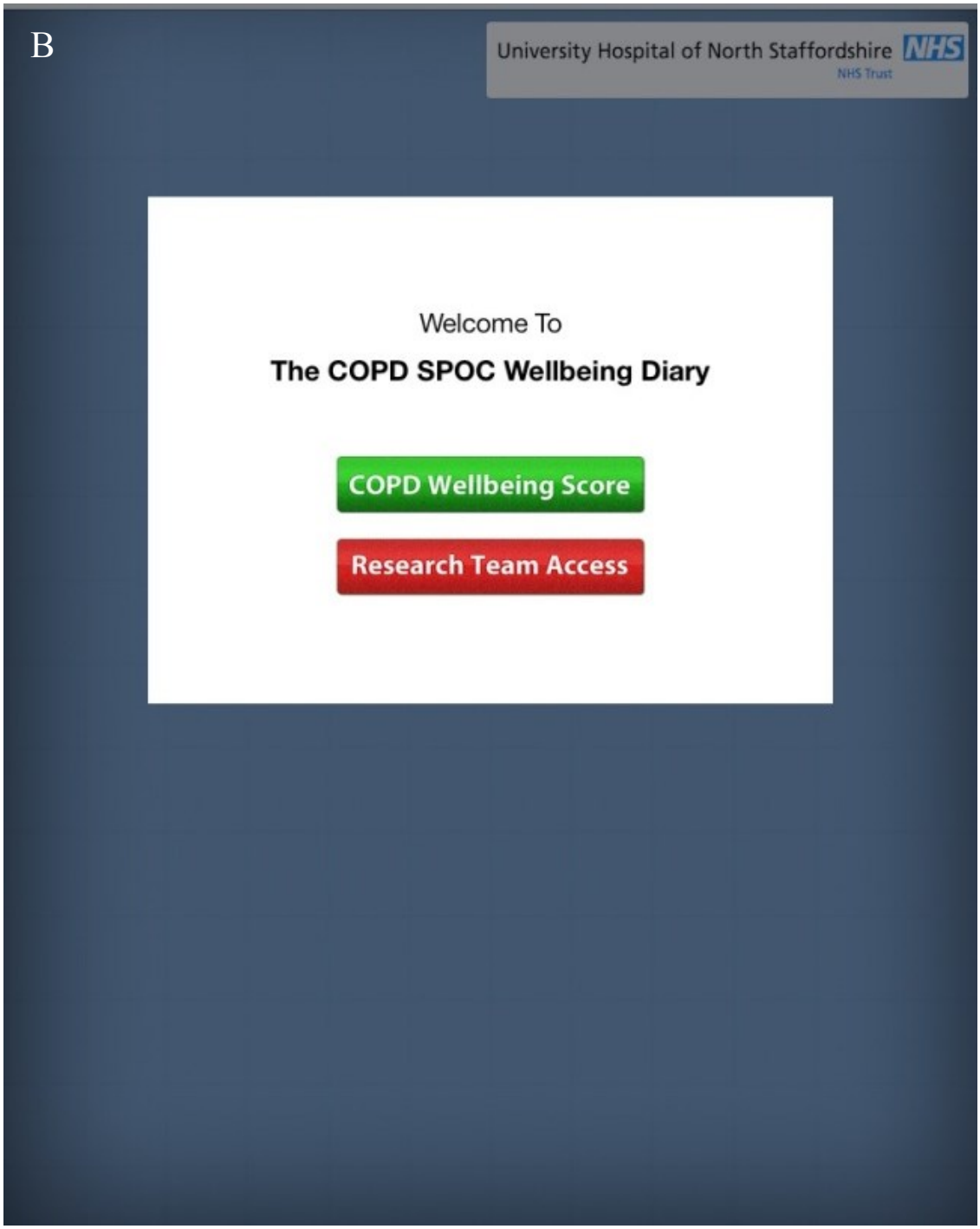


Figure 4.1B: Electronic Wellbeing and Self-Assessment diary log-in screen shots.

B: with a welcome message and two buttons. For the COPD patients to complete the diary they would select the green button "COPD Wellbeing Score". The clinical research team press the red "Research Team Access" button and enter a password-protected "page" where they compete the HRO.

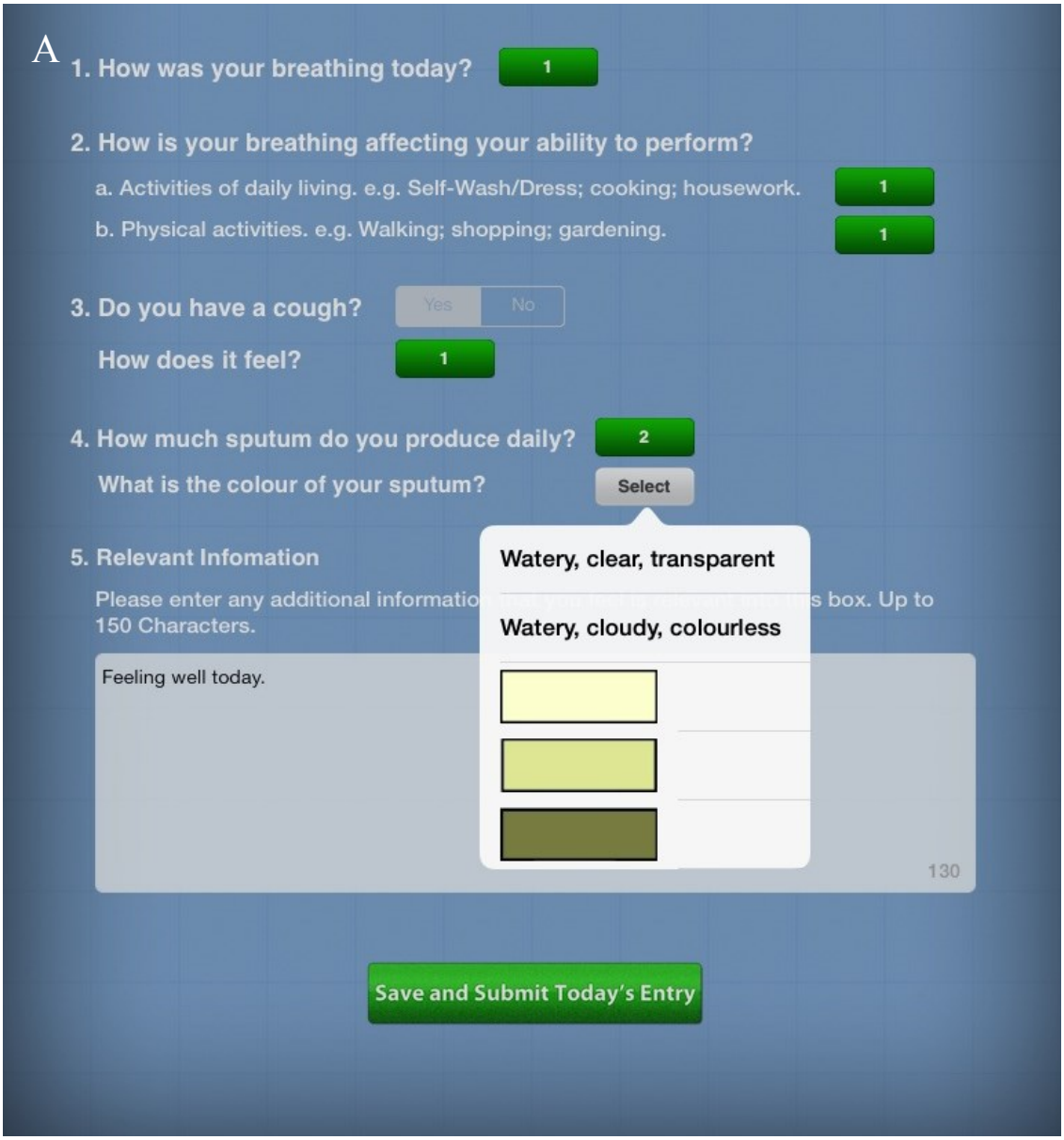


Figure 4.2A: COPD Wellbeing Scores in the COPD Wellbeing and Self-Assessment Diary.

These "screen-shots" show the COPD Wellbeing Score section which COPD patients are asked to complete daily. Each question is answered by pressing the grey "Select" button. This will then turn green and an appropriate score is assigned to the response. There is a free text section (white rectangular box) and the green button at the bottom ("Save and Submit Today's Entry") allows for the completed diary entry to be submitted.

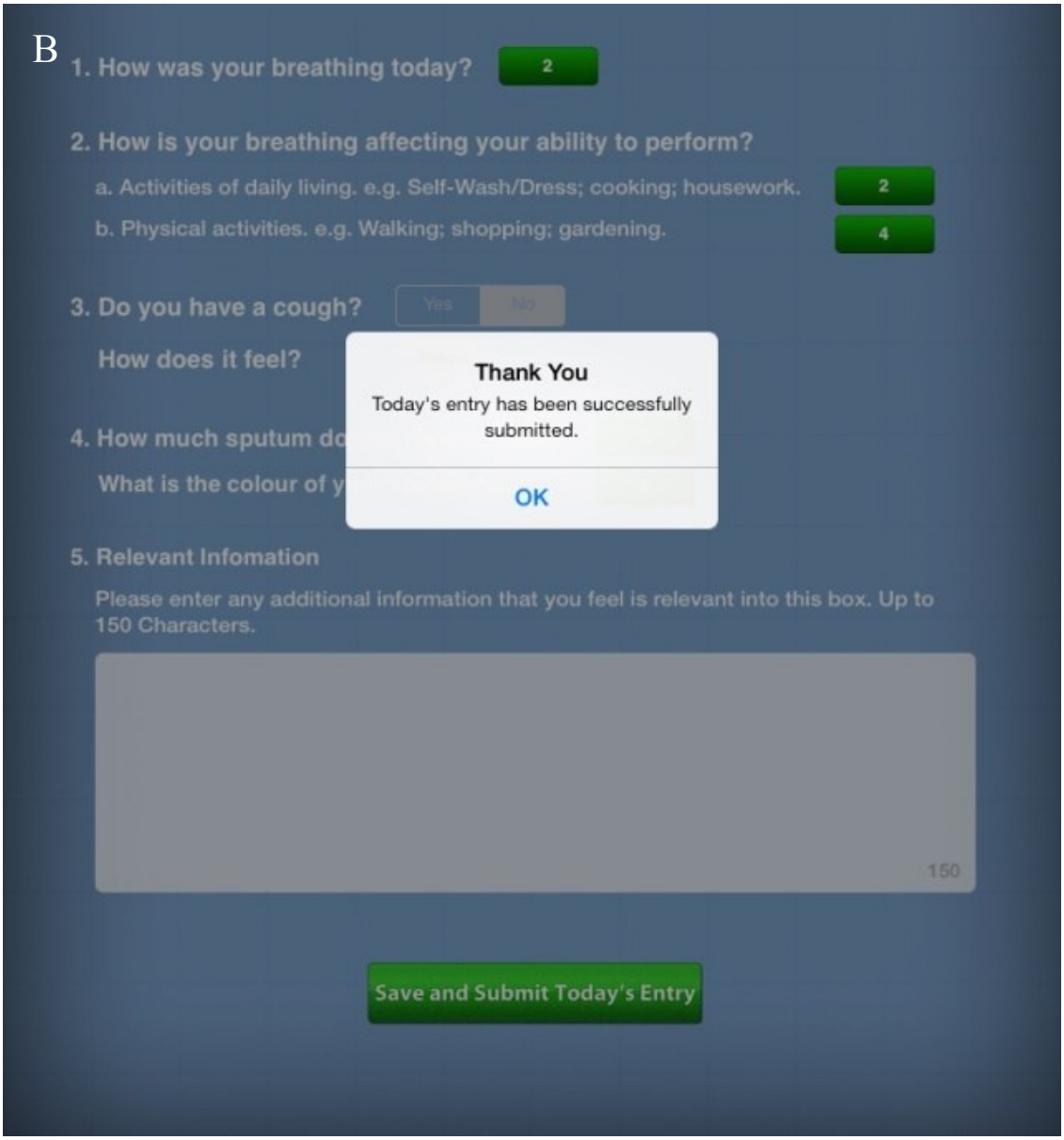


Figure 4.2B: COPD Wellbeing Scores in the COPD Wellbeing and Self-Assessment Diary.

This figure (B) shows the "Thank You" message after a diary entry has been successfully completed and submitted. This information is then transmitted to the host website once the green "Save and Submit Today's Entry" button is pressed.

4.2.1.2.2.4. Research team section screen.

The HRO (research team section) was only accessible by members of the research team and allowed demographic, clinical and physiological data entry (Figure 4.3). This will be password protected and only accessible by the research team. If during monitoring, patients were undergoing an exacerbation, the research team could press on an exacerbation button at the top of the page. This then allowed all recorded COPD Wellbeing Scores during the event to be flagged as red on the host website (thereby differentiating them from scores recorded during the stable phase) (Figure 4.4). On patient recovery, the research team would then deselect the option.

Pressing submit will complete the entry; however the research team can go back and add additional entries as this component of the electronic diary will not "lock-out" post entry submission. This section will also contain the log-out button so that COPD patients cannot "accidently" exit the electronic Wellbeing and Self-Assessment diary. The previous submitted entry also appears to ensure continuity with COPD patient details submitted at the previous visit. This is particularly important for the free-text section which will have a clinical log of the patient's condition.

1. COPD History: Duration (Years)
2. Smoking Habit: Never Smoker, Current Smoker, Ex-Smoker
3. Shortness of breath at rest: Y/N; on exertion: Y/N
4. MRC Score: 1 to 5
5. BMI: variable
6. Other Respiratory Illness Y/N
    a. TB, Bronchiectasis.
7. Other known medical conditions
    a. Heart failure, MI, Angina, Diabetes, Hypertension
8. Respiratory medications.
9. Number of hospital admissions for COPD in the past 12 months.
10. How many acute exacerbations in the last 12 months?
11. Free text section for further clinical information and the assessment of gum disease.

Figure 4.3: Research team structured clinical log.

The research team's section incorporates a structured log with the following questions (1 to 10) all of which will need to be populated before an entry can be submitted plus a free text section (11). This section will automatically populate with the previous entry that was submitted. Y = yes; N = no; TB = tuberculosis; MI = myocardial infarction. Essentially the log serves as a portable electronic medical history of the patient.

A

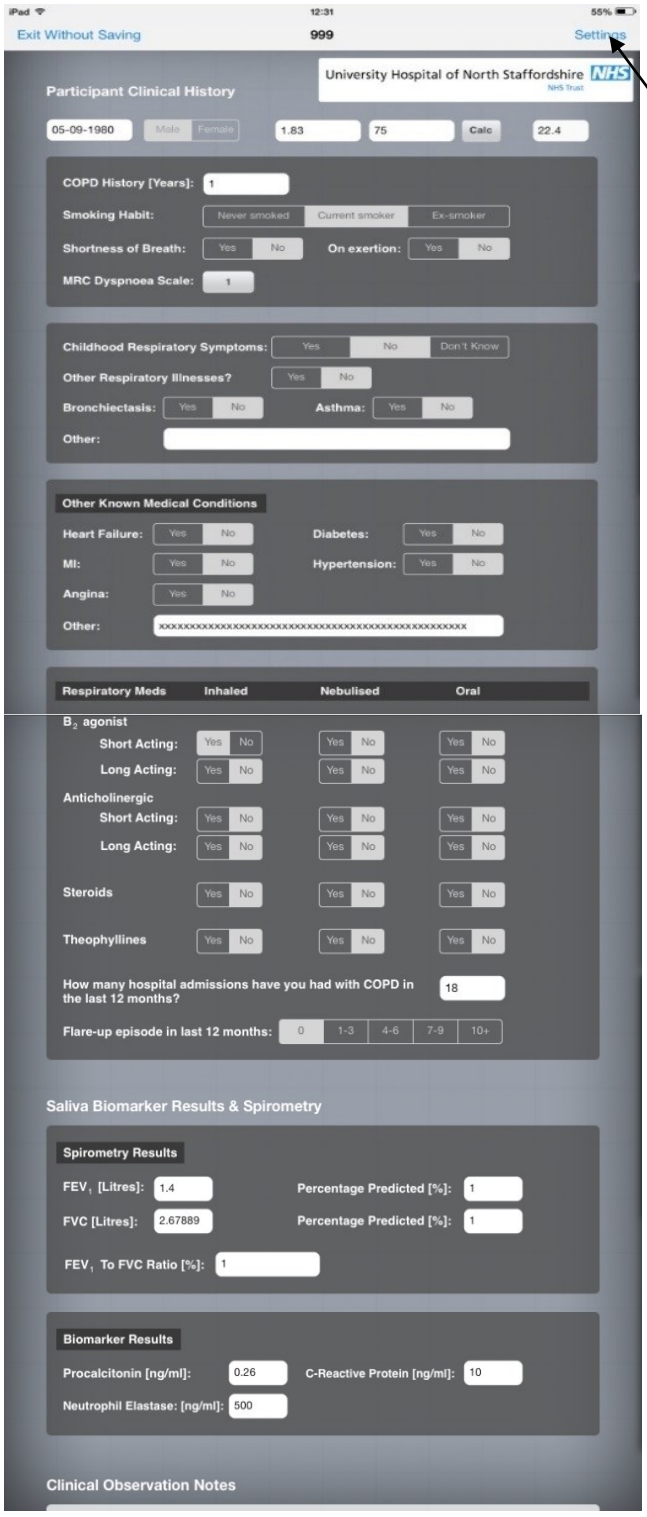


Figure 4.4A: The Research team section screenshots.

Screenshots of the clinical research team section which allows for a comprehensive clinical history to be documented. Once a response has been selected the dark grey button turns light grey. The exacerbation menu can be accessed by pressing setting (S) on the top bar.

B

Figure 4.4B: The Research team section screenshots.

This figure highlights the exacerbation menu where pressing the red exacerbation button (B) will flag all entries of the COPD Wellbeing Score as an exacerbation on the host-website. This figure also highlights the log-out button (C) and the patient reminder button (D).

4.2.1.2.3. Host website components

In parallel to the development of the Wellbeing and Self-Assessment Diary App, a dedicated password-protected website was constructed to host, monitor and store submitted data. The website had its own domain entry; functionality also allowed registration of unique COPD patient profiles and graphical display of patients' metrics over time to help identify clinical deterioration.

4.2.1.3. Development and publication

Over a period of 4 months the electronic Wellbeing and Self-Assessment diary and its website were constructed by the software developers, tested by myself and the developers and refined, ready for use in the community-based longitudinal study described later in this chapter. Prototype electronic diaries were "published" to allow for critique and refinement; with Version 1.0 of the electronic Wellbeing and Self-Assessment Diary appearing on the Apple App Store (Apple, USA) 07th January 2013 for downloading onto an iPad at no financial cost. The name given to the electronic Wellbeing and Self-Assessment diary "App" was (COPD-SPOC); this term was used following the recommendation of 23 characters for App names by the Apple store (Apple, USA). The host-website was also published on the worldwide web on the 07th January 2013. URL: http://nstaffs.pipeten.co.uk/index.php/nscontrol677/mlogin

4.2.2. Longitudinal study design

From November 2012 to July 2014 individuals with COPD, confirmed by spirometry according to GOLD criteria (GOLD, 2016), were recruited consecutively from the Directorate of Respiratory Medicine's research and outpatient clinic database (Chapter 2, Page 88). These COPD patients satisfied criteria of frequent exacerbators (Wedzicha et al., 2013) (defined as greater than or equal to two exacerbations per annum) who self-managed their condition and had a course of "rescue" medications (antibiotics and steroids) at home which they normally used to initiate treatment if they felt they were about to have an acute exacerbation. Patients had to be clinically stable and acute exacerbation free for at least six weeks prior to study enrolment. This was a community-based study where COPD patients were monitored longitudinally in their own home from enrolment at stable baseline through the exacerbation period and two weeks' post-exacerbation recovery. Exacerbation length was defined as the period between the date on which an exacerbation was confirmed and the date on which the COPD patient reported a return to their normal breathing (Johnston et al., 2013) and completed treatment (antibiotics and steroids). Any patient who felt they needed to recommence treatment for a further deterioration of their COPD (re-exacerbation) within the two-week post-exacerbation-recovery phase continued to be monitored until they reported a return to their usual self. In this way exacerbations and recovery were patient-defined. COPD patients were provided with an iPad (Apple, USA) pre-installed with the electronic Wellbeing and Self-Assessment diary (COPD-SPOC App) and a unique user-login identification number, which would allow diary entry and completion of the self-assessment. The iPad (Apple, USA) had 3G mobile data transmission and reception capacity (Vodafone, UK) in order to allow transmission of all submitted data to the host-website. Patients were also encouraged to enter a free-text comment as often as possible. A paper-based version of the electronic Wellbeing and Self-Assessment diary was available (Appendix 1, Page 485); however none of the enrolled

COPD patients requested a paper-based diary either at enrolment or by converting from the electronic diary to a paper-based diary during the study. Patients provided written feedback on the diary which will be further explored and discussed in Chapter 5, Page 361.

At visit one, conducted by myself and a specialist respiratory nurse (research team) in the patients' home, a comprehensive HRO was entered into the research section of the electronic Wellbeing and Self-Assessment diary (Section 4.2.1.2.2.4, Page 265). This included COPD patient demographics, a full clinical history, duration of COPD diagnosis, smoking history, MRC dyspnoea score, childhood and other respiratory diseases, co-morbidities and medications. Oral hygiene was checked and recorded. Prior to enrolment patients were instructed on the saliva collection protocol (Chapter 2, Figure 2.28, Page 209) and a hard copy of this protocol was also provided at visit one. Patients were asked to provide 2mls of unstimulated whole saliva via passive drool into an ice-cooled 15ml marked centrifuge tubes (Nunc, Denmark). Spirometry was performed using a portable Koko Legend spirometer (nSpire, USA).

During the first visit, each patient received a "walk-through" on how to use the iPad and installed electronic Wellbeing and Self-Assessment diary; specifically how to complete and submit the COPD Wellbeing Score section (Figure 4.2A, Page 258) once a day, preferably aiming to complete their entry at the same time each day. They were informed that their diary scores would be remotely monitored daily by the research team and that a change in symptoms, or diary non-completion, would trigger contact by myself or the nurses. The patients could also initiate direct mobile contact with then team between the hours of 08:00 to 20:00 Monday to Sunday if they so wished or via the free-text section in the electronic diary.

Following the first visit, patients were followed up weekly in their own home by the research team to obtain a 2ml sample of saliva, perform spirometry and update the HRO. The only deviation to this protocol was patients who reported they felt unwell or feeling they were about to have an acute exacerbation of COPD. In these circumstances they were advised to contact the research team so that an immediate visit could be organised outside of the study hours (08:00 to 20:00) within 12 hours. Patients had been taught to produce and deposit a 2ml sample of saliva prior to the commencement of any treatment. Spare saliva collectors and plastic envelopes were provided to each COPD patient at enrolment; collected samples were kept in their home freezer and collected by the team during the "emergency visit", when spirometric and full clinical assessment would also be repeated. The study received prior approval from NRES Committee North West - Greater Manchester South, 12/NW/0623; all participants gave informed written consent.

4.2.2.1. Unstimulated whole saliva collection and processing

Each participant received verbal instructions and a printed protocol for saliva collection (Chapter 2, Figure 2.28, Page 209). Briefly, patients were asked to abstain from alcohol for at least 12 hours; fast for 30 minutes; refrain from brushing their teeth and smoking for 30 minutes, prior to providing saliva samples. Oral hygiene was checked and a mucosal examination was performed at each visit. All visit saliva samples were collected at the same time of day for each COPD patient as far as practically possible. Immediately before collection COPD patients rinsed their mouths with 10mls tap water; they then sat in an upright position, tilted their heads forward, and allowed saliva to pool in the mouth before passively drooling into ice-cooled collectors up to a pre-marked volume of 2mls.

Collected saliva samples were transported on ice and stored within 4 hours of collection in the Guy Hilton Research Centre Freezer Room (Keele University, UK) at -80^{o}C until analysis. Pre-saliva analysis sample preparation involved sample thawing and centrifugation at 3000rpm for 15 minutes (Chapter 2, Page 91). All saliva sample measurements were undertaken within three months of storage; all biomarker assays were performed in duplicate. Random saliva samples were tested for blood contamination with an 8-parameter urine regent strip (Siemens, Germany) using a modified testing procedure (Chapter 2, Page 200).

4.2.2.2. Analysis of biomarkers in saliva

Protocols for analysis of CRP, PCT and NE levels in saliva were established in Chapter 2, Page 83. Briefly, CRP was measured in 15ul of saliva using a Salivary ELISA Kit (Salimetrics Europe, UK), (Chapter 2, Page 90) which has a range of quantification of 0.10 to 30ng/ml. Salivary PCT and NE were measured using in-house modified commercial serum based ELISAs. The PCT was determined in 100ul of saliva diluted 1:2 in PBS-T using a VIDAS BRAHMS PCT kit on the mini-VIDAS instrument (bioMérieux, France) (Chapter 2, Page 112) which has a range of quantification of 0.10 to 400ng/ml. The NE was measured in 7.0ul of saliva diluted 1:200 in ELISA wash buffer using PMN-Elastase ELISA Kit (Immundiagnostik, Germany), (Chapter 2, Page 149), which has a range of quantification of 2.2 to 2000ng/ml.

4.3. Statistical Analysis

The statistical tests employed are discussed in Chapter 2, Page 86. Specific extra tests for this study are documented in this section. A power calculation was conducted during the preliminary study design process which demonstrated that a minimum sample size of 40 COPD patients was required to detect a significant change in baseline salivary biomarker levels for prediction of the onset of an acute exacerbation of COPD. Thus minimum target patient recruitment for this study was set at 40 COPD patients.

To conduct preliminary analysis of the longitudinal data set (PRO, saliva biomarkers and spirometry) the data was split into four distinct classifications (Table 4.1). These divisions made data processing and interpretation easier and also facilitated identification of any early signals/subtle changes in the period leading to the onset of an acute COPD exacerbation (prodromal phase).

Table 4.1: Data classification definitions.

	COPD Disease Phase			
	***Stable**	**Prodromal**	****Exacerbation**	**Post-Exacerbation-Recovery**
Definition	**The period from enrolment to 8 days prior to the onset of an exacerbation**	**The period between 7 to 1 days prior to the onset of an exacerbation**	**The period from exacerbation onset and start of treatment (Day 0) to treatment completion and when patients felt their breathing had normalised (Day 7)**	**The immediate period (14 days) following treatment completion**

*COPD patients had to be stable for at least 6 weeks prior to enrolment into the community-based longitudinal study.

**exacerbation phase onset was COPD patient-defined; the first day (Day 0) of this phase was when patients felt they needed to commence treatment for an acute exacerbation of COPD. The length of the exacerbation phase for all COPD patients was set at 7 days, equivalent to the number of days of their usual prescribed course of treatment with antibiotics (amoxicillin 500mg three times a day or doxycycline 100mg once a day) and prednisolone 30mg a day.

COPD patients' stable phase data collected across multiple time-points (PROs, spirometry and salivary biomarkers) were used for initial data analyses across the 4 phases. Latent class growth analysis (LCGA) was performed on the three salivary biomarkers (log transformed) and spirometric values (FEV_1 and FVC). Change point analysis (CPA) with Cumulative sum charts (CUSUM) (where needed) was used to analyse the COPD Wellbeing Scores. Index exacerbation data were grouped into principal components using factor analysis (Bafadhel et al., 2011b) and exacerbation salivary biomarkers, using area under the curve (AUC) and receiver operating characteristics (ROC), were split into clinically meaningful thresholds. A

Bonferroni correction was applied in cases of multiple comparison testing. A p-value of less than 0.05 was considered statistically significant.

4.3.1. Latent class growth analysis

Latent class growth analysis is a statistical technique that groups a variable repeatedly measured longitudinally into hypothetical trajectory classes. Latent class growth analysis (LCGA) has been increasingly recognised for its usefulness in identifying homogenous sub-populations within a larger heterogeneous population, and particularly for the identification of meaningful individual sub-population clusters based on their trajectories over time (Jung and Wickrama, 2008). Hence this analysis could be applied to COPD patient populations to determine sub-population clusters based on common longitudinal variability of a target variable. This approach could provide meaningful information on COPD patients as certain parameters for example: FEV_1 do not manifest a monotonous decline over time (Casanova et al., 2014). Thus grouping COPD patients based on a variable change over time may provide a more comprehensive assessment of the particular variable in question for example: FEV_1.

LCGA was applied to better understand whether clusters existed for FEV_1, FVC and all three salivary biomarkers within the stable prodromal phase data. The baseline co-variates (Age, Gender, BMI, FEV_1, Total Co-morbidities) for these identified COPD patient clusters were then tested using multinomial analysis to explore whether significant differences in these characteristics existed between the clusters.

LCGA is an iterative process where calculation occurs in a step-wise approach with increasing cluster numbers; meaning that the target population would be analysed assuming that just 1 sub-population cluster exists then 2 sub-population clusters and so on. To determine whether

the optimal number of sub-population clusters have been chosen a goodness-of-fit and posterior probabilities tests were employed. This is important as a model can become increasingly complex and thus prone to error when too many parameters (in this chapter: clusters) are applied. I utilised two goodness of fit tests: Bayesian information criterion (BIC) and Bootstrap log-likelihood (BLRT). The BIC value determines the goodness of fit for the model in which the lower the value, the more optimal the number of clusters (Nylund et al., 2007). The BLRT attaches a statistical significance on whether an increased number of clusters (n compared to n-1) in the model results in a significant improvement in the allocation of subjects to their respective clusters (McLachlan and Peel, 2004). The results from these tests generate a statistical significance of whether increasing the number of clusters would improve sub-population cluster membership. The BIC value and p-value can be used as a guide in conjunction with posterior probabilities to decide the overall number of sub-population clusters (Posada and Buckley, 2004).

In using the above approach, it is first important to understand the way LCGA assigns individuals to a particular sub-population cluster. When a LCGA model is calculated each individual (for example, COPD patients in this study) are assigned to a cluster based on the trajectory of a target value (for example, salivary biomarker or spirometric indices in this study) over time. Cluster membership is however assigned based on the highest posterior probability across the number of clusters being tested.

For example: If COPD patient "x" is being tested in a 3 cluster LCGA their posterior probability membership for each cluster may be as follows: Cluster 1: 75%, Cluster 2: 15%, Cluster 3: 20%. The LCGA analysis would assign this COPD patient as belonging to cluster 1

based on the highest posterior probability (expressed as a percentage). However, this COPD patient "x" may also share features with the other 2 sub-population clusters.

Thus looking at the posterior probabilities for cluster membership can help to decide the optimal number of clusters to create as homogenous a sub-population as possible (Rzewuska et al., 2015).

4.3.2. Change point analysis (CPA)

Change point analysis (CPA) is a statistical technique that aims to identify a time-point when a longitudinally repeated measured variable changes its probability distribution. This allows for the location of multiple change points in trajectory of real time life data sets and is sensitive to subtle changes. The analysis is a combination of CUSUM (a sequential analysis technique) and bootstrapping which assigns a confidence interval for each change. The technique is superior to CUSUM in isolation and other methods for example moving averages and can be applied to non-parametric data (Gavit et al., 2009). This mathematical technique will be applied to the daily COPD Wellbeing Score across the 4 defined COPD phases (Table 4.1), but not to salivary biomarkers or spirometric indices due to the reduced number of data points as these parameters were quantified weekly.

4.3.3. Factor analysis

Factor analysis was conducted on the index exacerbation values for spirometry, PROs and salivary biomarkers to understand whether the principal components of an exacerbation can be grouped together.

4.4. Results

In total 60 COPD patients passed the inclusion/exclusion criteria and were recruited into the study; subsequently 3 patients were withdrawn due to illness and 2 due to non-compliance with the study protocol. The remaining 55 COPD patients were all ex-smokers (greater than 20 pack year history; and stopped smoking for over 1 year) and represented various stages of disease severity (GOLD Stage I, 7; Stage II, 24; Stage III, 19; Stage IV, 5). The cohort characteristics are shown in Table 4.2. Total duration of all COPD patients in the community-based longitudinal study was 14.3 ± 9.9 weeks. All patients (apart from 6) experienced at least one exacerbation during the study period.

These 55 COPD patients (Table 4.3) were sub-divided into two cohorts: 49 exacerbators and 6 non-exacerbators who remained stable throughout their participation in the community-based longitudinal study. As noted above, despite all recruited COPD patients having reported a minimum of two exacerbations in the preceding 12 months prior to enrolment, there were 6 COPD patients who failed to exacerbate. Furthermore, some of the patients who had an acute event, exacerbated early on in the study period and therefore had a reduced recorded data-length for their stable phase (Table 4.4, Page 282).

Table. 4.2: Total study population baseline and stable phase characteristics.

	COPD Patients					
	(n = 55)					
Demographics		**I**	**II**	**III**	**IV**	***p-value**
	(n = 55)	**(n = 7)**	**(n = 24)**	**(n = 19)**	**(n = 5)**	
Age, [a]years	**68.4 ± 7.8**	70.1 ± 5.5	66.3 ± 8.2	70.5 ± 7.9	67.8 ± 7.4	=0.227
Gender, Male (Female)	**27 (28)**	2 (5)	13 (11)	10 (9)	2 (3)	=0.214
Duration of COPD, [a]years	**8.1 ± 6.5**	10.9 ± 10.1	6.6 ± 4.4	9.4 ± 7.7	6.8 ± 3.1	=0.732
$FEV_{1,}$ [a] % predicted	**53.6 ± 18.2**	86.4 ± 6.6	59.9 ± 9.0	40.6 ± 4.2	25.5 ± 7.5	**<0.001**
FVC, [a] % predicted	**77.8 ± 14.6**	102.6 ± 10.3	80.0 ± 9.1	72.0 ± 7.3	54.8 ± 8.7	**<0.001**
BMI, [a](kg/m^2)	**27.0 ± 5.4**	29.1 ± 5.2	28.3 ± 5.5	24.9 ± 4.9	25.5 ± 5.3	=0.086
MRC Score, [b] n	**3.00, 1.00**	3.00, 0.50	3.00, 1.50	3.00, 1.00	3.00, 2.75	=0.997
Exacerbations in the last 1 year, [a]n	**4.3 ± 2.4**	4.9 ± 3.3	4.3 ± 1.1	4.2 ± 3.6	4.2 ± 1.3	=0.940
Co-morbidities						
None	**13**	3	5	3	2	
Cardiovascular	**12**	1	7	2	2	
Type 2 Diabetes Mellitus	**7**	2	2	2	1	
Hypertension	**21**	3	9	7	2	
Gum Disease	**1**	0	1	0	0	
Other	**23**	3	9	9	2	
COPD Treatment						
β_2-Agonists, Short Acting, (Long Acting)	**54, (47)**	7, (5)	24, (19)	19, (19)	4, (4)	
Anticholinergic, Short Acting, Long Acing)	**4, (40)**	0, (4)	1, (18)	1, (15)	2, (3)	
Inhaled Steroid	**48**	5	20	18	5	
Oral Theophyllines	**14**	1	6	4	3	

Data presented as a = mean ± SD unless and b = median, IQR. *P-value represents significance across COPD severity as defined by GOLD (I = 7, II = 24, III = 19 and IV = 5) by one-way ANOVA.

Table. 4.3: Exacerbation and non-exacerbator cohort characteristics.

Demographics	COPD Patients (n = 55)		
	Exacerbators (n = 49)	Non-Exacerbators (n = 6)	p-value
Age, [a]years	68.2 ± 8.0	70.3 ± 6.4	=0.524
Gender, Male (Female)	25 (24)	2 (4)	=0.544
Duration of COPD, [a]years	7.8 ± 6.4	10.5 ± 7.5	=0.350
***FEV_1, [a](% predicted)**	50.4 ± 16.8	63.4 ± 27.9	=0.131
***FVC, [a](% predicted)**	76.9 ± 14.3	84.9 ± 16.7	=0.209
BMI, [a] (kg/m^2)	27.3 ± 5.5	24.4 ± 4.2	=0.213
MRC Score, [b] n	3.0, 1.0	3.0, 0.0	=0.148
Exacerbations in the last 1 year, [a]n	4.6 ± 2.4	2.3 ± 0.5	**<0.021**
Exacerbations in the study, [a]n	1.3 ± 0.5	0	
Co-morbidities			
None	11	2	
Cardiovascular	12	0	
Type 2 Diabetes Mellitus	7	0	
Hypertension	19	2	
Gum Disease	1	0	
Other	20	3	
COPD Medications			
β_2 Agonists, Short Acting, (Long Acting)	48, (43)	6, (4)	
Anticholinergic, Short Acting, (Long Acting)	3, (36)	1, (4)	
Inhaled Steroid	45	3	
Oral Theophylline	13	1	

Data presented as a = mean ± SD and b = median, IQR. P-value represents significance between COPD patients who exacerbated and those who remained stable throughout their participation in the study. *FEV_1 values represent the stable phase data.

Table 4.4: Total length of pre-exacerbation phase in the exacerbation cohort.

*Total recruited COPD patients	Length of time: Stable phase	Length of time: prodromal phase	Total length of time: **Pre-exacerbation phase
49	13 days	7 days	20 days
39	28 days	7 days	35 days
29	42 days	7 days	49 days
19	74 days	7 days	81 days
9	251 days	7 days	258 days

*COPD patients who had suffered at least one exacerbation during the community-based longitudinal study (n = 49) **Pre-exacerbation phase is defined as the stable plus prodromal phase.

Although the exacerbation phase can be variable in practice, all COPD patients reported a return to normal after completing treatment (antibiotics and steroids) on Day 7 of the exacerbation phase. Following this, each COPD patient had a minimum 2-week post-exacerbation-recovery phase as this was pre-set in the study protocol. Therefore any COPD patients who re-exacerbated within this two-week time-frame remained in the study until they had completed further treatment and then achieved a 2 week post-exacerbation-recovery phase.

In this study, a sub-population of COPD patients underwent further acute exacerbations (n = 15), termed a re-exacerbation within the index post-exacerbation-recovery phase at 10.9 ± 7.7 days, requiring further treatment with antibiotics and steroids (Table 4.5). There was a significantly lower FEV_1 at stable baseline ($p<0.02$ by independent t-test) in this COPD patient sub-population (exacerbation cohort). None of the other parameters was significantly different between the 2 sub-populations (age: p=0.135, BMI: p=0.236, FVC: p=0.369 and total co-morbidities: p=0.273).

The length of study participation for the COPD patients in the exacerbation cohort (n = 49) was 13.0 ± 8.5 weeks whilst for the non-exacerbation cohort (n = 6) was 25.3 ± 14.8 weeks. The non-exacerbator patients were kept in the study for longer to allow sufficient time for them to have an acute event as well as to collect background data on biomarker levels and other metrics over time. As such, this cohort, albeit small, provided a much needed control group for the exacerbation cohort and allowed cross-comparison.

Cardiovascular disease and hypertension were the commonest co-morbidities present in 27 out of 55 COPD patients; all co-morbidities remained stable throughout the study. All salivary biomarker level, spirometric index and PRO (COPD Wellbeing score) data were cross-referenced to the HRO data recorded by the research team in the electronic COPD Wellbeing and Self-Assessment diary to ensure there were no factors present that could bias the results. No changes in medications or co-morbidity status occurred for all COPD patients during their participation in the study; the only treatment change occurred when COPD patients were undergoing an acute exacerbation of COPD. None of the participating patients were admitted to hospital as a result of their exacerbations. There was no significant blood contamination in any of the tested saliva samples.

Table. 4.5: Single and re-exacerbator cohort characteristics.

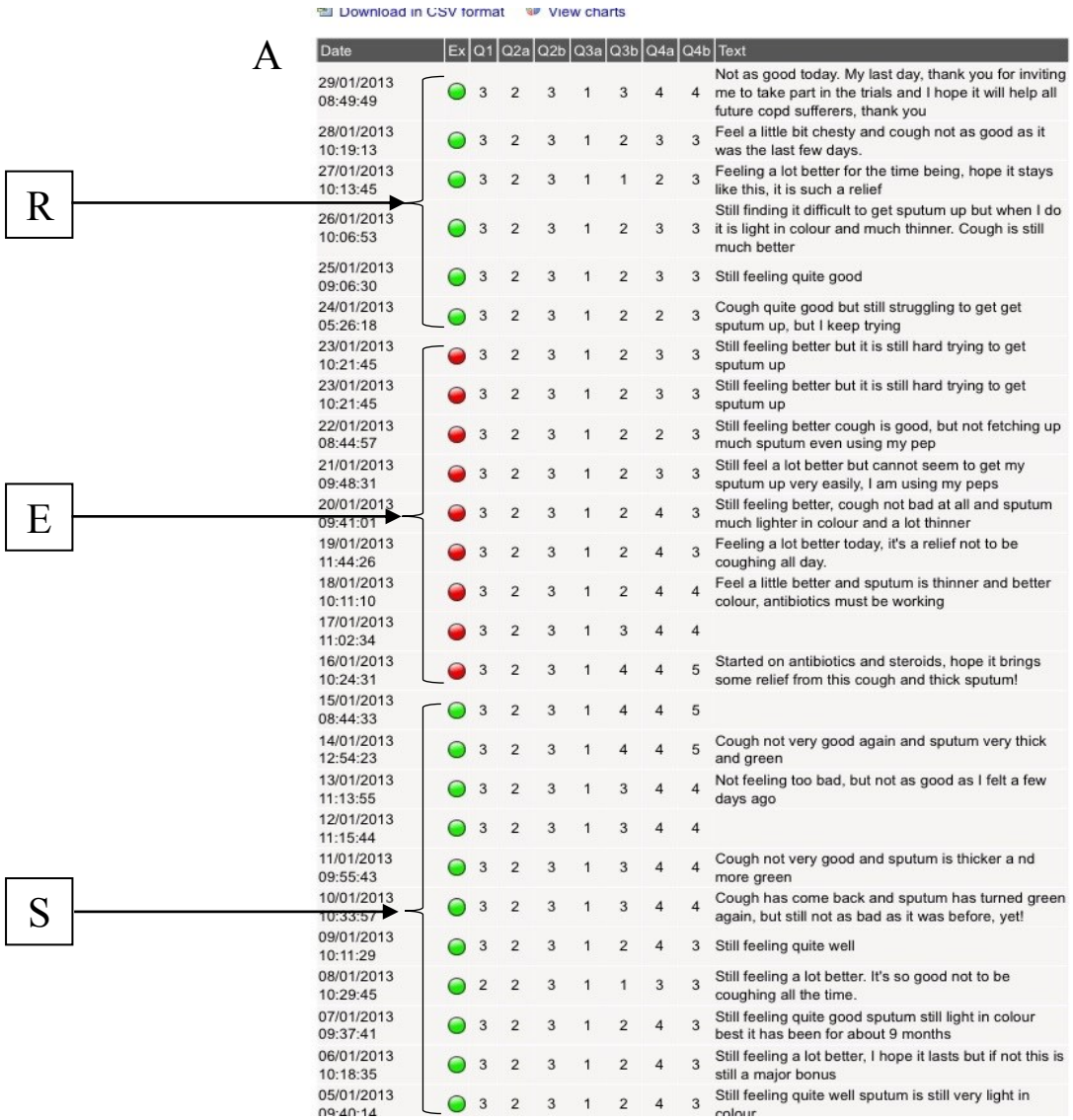
Demographics	COPD Exacerbators Cohort (n = 49)		p-value
	Single Exacerbators (n = 34)	Re-exacerbators (n = 15)	
Age, [a]years	69.3 ± 7.5	65.6 ± 8.7	<0.136
Gender, Male (Female)	19 (15)	5 (10)	<0.452
Duration of COPD, [a]years	8.4 ± 6.3	6.5 ± 6.8	<0.352
***FEV_1, [a](% predicted)**	55.38 ± 17.24	45.7 ± 13.79	**<0.017**
***FVC, [a](% predicted)**	78.2 ± 15.3	74.2 ± 11.6	<0.287
BMI, [a] (kg/m^2)	27.9 ± 5.9	25.9 ± 4.2	<0.237
MRC Score, [b] n	3.0, 1.0	3.0, 1.5	<0.154
Exacerbations in the last 1 year, [a]n	4.6 ± 2.4	5.2 ± 3.0	<0.215
Exacerbations in the study, n	1	2	
Co-morbidities			
None	8	2	
Cardiovascular	11	0	
Type 2 Diabetes Mellitus	4	0	
Hypertension	13	2	
Gum Disease	1	0	
Other	16	3	
Total Co-morbidities, [a]n	1.4 ± 1.1	1.1 ± 0.7	<0.274
COPD Medications			
β_2 Agonists, Short Acting, (Long Acting)	33, (30)	6, (4)	
Anticholinergic, Short Acting, (Long Acting)	3, (22)	1, (4)	
Inhaled Steroid	31	3	
Oral Theophylline	9	1	

Data presented as a = mean ± SD and b = median, IQR. P-value represents significance between the two groups. *FEV_1 values represent the stable phase data.

4.4.1. Patient reported outcomes (PRO)s

4.4.1.1. The capture of electronic PROs

PRO (COPD Wellbeing Scores) were captured using the electronic Wellbeing and Self-Assessment diary (Figure 4.2, Page 263). Once submitted these can then be viewed on the host-website by the research team both as the individual component scores and graphically as line charts (Figure 4.5) to allow COPD surveillance and identify deteriorations in health status.



Date	Ex	Q1	Q2a	Q2b	Q3a	Q3b	Q4a	Q4b	Text
29/01/2013 08:49:49		3	2	3	1	3	4	4	Not as good today. My last day, thank you for inviting me to take part in the trials and I hope it will help all future copd sufferers, thank you
28/01/2013 10:19:13		3	2	3	1	2	3	3	Feel a little bit chesty and cough not as good as it was the last few days.
27/01/2013 10:13:45		3	2	3	1	1	2	3	Feeling a lot better for the time being, hope it stays like this, it is such a relief
26/01/2013 10:06:53		3	2	3	1	2	3	3	Still finding it difficult to get sputum up but when I do it is light in colour and much thinner. Cough is still much better
25/01/2013 09:06:30		3	2	3	1	2	3	3	Still feeling quite good
24/01/2013 05:26:18		3	2	3	1	2	2	3	Cough quite good but still struggling to get get sputum up, but I keep trying
23/01/2013 10:21:45		3	2	3	1	2	3	3	Still feeling better but it is still hard trying to get sputum up
23/01/2013 10:21:45		3	2	3	1	2	3	3	Still feeling better but it is still hard trying to get sputum up
22/01/2013 08:44:57		3	2	3	1	2	2	3	Still feeling better cough is good, but not fetching up much sputum even using my pep
21/01/2013 09:48:31		3	2	3	1	2	3	3	Still feel a lot better but cannot seem to get my sputum up very easily, I am using my peps
20/01/2013 09:41:01		3	2	3	1	2	4	3	Still feeling better, cough not bad at all and sputum much lighter in colour and a lot thinner
19/01/2013 11:44:26		3	2	3	1	2	4	3	Feeling a lot better today, it's a relief not to be coughing all day.
18/01/2013 10:11:10		3	2	3	1	2	4	4	Feel a little better and sputum is thinner and better colour, antibiotics must be working
17/01/2013 11:02:34		3	2	3	1	3	4	4	
16/01/2013 10:24:31		3	2	3	1	4	4	5	Started on antibiotics and steroids, hope it brings some relief from this cough and thick sputum!
15/01/2013 08:44:33		3	2	3	1	4	4	5	
14/01/2013 12:54:23		3	2	3	1	4	4	5	Cough not very good again and sputum very thick and green
13/01/2013 11:13:55		3	2	3	1	3	4	4	Not feeling too bad, but not as good as I felt a few days ago
12/01/2013 11:15:44		3	2	3	1	3	4	4	
11/01/2013 09:55:43		3	2	3	1	3	4	4	Cough not very good and sputum is thicker a nd more green
10/01/2013 10:33:57		3	2	3	1	3	4	4	Cough has come back and sputum has turned green again, but still not as bad as it was before, yet!
09/01/2013 10:11:29		3	2	3	1	2	4	3	Still feeling quite well
08/01/2013 10:29:45		2	2	3	1	1	3	3	Still feeling a lot better. It's so good not to be coughing all the time.
07/01/2013 09:37:41		3	2	3	1	2	4	3	Still feeling quite good sputum still light in colour best it has been for about 9 months
06/01/2013 10:18:35		3	2	3	1	2	4	3	Still feeling a lot better, I hope it lasts but if not this is still a major bonus
05/01/2013 09:40:14		3	2	3	1	2	4	3	Still feeling quite well sputum is still very light in colour

Figure 4.5A: Host-website screenshot.

This screenshot of the host-website represents the research teams' view of the electronic Wellbeing and Self-Assessment diary; specifically the COPD Wellbeing Scores submitted by patients during the community-based study. A = This display highlights the recorded components of the COPD Wellbeing scores, the traffic light alerts on the left of the submissions: red = exacerbation and green = stable; the free-text entry is to the right of the submitted scores. In this particular patient, the stable (S), exacerbation (E) and post-exacerbation recovery (R) phases are demonstrated. The data can also be displayed longitudinally as a chart (Figure 4.5B) highlighting the fluctuations in the COPD patients' symptoms.

B

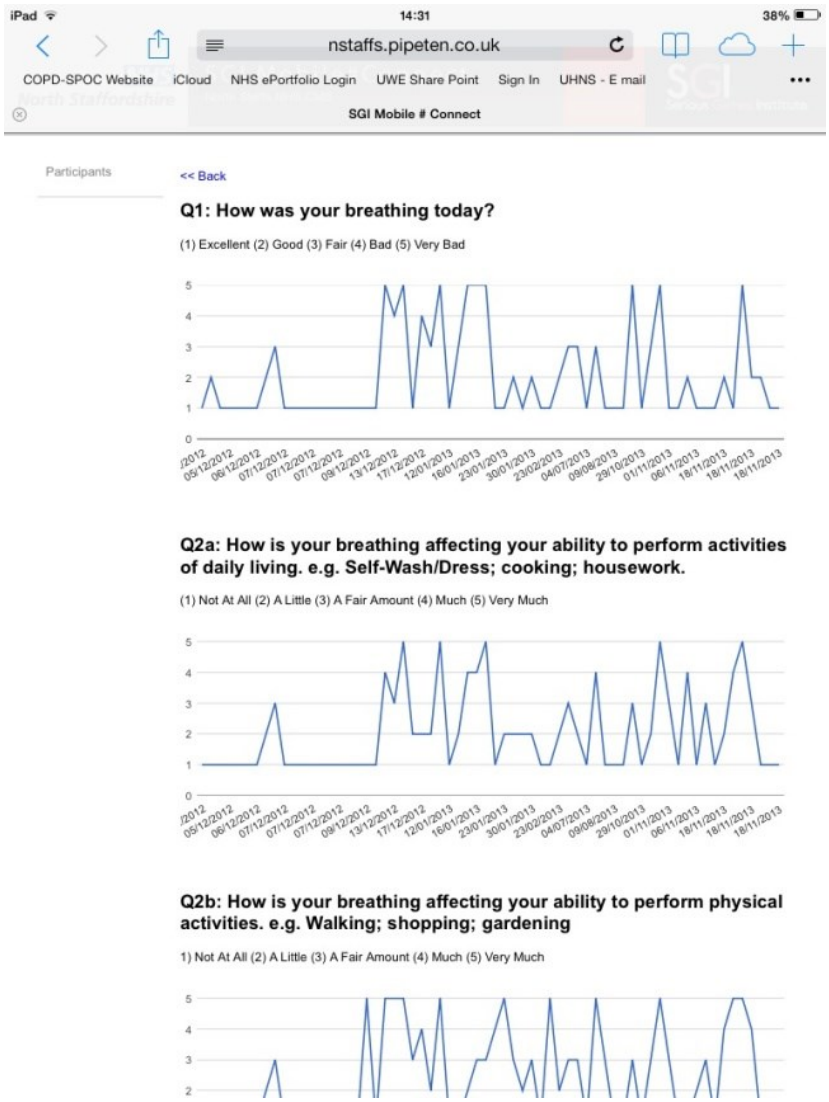


Figure 4.5B: Host-website screenshot.

This screenshot of the host-website represents the research teams' view for the electronic Wellbeing and Self-Assessment diary specifically the graphical display for the COPD Wellbeing Scores that have been submitted by the COPD patients in the community based study. This displays longitudinally the fluctuations in this COPD patients' symptoms which are represented in scores in Figure 4.5A.

4.4.1.2. Results

Out of a total of 5461 possible unique daily electronic Wellbeing and Self-Assessment diary entries, 5371 were completed and transmitted successfully to the host-website (98.4%). The 90 data diary entries that were not completed were due to COPD patient non-compliance despite prompting by the research team.

4.4.1.3. PRO analysis

All symptom data were split into the previously discussed 4 COPD disease phases (Table 4.1, Page 275) and analysed accordingly. The stable-prodromal-phase total COPD Wellbeing Scores in the patient exacerbator cohort (n = 49) were also compared with the control cohort (n = 6).

Unlike saliva and spirometry which were performed weekly, the wellbeing diary scores were collected daily. For each wellbeing component, the score range was set from 1 to 5, with 5 reflecting worst status. To analyse these COPD Wellbeing Scores, CPA was used, with CUSUM charting where indicated, to understand the day to day score fluctuations and changes in trajectories. Firstly, each of the individual components which were assigned an individual score (Figure 2.29, Page 212) to establish a median stable phase score for each component of the COPD Wellbeing score (breathing, ADL, cough, sputum production and colour) for each COPD patient. This score was considered the baseline burden for that component of the COPD Wellbeing score and was then deducted from the daily scores across all four phases to standardise the results for each COPD patient. Thus a COPD patient who symptomatically is in the stable phase of their COPD would score less than or equal to 0 (normal or reduced symptom burden), and a COPD patient with worsening symptoms would have a total score of greater than or equal to 1 (worsening in symptom burden). The component scores were then

summed to create a total component scores (termed total COPD Wellbeing Score); this allowed statistical analysis across the entire COPD patient population. If significant differences existed in the stable phase COPD Wellbeing score these would then be divided into sub-populations; the assignment of COPD patients into these sub-populations would form the first component of a multidimensional score (Section 4.4.4, Page 350) based on PROs, spirometry (Section 4.4.2, Page 299) and salivary biomarkers (Section 4.4.3, Page 313).

4.4.1.4. Stable and prodromal phases

The COPD Wellbeing score in the preceding five-week period (stable plus prodromal phase) towards an exacerbation phase demonstrated a single stable trajectory of symptoms up to 4 days prior to the onset of an acute exacerbation. At this time point there was a significant positive change (increased symptom burden) in the trajectory of the COPD Wellbeing scores in all COPD subjects who exacerbated ($p<0.01$ by CPA). The mean change in the COPD Wellbeing score at that time point is from 0.17 to 1.32 (Figure 4.6). Cross-analysis of the median stable phase total COPD Wellbeing Score between the exacerbator (17, 6) cohort (n = 49) and non-exacerbator (14, 5) cohort (n = 6) revealed no significant difference ($p=0.097$ by Mann-Whitney U test). Using the CPA data, the exacerbation cohort (n = 49) was split into 2 separate groups according to the lead time interval in symptom change: (a) Patients who had experienced a rise in COPD Wellbeing scores at 4 days and more from the onset of an acute exacerbation of COPD (Group 1: n = 42) and (b) Patients who experienced a change in scores 3 days or closer to the exacerbation onset (Group 2: n = 7) (Table 4.6). The purpose of this split was to create a multidimensional score based on COPD patients' phenotypes (Section 4.4.4, Page 350).

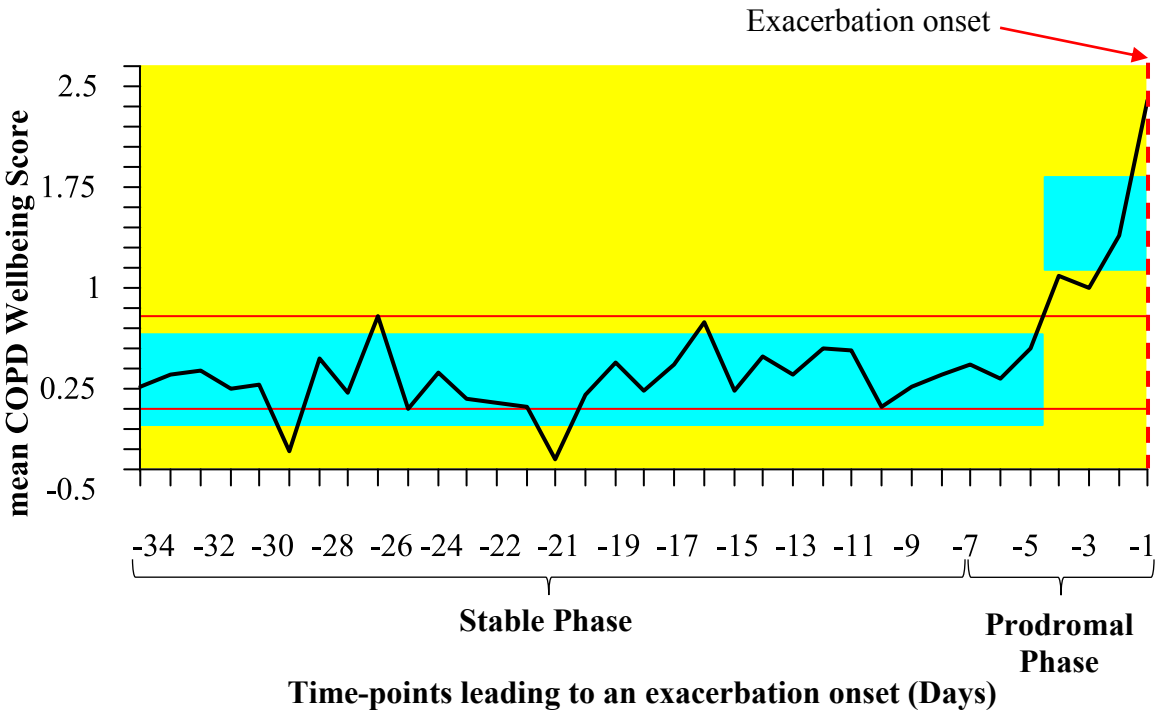


Figure 4.6: Stable and prodromal phase COPD Wellbeing scores.

A line chart (black) showing the mean COPD Wellbeing scores of those patients who exacerbated (n = 49) in the 5 immediate weeks preceding the onset of an exacerbation. The two red lines represent control limits for the maximum range within which the COPD Wellbeing Score is expected to vary over assuming no change has occurred. These control limits are calculated as mean Scores ± 2 SD. Points outside the control limits indicate a clinically relevant change has occurred; for display, the shift in the blue band indicates the statistically significant change in the trajectory. A clear change was seen at time point -4 days prior to the onset of the exacerbation ($p<0.01$ by CPA).

Table 4.6: COPD Wellbeing Score sub-populations based on stable-prodromal data.

COPD Patient	Symptom Group Membership
1	1
2	1
3	1
4	1
5	1
6	1
7	1
8	1
9	1
10	2
11	1
12	1
13	2
14	2
15	1
16	1
17	1
18	1
19	1
20	1
21	1
22	1
23	1
24	1
25	1
26	1
27	1
28	1
29	1
30	1
31	1
32	1
33	2
34	2
35	1
36	1
37	1
38	1
39	2
40	1
41	1
42	1
43	1
44	1
45	2
46	1
47	1
48	1
49	1

4.4.1.5. Exacerbation phase

Next the change in COPD Wellbeing scores was determined within the defined exacerbation phase, during which all exacerbator patients (n=49) took their standby 7-day course of antibiotics (amoxicillin 500mg three times a day or doxycycline 100mg a day) and prednisolone 30mg a day. Overall during the exacerbation phase there was a steady consistent fall in symptom burden with no statistically significant shift in trajectory identified (Figure 4.7). Sub-analysis of the CUSUM chart showed that the COPD Wellbeing scores begin to fall below the mean exacerbation phase score (and hence patient start returning to their/normal self) at day 3 post-exacerbation onset (Figure 4.8).

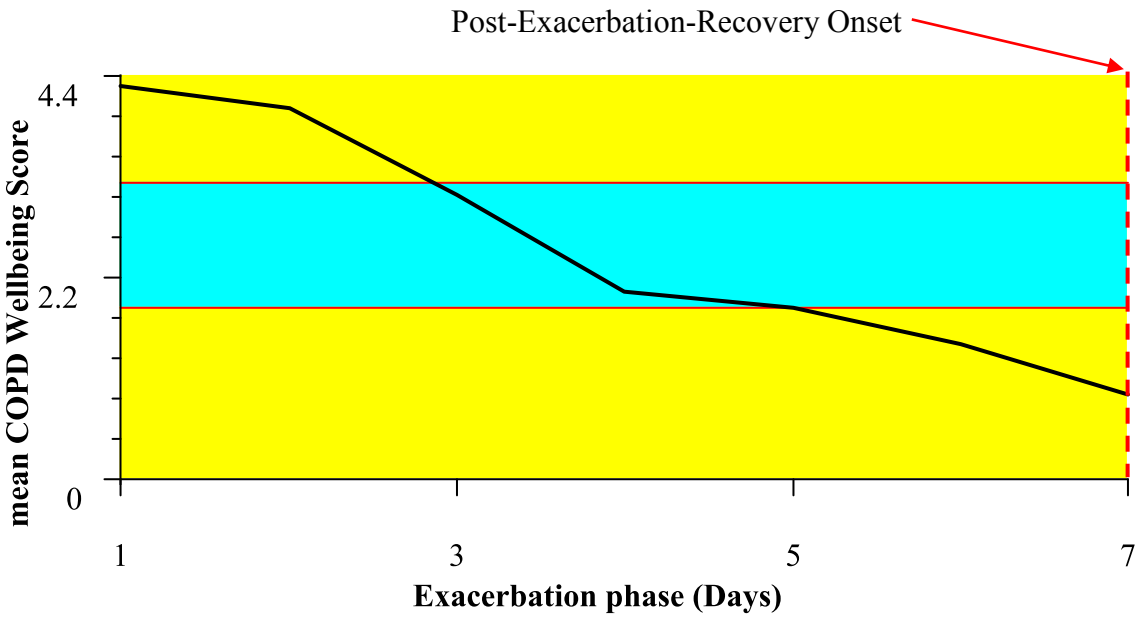


Figure 4.7: Exacerbation phase COPD Wellbeing Score.

The line chart demonstrates the change in mean COPD Wellbeing scores during the exacerbation phase (n = 49). During this phase, patients are receiving treatment with steroids and antibiotics. The two red lines represent control limits for the maximum range in which the COPD Wellbeing Scores are expected to vary over assuming no change has occurred. These control limits are calculated as mean Scores ± 2 SD. Points outside the control limits indicate a clinically relevant change has occurred; for display a shift in the blue band reflects a statistically significant change in the trajectory. The chart demonstrates a decrease in symptom burden with mean scores returning towards baseline in a stable trajectory.

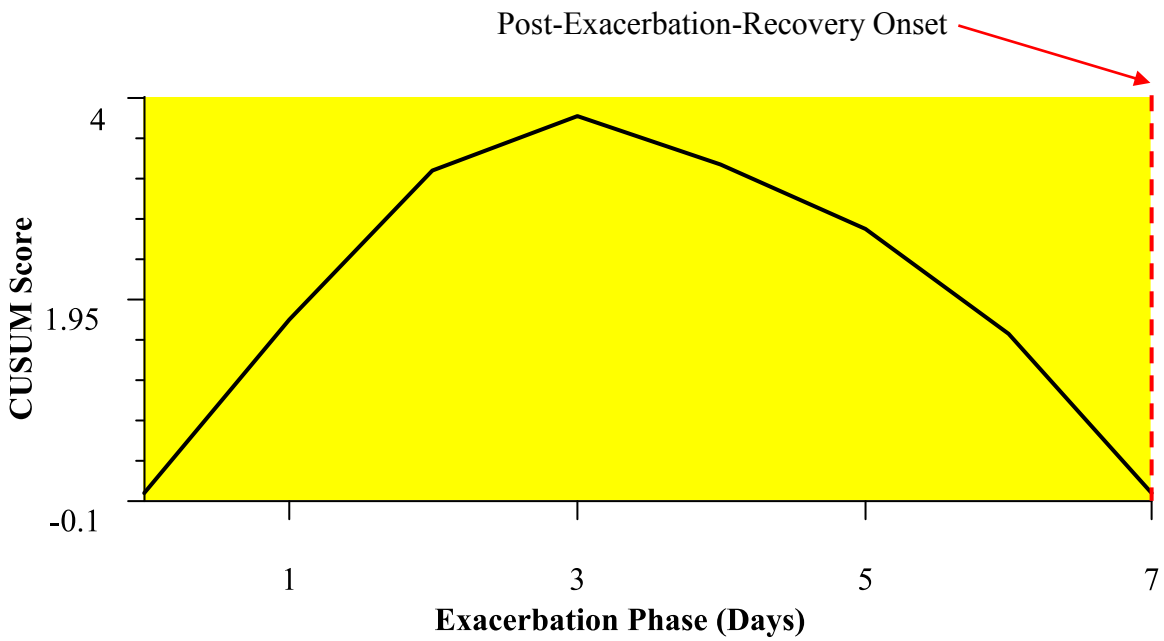


Figure 4.8: Cumulative Sum chart of COPD Wellbeing score.

This line chart represents the cumulative sum (CUSUM) chart used in the change point analysis (CPA) for the exacerbation phase. A positive gradient indicates COPD Wellbeing scores above the average, whereas a negative gradient indicates reducing symptom scores towards baseline. Although the CPA did not demonstrate a statistically significant change in the trajectory of the COPD Wellbeing Score, the CUSUM for the exacerbation phase demonstrates that the score starts to return to baseline at around day 3 of the exacerbation phase (also reflected as day 3 post-start of treatment).

4.4.1.6. Post-exacerbation-recovery phase

Within the post-exacerbation-recovery phase there appears to be a statistically significant worsening in mean COPD Wellbeing Scores (0.76 to 0.98) at day 10 ($p<0.05$) (Figure 4.9). This positive shift however may reflect the sub-population of COPD patients who re-exacerbated and correlates with the observed mean re-exacerbation time-point (10.9 ± 7.7 days) post completion of treatment in the re-exacerbator cohort.

The advantages of using the CPA approach to analyse the complex dynamics of the symptom data is highlighted in Figure 4.9. Although the mean COPD Wellbeing Scores have not exceeded the expected mean variability defined by the confidence intervals (red lines), it does demonstrate that there is a statistically significant change in the trajectory of the scores which would not have been identified through conventional analytical techniques (Section 4.3.2, Page 278).

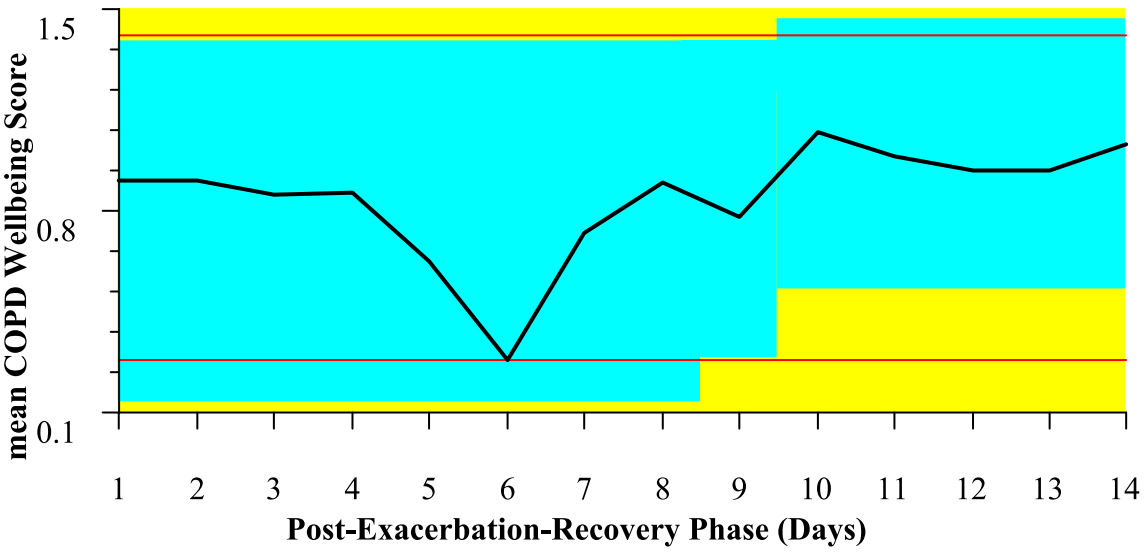


Figure 4.9: Post-Exacerbation-Recovery COPD Wellbeing Score.

The line chart demonstrates the change in mean COPD Wellbeing scores in the post-exacerbation-recovery phase following an index exacerbation of COPD. During this phase, patients had returned to using their usual medications. The two red lines represent control limits which are the maximum range that the values COPD Wellbeing Score are expected to vary within assuming no change has occurred. These control limits are calculated as mean Scores ± 2 SD. Points outside the control limits indicate a clinically relevant change has occurred; for display, the shift in the blue band reflects a statistically significant change in the trajectory at around day 10 ($p<0.05$)

4.4.1.7. COPD sub-population of re-exacerbators

Stable baseline COPD Wellbeing scores, index exacerbation score at day 1 and the index post-exacerbation recovery score at day 1 were compared between the single exacerbator group (n = 35) and the re-exacerbator group (n = 14). COPD Wellbeing Scores were not significantly different between the single and re-exacerbation groups in the stable phase (p=0.922); at day 1 of the index exacerbation phase (p=0.278); or at day 1 of the post-exacerbation-recovery phase (p=0.564) (Table 4.7).

Table 4.7: Comparison of COPD Wellbeing Scores between single exacerbator and re-exacerbator patients.

COPD Wellbeing Score	Single Exacerbators (n = 35)	Re-Exacerbators (n =14)	p-value
Stable Phase	17, 6	16, 4	p=0.922
Exacerbation Phase	19, 4	23, 4	p=0.278
Post-Exacerbation-Recovery Phase	17, 6	17, 5	p=0.564

Data are presented as median, IQR.

Change point analysis (CPA) of the post-exacerbation-recovery phase for the entire group (n = 49) identified a significant change in trajectory of the COPD Wellbeing Score at day 10 post-exacerbation-recovery phase (Figure 4.9). However, analysis of the COPD Wellbeing Score at day 10 post-exacerbation-recovery between the 2 groups is not statistically significant (p<0.467 by Mann-Whitney U test). This result is unusual but perhaps highlights the importance of daily symptom monitoring to isolate the change in symptom burden trajectorially for each patient.

In conclusion, PRO analysis on symptoms revealed an increase in the COPD Wellbeing score at around 4 days prior to an exacerbation onset. Recovery whilst on treatment appears to be uniform, with a clear improvement in the COPD Wellbeing Score after 3 days (although this was not statistically significant). Additionally, in the post-exacerbation recovery phase following treatment completion, there appears to be a significant but subtle shift in symptoms at day 10.

4.4.2. Spirometry

Spirometry was conducted on all COPD patients at study enrolment and then weekly during the study course. As described 49 out of 55 COPD patients reported at least one acute exacerbation of COPD during the study. As with PRO analysis, spirometry data was split into 4 phases according to the criteria in Table 4.1, Page 275. This allowed comparative analysis of spirometric metrics across the various phases, particularly the stable-prodromal period, to determine any signals predictive of the onset of an acute exacerbation. With respects to the COPD patients who did not exacerbate (n = 6), their stable phase spirometric data acted as a control.

4.4.2.1. Forced expiratory volume in one second (FEV_1)

Analysis across the 4 phases in COPD patients who experienced at least one exacerbation (n = 49) showed a statistically significant difference in FEV_1 values ($p<0.001$ by repeated measures ANOVA. Using post-hoc analysis, a significant reduction in FEV_1 values was observed as expected between the stable and exacerbation phases respectively ($p<0.006(\$)$ by paired t-test); but no statistically significant difference between stable and prodromal or post-exacerbation-recovery phases ($p=0.154$ and $p=0.999$ respectively). A statistically significant difference was noted between prodromal and post-exacerbation recovery FEV_1 values ($p<0.038(\$)$), but not between the prodromal and exacerbation phases ($p=0.287$). A significant improvement in FEV_1 was also observed, as would be expected, between exacerbation and post-exacerbation-recovery phases ($p<0.001(\$)$) (Figure 4.10, Table 4.8). Cross analysis between the exacerbator (n = 49) and non-exacerbator (n = 6) control cohorts demonstrated no statistically significant difference in stable phase FEV_1 ($p=0.133$, by independent t-test). Although the non-exacerbator cohort appears to have a much higher stable phase FEV_1, it is important to note the large SD and difference in sample sizes between the 2 cohorts.

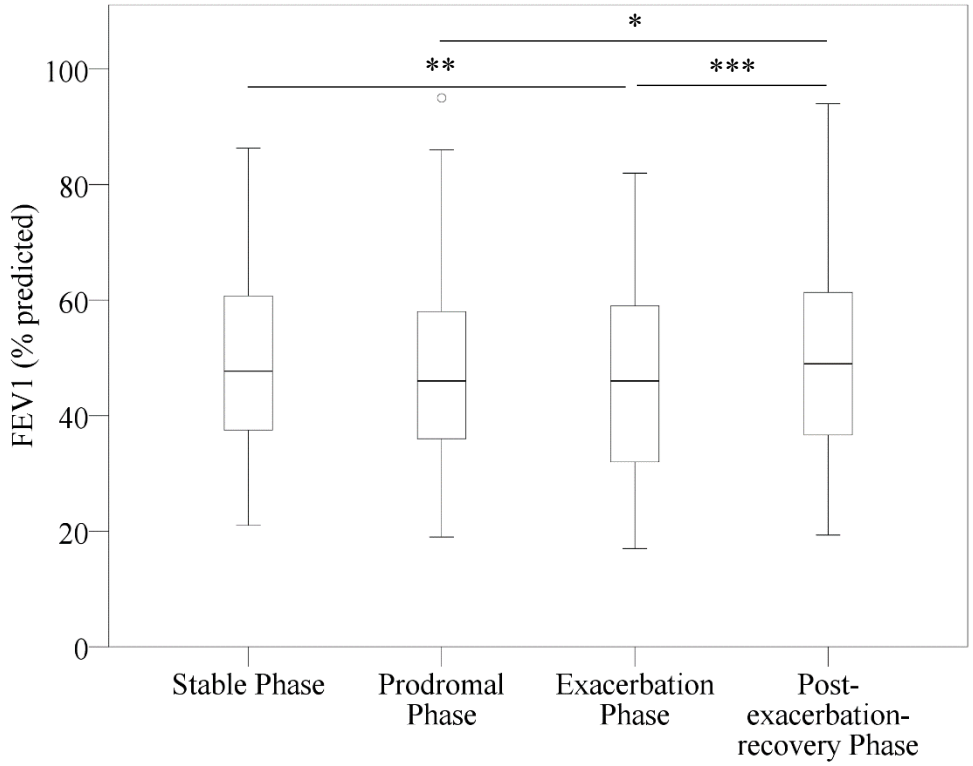


Figure 4.10: Forced expiratory volume in one second across the 4 defined COPD phases.

Box and whisker plots for Forced expiratory volume in one second (FEV_1) across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery for COPD patients who had at least one exacerbation (n = 49). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days post treatment completion. There was a significant statistical difference across the 4 phases ($p<0.001$ by Repeated Measures ANOVA). Post-hoc analysis showed a significant difference between the stable and exacerbation phases (**$p<0.006$($) by paired t-test); between the prodromal and post-exacerbation-recovery phases (*$p<0.038$); and the exacerbation and post-exacerbation-recovery phases (***$p<0.001$($)) but not between prodromal and exacerbation phases ($p<0.288$). There was no statistically significant difference between the stable phase and both prodromal and post-exacerbation-recovery phases ($p=0.154$ and $p=0.999$ respectively).

Table 4.8: FEV_1 values across the whole COPD study cohort.

	FEV_1 (% predicted)			
COPD patients (n = 55)	**Stable Phase**	**Prodromal Phase**	**Exacerbation Phase**	**Post Exacerbation Recovery Phase**
Exacerbators (n = 49)	50.4 ± 18.7	48.9 ± 19.5	46.0 ± 18.8	51.4 ± 19.3
Non-Exacerbators (n = 6)	63.4 ± 27.9	n/a	n/a	n/a

Data presented as mean ± SD.

4.4.2.1.1. Latent class growth analysis (LCGA)

Using the exacerbator cohort (n = 49) data, LCGA was performed on FEV_1 values across the stable-prodromal period at 5 different time points, each 1 week apart from each other, progressing towards an acute exacerbation of COPD (n = 49). This time-frame was consistent with the analysis performed on the equivalent time period for the COPD Wellbeing Scores (Section 4.4.1.4, Page 289). Exacerbator data was chosen as there was a fixed time-point to anchor the end of their stable-prodromal phase values and the commencement of an acute event. For the COPD patients who did not exacerbate in the study (n = 6), as would be expected there was no option to determine the location of their equivalent phase comparable to the exacerbator cohort.

An iterative LCGA using the BIC and BLRT p-value was conducted to determine the pilot number of sub-population clusters (Table 4.9) for COPD patients' stable and prodromal phase FEV_1 values. Although the BIC reduced from 2 to 3 sub-population clusters, there was not a statistically significant improvement in goodness-of-fit (p=0.314 by BLRT). The posterior probability matrix at 2 sub-population clusters (Table 4.10) demonstrated a high level of homogeneity between the 2 clusters; thus 2 sub-population clusters were chosen for stable

phase FEV_1 values. The LCGA populated the separate sub-population clusters with the following number of COPD patients: Cluster 1 = 14, Cluster 2 = 35. The membership of each COPD patient to their respective cluster is shown in Table 4.11. Overall, each cluster formed 2 discrete sub-populations of COPD patients with an inter-cluster overlap of 1% for cluster 1 and 5% for clusters 2. Each sub-population cluster was defined as "moderate" or "severe" range based on the established GOLD criteria (GOLD 2016). These sub-population clusters exhibited 2 unique stable phase FEV_1 value trajectories (Figure 4.11).

Table 4.9: LCGA Goodness of fit tests for stable-prodromal FEV_1 data.

LCGA	BIC	BLRT (p-values)
1 cluster	1824	n/a
2 cluster	1672	**p<0.049**
3 cluster	1558	p=0.314

The BIC value represents the goodness-of-fit test for the LCGA analysis; basically the lower a value, the better the model. The p-value determines whether there is a significant improvement in the model with "n" clusters compared to "n-1". Of interest is the finding of a minimal improvement in the BIC from 2 to 3 clusters and the lack of statistical significance (p=0.314) when increasing the clusters from 2 to 3. LCGA = latent class growth analysis, BIC = Bayesian information criterion, BLRT = Bootstrap log-likelihood.

Table 4.10: Homogeneity of COPD patients cluster membership for stable-prodromal FEV_1 data.

Cluster	1 (n = 14)	2 (n = 35)
1	1%	99%
2	95%	5%

Table 4.11: Exacerbator COPD patient cluster membership for stable-prodromal FEV_1 data.

COPD Patient	Cluster Membership
1	2
2	2
3	2
4	1
5	2
6	2
7	1
8	2
9	2
10	2
11	1
12	2
13	1
14	2
15	1
16	2
17	2
18	1
19	2
20	1
21	2
22	1
23	2
24	2
25	2
26	2
27	2
28	1
29	2
30	2
31	2
32	1
33	2
34	2
35	1
36	2
37	2
38	2
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41	2
42	2
43	1
44	1
45	2
46	2
47	2
48	2
49	1

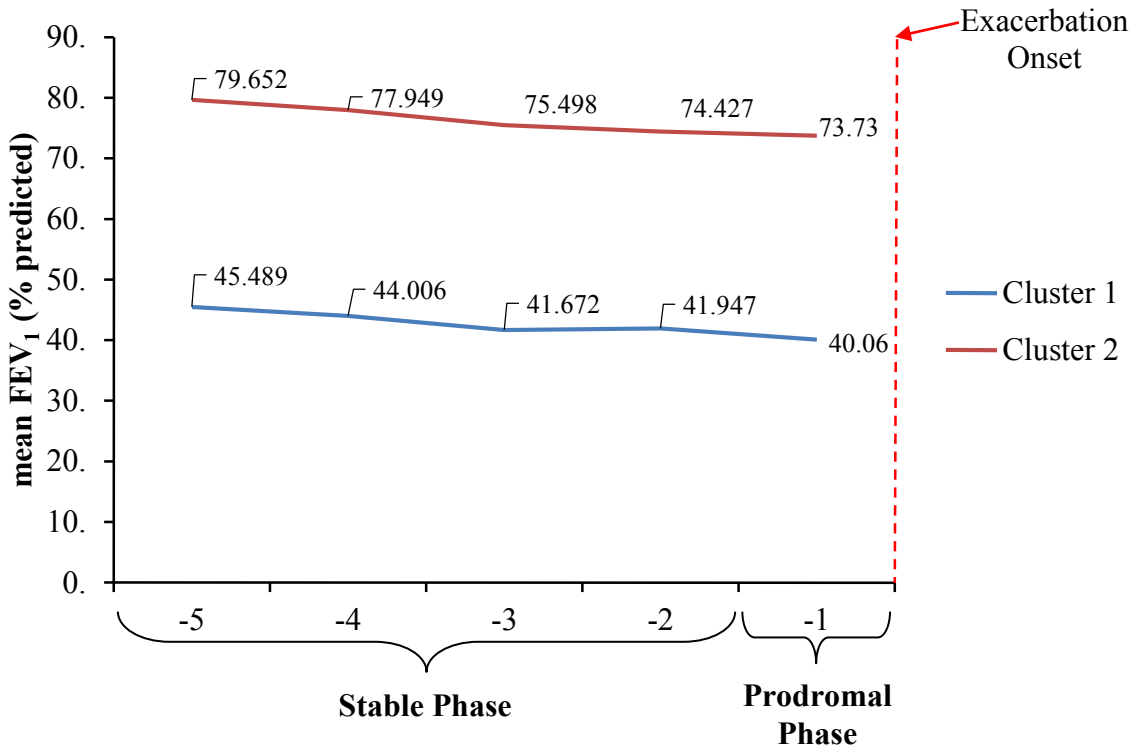

Figure 4.11: Forced expiratory volume in one second clusters in the stable-prodromal period.

This line chart represents the latent class growth analysis for the mean percentage predicted forced expiratory volume in one second (FEV_1) values for COPD patients who experienced at least one acute exacerbation of COPD (n = 49) in the stable and prodromal phases. This period is defined as 5 weeks prior to the onset of an exacerbation and each interval represents 1 week. Cluster 1 = 14 and Cluster 2 = 35 COPD patients. The values represent the mean FEV_1 at each time-point for the respective clusters.

4.4.2.1.2. Baseline characteristics associated with cluster membership.

A further separate sub-analysis was conducted to explore whether there was any significant difference between the 2 sub-population clusters for a panel of known COPD co-variates. Overall there was no significant difference between the "moderate" and "severe" clusters for: age, gender, BMI and total co-morbidities ($p=0.718$, $p=0.631$, $p=0.335$ and $p=0.326$ respectively by Binomial Analysis); although as perhaps to be expected FVC (% predicted) was significantly lower in the "severe" cluster ($p<0.02$) (Table 4.12). Analysis between these 2 clusters and the control cohort demonstrated a significant difference for FVC ($p<0.032$ by one-way ANOVA), but no difference in age ($p=0.718$), gender ($p=0.631$), BMI ($p=0.335$) and total co-morbidities ($p=0.326$) respectively. Sub-population cluster 1 "moderate" had the lowest number of COPD patients (n = 15). Sub-population cluster 2 "severe" consisted of the highest number of COPD patients (n = 35), which was to be expected as the majority of COPD patients in the study had severe disease status.

The above results suggest two unique trajectories of FEV_1 during the stable-prodromal phase for the studied COPD patients. Interestingly GOLD classification for COPD disease severity (Vestbo et al., 2013) sets four FEV_1 thresholds; within this patient cohort there is no indication to increase the classification of COPD patients based on sub-population clusters by more than 2.

These 2 identified clusters will be used later in a corollary analysis (Section 4.4.4, Page 350) to form a multidimensional score of COPD patients encompassing symptoms, spirometry and salivary biomarkers.

Table 4.12: Covariates comparison between the 2 patient exacerbator clusters for FEV_1 and control non-exacerbator cohort.

Baseline Covariates	COPD Sub-Population Clusters for FEV_1 Exacerbation Cohort (n = 49)			COPD Control Group Non-Exacerbation Cohort	
	1 (n = 14)	2 (n = 35)	*p-value	(n = 6)	**p-value
Age, [a]years	69.1 ± 6.3	67.8 ± 8.6	p=0.828	70.3 ± 6.4	p=0.718
Gender Male, (Female)	8,7	17, 18	p=0.430	2, 4	p=0.631
BMI, [a](kg/m^2)	28.3 ± 6.1	26.9 ± 5.3	p=0.915	24.4 ± 4.2	p=0.335
Total Co-Morbidities, [a]n	1.5 ± 1.2	1.2 ± 0.9	p=0.511	0.8 ± 0.8	p=0.326
FVC [a](% predicted)	84.4 ± 14.7	74.0 ± 13.1	**p<0.02**	85.0 ± 16.9	**p<0.032**

Data presented as a = mean ± SD. *P-value indicated the statistical significance between the 2 patient exacerbator sub-population clusters (n = 49) and the control non-exacerbator cohort (n = 6) by Binomial logistic regression: **p-value indicates the statistical significance between the same groups by one-way ANOVA.

4.4.2.2. Forced vital capacity (FVC)

Analysis across the 4 study-defined phases in COPD patients who experienced at least one acute exacerbation (n = 49) revealed a statistically significant difference in FVC ($p<0.001$ by repeated measures ANOVA). Post-hoc analysis showed a significant reduction between the stable and prodromal phase FVC values ($p<0.018$($) by independent t-test) and between the stable phase and exacerbation phase values ($p<0.001$($)). There was no statistically significant difference between the stable and post-exacerbation-recovery phase FVC values ($p=0.999$). A significant reduction was also demonstrated between the prodromal and exacerbation phase FVC values ($p<0.034$($)), but not between the prodromal and post-exacerbation-recovery phase FVC values ($p=0.076$). A significant improvement was observed in FVC between the exacerbation and post-exacerbation-recovery phases ($p<0.001$($)) (Figure 4.12), (Table 4.13). Cross-analysis between the exacerbator (n = 49) and non-exacerbator (n = 6) cohorts demonstrated no statistically significant difference in stable phase FVC ($p=0.141$, by independent t-test). Although there appeared to be a large difference in mean stable FVC between these 2 cohorts, it is important to note the wide standard deviation ranges and the large difference in sample sizes between them.

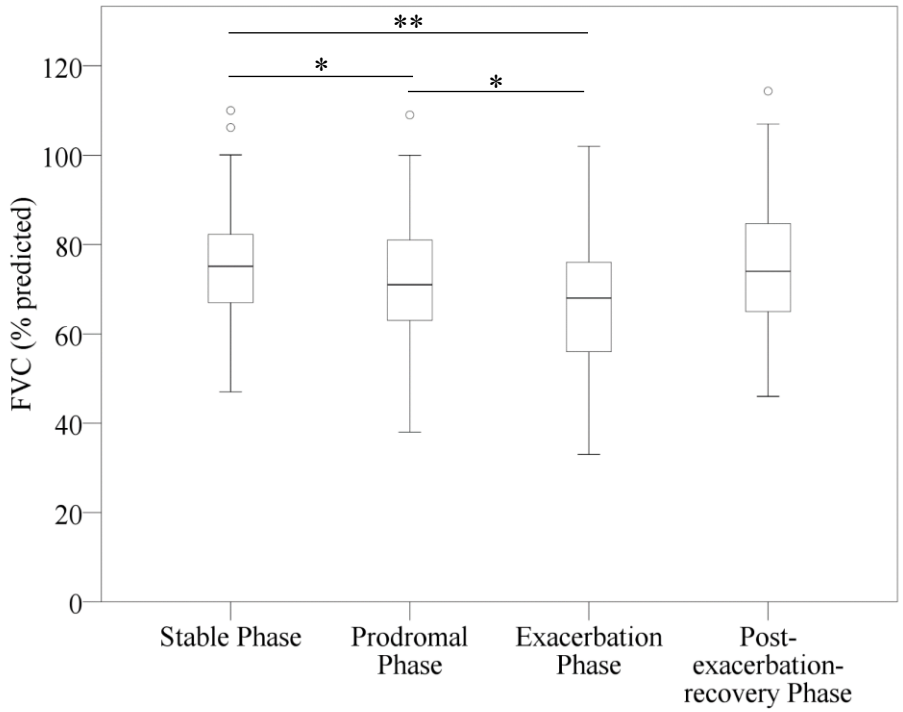

Figure 4.12: Forced vital capacity across the 4 defined COPD phases.

Box and whisker plots for forced vital capacity (FVC) across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery for the COPD patients who exacerbated (n = 49). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days post treatment completion. Outliers are identified by ^{o} (1.5 x the interquartile range). There was a significant statistical difference in FVC across the 4 phases ($p<0.001$ by repeated measures ANOVA). Post-hoc analysis showed a significant reduction between the stable and prodromal phase FVC values (*$p<0.018$($) by independent t-test) and between the stable phase and exacerbation phase values (**$p<0.001$($)). There was no statistically significant difference between the stable and post-exacerbation-recovery phase FVC values ($p=0.999$). A significant reduction was also demonstrated between the prodromal and exacerbation phase FVC values (*$p<0.034$($)) but not between the prodromal and post-exacerbation-recovery phase FVC values ($p=0.076$). Significant improvement was observed in FVC between the exacerbation and post-exacerbation-recovery phases ($p<0.001$($)).

Table 4.13: FVC values across the whole study cohort.

	FVC (% predicted)			
COPD patients (n = 55)	**Stable Phase**	**Prodromal Phase**	**Exacerbation Phase**	**Post Exacerbation Recovery Phase**
Exacerbators (n = 49)	75.3 ± 14.6	71.8 ± 15.3	67.2 ± 16.2	75.4 ± 15.3
Non-Exacerbators (n = 6)	84.9 ± 16.7	n/a	n/a	n/a

Data presented as mean ± SD

4.4.2.2.1. Latent class growth analysis

Utilising data from the exacerbator cohort (n = 49), latent class growth analysis (LCGA) was performed on FVC values across the stable-prodromal period at 5 different time points, each 1 week apart from each other progressing towards an acute exacerbation of COPD.

As before, an iterative LCGA was conducted using the BIC and BLRT p-value to determine the pilot number of sub-population clusters (Table 4.14) for COPD patients' stable-prodromal phase FVC values. Although the BIC value reduced from 1 to 2 sub-population clusters this was not statistically significant (p=0.286 by BLRT). Thus for FVC in the COPD exacerbator group (n = 49) there are no discrete sub-population clusters (Figure 4.13).

This result is interesting and can be explained by understanding how the LCGA clusters COPD patients. LCGA assigns COPD patients to a particular sub-population cluster not only on the target variable, in this case FVC, but also the change in that target variable over time. Thus although the percentage predicted FVC levels across the COPD patients falls within a wide range (Table 4.2, Page 280), the rate of change of FVC at different points lacks enough variability to classify the COPD patients into further sub-population clusters unlike FEV_1. Accordingly, FVC will not be included in the final correlative analysis as it appears not to provide any further information on the sub-populations within the exacerbation cohort (n = 49).

Table 4.14: LCGA Goodness of fit tests for stable-prodromal FVC data.

LCGA	BIC	BLRT (p-values)
1 cluster	1728	n/a
2 cluster	1679	p=0.286

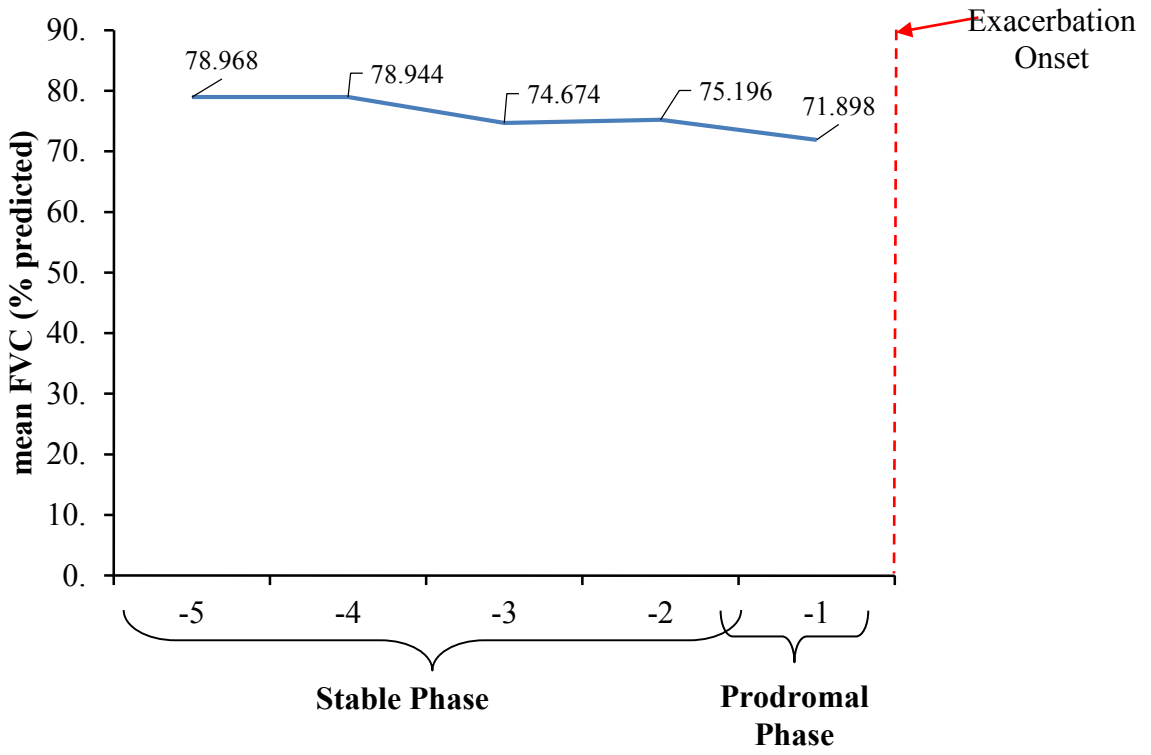

Figure 4.13: Forced vital capacity cluster across stable-prodromal phases.

This line chart represents the latent class growth analysis for the mean percentage predicted forced vital capacity (FVC) values for COPD patients who experienced at least one acute exacerbation of COPD (n = 49) in the stable and prodromal phases. This period is defined as 5 weeks prior to the onset of an exacerbation and each interval represents 1 week. The values represent the mean FVC at that time-point for the respective clusters. There were no identifiable distinct sub-population clusters for FVC in this COPD exacerbator cohort.

4.4.2.3. COPD sub-population: re-exacerbators

The next analysis looked to determine whether stable phase FEV_1, FVC and the index exacerbation and post-exacerbation-recovery spirometry values were significantly different between the single-exacerbator and re-exacerbator groups (Table 4.15). Overall stable phase FEV, values were significantly lower in the sub-population who re-exacerbated compared to those who remained stable ($p<0.017$ by independent t test). Utilising the LCGA sub-population clusters for FEV_1 (Table 4.11, Page 303) there was a significant difference between the total number of COPD patients who re-exacerbated between the two clusters ($p<0.001$ by Mann Whitney U Test).

Table 4.15: Spirometry values between single and re-exacerbators

	COPD Patients (n = 49)		
Spirometry (% predicted)	**Single Exacerbators (n = 34)**	**Re-Exacerbators (n = 15)**	**p-value**
Stable Phase: FEV_1	55.4 ± 17.2	45.7 ± 13.8	**p<0.017**
Exacerbation Phase: FEV_1	50.2 ± 17.8	41.9 ± 14.7	p=0.110
Post-exacerbation-recovery Phase FEV_1	54.6 ± 19.6	44.0 ± 16.6	p=0.069
Stable Phase: FVC	78.2 ± 15.3	74.2 ± 11.6	p=0.286
Exacerbation Phase FVC	70.5 ± 15.0	67.9 ± 15.1	p=0.904
Post-exacerbation-recovery Phase FVC	77.1 ± 15.9	70.9 ± 12.8	p=0.999

Data presented as mean ± SD.

4.4.3. Salivary biomarker levels.

Salivary CRP, PCT and NE levels were measured in all the studied 55 COPD patients. Analyte measurements were carried out using a total of; (1) 20 Salimetrics CRP ELISA kits (Salimetrics, USA) with an intra- and inter-assay CV of 9.6% and 14.5% respectively; (2) Thirty VIDAS B.R.A.H.M.S PCT (bioMérieux, France) with an intra- and inter-assay CV of 9.4% and 12.1% respectively; and (3) Twenty PMN Elastase ELISA kit (Immundiagnostik, Germany) with an intra-assay CV of 4.0% and inter-assay CV of 9.3% respectively.

49 out of 55 COPD patients reported at least one acute exacerbation. Out of these 49 COPD patients, 47 patients experienced a rise in at least 1 salivary biomarker; 40 COPD patients experienced a rise in at least 2 salivary biomarkers; and 20 COPD patients experienced a rise in all 3 salivary biomarkers during the prodromal and exacerbation phases.

The study protocol involved weekly saliva sampling; when a patient felt that they were about to have an exacerbation, a saliva sample was taken on the same day in the provided collector tubes and kept in their freezer until collected by the research team as soon as possible within 12 hours. Saliva prodromal phase samples from the exacerbator cohort were therefore from varying time points (5.24 ± 1.93 days) up to 7 days prior to the onset of an exacerbation.

As before, salivary biomarker levels were split into the 4 previously described phases (Table 4.1, Page 275) to aid analysis and to determine whether saliva prodromal phase biomarker data differed from stable baseline so as to be predictive of an exacerbation onset. The non-exacerbator COPD cohort (n = 6) acted as the control group and their stable phase salivary biomarker levels were cross-compared to the exacerbator cohort (n = 49).

4.4.3.1 Salivary C-reactive Protein (CRP)

Analysis across the 4 defined phases in the exacerbator cohort (n = 49) showed a statistically significant change in salivary CRP levels ($p<0.001$ by Freidman's Two-way ANOVA). Post-hoc analysis further demonstrated that salivary CRP levels were significantly increased during an exacerbation (4.20, 6.33 ng/ml) compared to stable phase (2.38, 1.54 ng/ml, $p<0.003$($) by Wilcoxon Signed Rank Test). Prodromal phase salivary CRP (2.91, 4.27 ng/ml), was also significantly higher than stable phase ($p<0.001$($)), with no significant difference found between post-exacerbation-recovery phase and the stable phase data salivary CRP ($p=0.208$). (Table 4.16, Figure 4.14). Salivary CRP levels in this exacerbator cohort were increased in the prodromal (mean sample collection: 5.3 ± 1.9 days) prior to the onset of an exacerbation), and exacerbation phases compared to stable phase in 42 out of 49 COPD patients (Figure 4.15). Cross-analysis between the exacerbators (n = 49) and non-exacerbators (n = 6) demonstrated no statistically significant difference in stable phase salivary CRP levels between the two cohorts ($p=0.282$, by Mann-Whitney U Test).

Table 4.16: Salivary CRP levels across the whole COPD study cohort.

	Salivary CRP (ng/ml)			
COPD patients (n = 55)	**Stable Phase**	**Prodromal Phase**	**Exacerbation Phase**	**Post Exacerbation Recovery Phase**
Exacerbators (n = 49)	2.4, 1.5	2.9, 4.3	4.2, 6.3	2.5, 2.9
Non-Exacerbators (n = 6)	1.7, 0.2	n/a	n/a	n/a

Data presented as median, IQR.

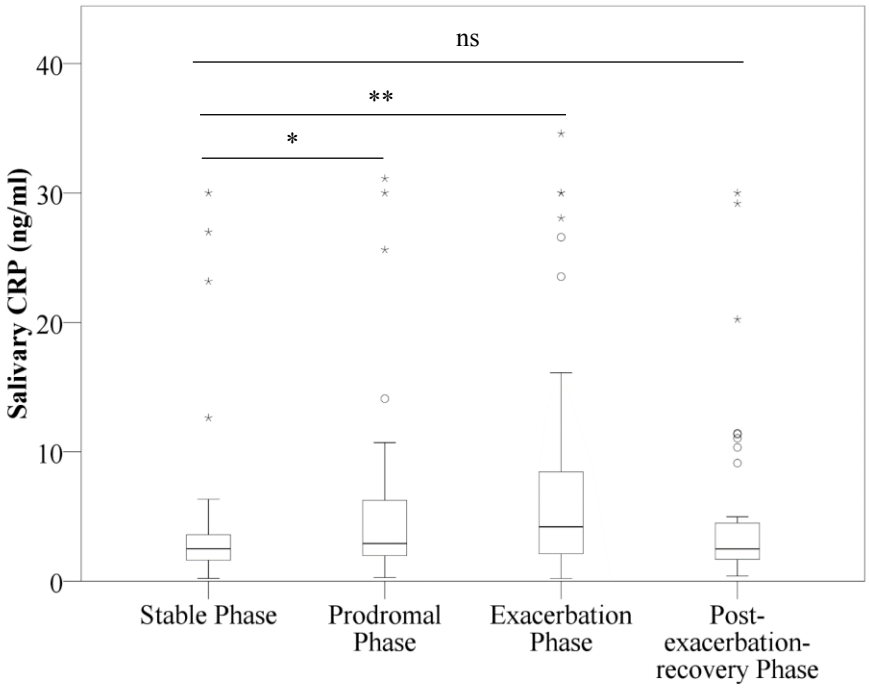

Figure 4.14: Salivary C-reactive protein (CRP) levels across the 4 defined COPD phases in the whole patient exacerbator group.

Box and whisker plots for salivary C-reactive protein (CRP) levels across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery for the whole exacerbator group (n = 49). Outliers are identified by º (1.5 x the interquartile range) and * (3 x interquartile range). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days' post treatment completion. There was a significant statistical difference across the 4 phases ($p<0.001$ by Freidman's Two-way ANOVA). Post-hoc analysis showed that salivary CRP levels were significantly increased during an exacerbation compared to stable phase (**$p<0.003$($) by Wilcoxon Signed Rank Test). Prodromal phase salivary CRP was also significantly higher than stable phase (*$p<0.001$($)), with no significant difference demonstrated between post-exacerbation-recovery phase and stable phase salivary CRP (ns: $p=0.208$).

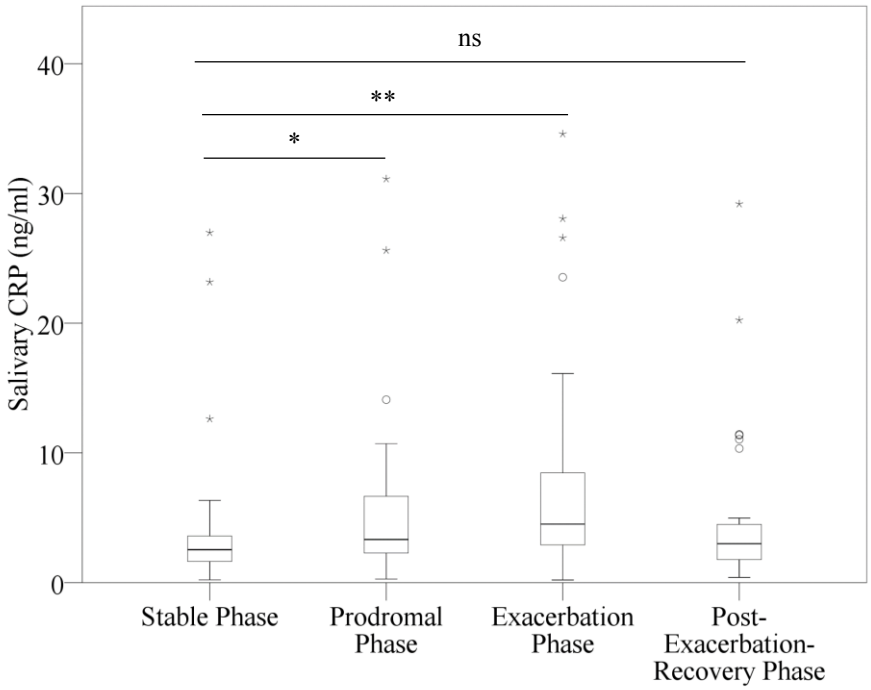

Figure 4.15: Salivary C-reactive protein (CRP) levels across the 4 defined COPD phases in patients who experienced raised analyte levels.

Box and whisker plots for salivary CRP levels across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery are shown only in those COPD patients who experienced a rise in CRP in either the prodromal and exacerbation phases (n = 42). Outliers are identified by ^{o} (1.5 x the interquartile range) and * (3 x interquartile range). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days' post treatment completion. The statistical analysis for this data set is identical to the whole patient exacerbator group (Figure 4.14). There was a significant statistical difference across the 4 phases ($p<0.001$ by Freidman's Two-way ANOVA). Post-hoc analysis showed that salivary CRP levels were significantly increased during an exacerbation compared to stable phase (**$p<0.003$($) by Wilcoxon Signed Rank Test). Prodromal phase salivary CRP was also significantly higher than stable phase (*$p<0.001$($)), with no significant difference demonstrated between post-exacerbation-recovery phase and stable phase salivary CRP (ns: $p=0.208$).

To understand whether a cut-off could be attributed to exacerbation phase salivary CRP levels, a further sub-analysis was conducted utilising an area under the curve (AUC) analysis with receiver operating characteristics (ROC) curves for the whole patient exacerbator group. Overall there was significant AUC = 0.70, $p<0.001$, 95% CI (0.58 to 0.80) (Figure 4.16). Sensitivity and specificity at 3 levels of salivary CRP for detecting the onset of acute exacerbation is shown in Table 4.17. These points were selected as they represented optimal salivary CRP values for sensitivity, specificity and both parameters combined.

Table 4.17: Sensitivity and specificity of exacerbation phase salivary CRP levels.

Salivary CRP (ng/ml)	Sensitivity	Specificity
0.39	98%	2%
2.91	70%	63%
6.21	37%	90%

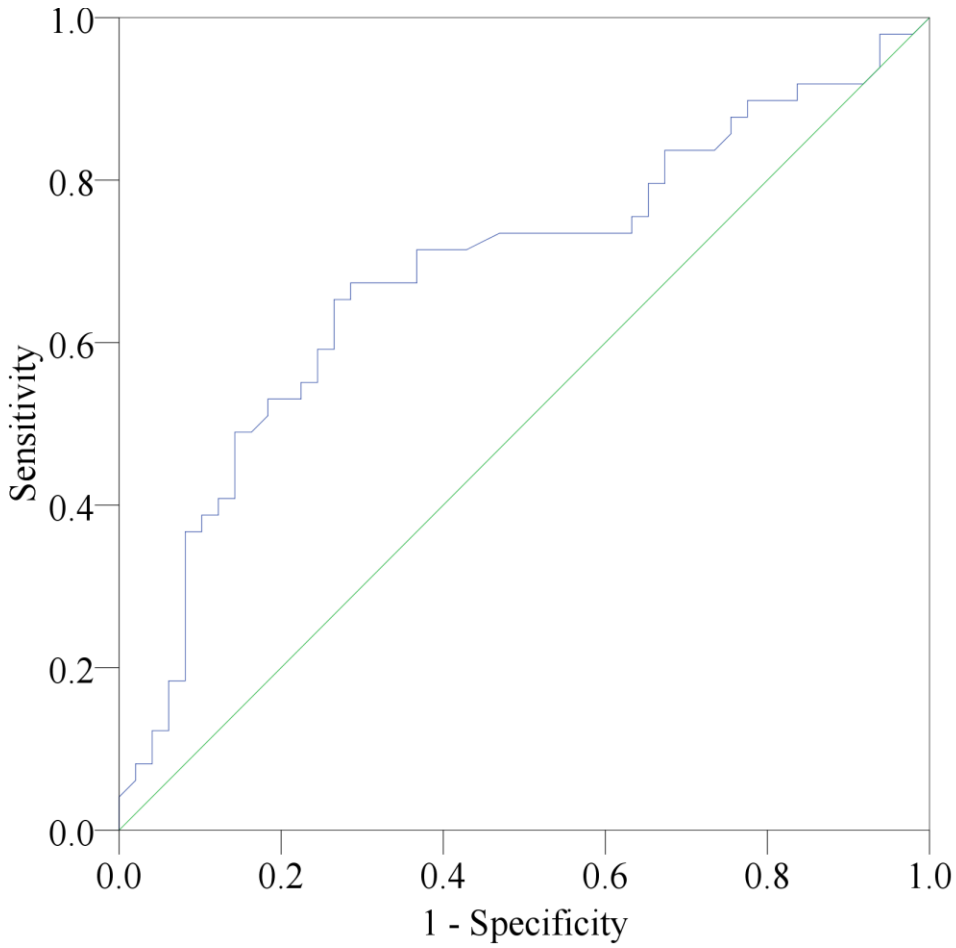

Figure 4.16: ROC curve of salivary CRP levels.

This ROC curve highlights the sensitivity and specificity of salivary CRP in distinguishing the onset of a COPD exacerbation. Overall there is a significant AUC = 0.70, $p<0.001$, 95% CI (0.58 to 0.80). This highlights that salivary CRP levels at the onset of an exacerbation can be used with moderate diagnostic accuracy.

4.4.3.1.1. Latent class growth analysis

Latent class growth analysis (LCGA) was performed on salivary CRP levels from the exacerbator patient cohort (n = 49) across the stable-prodromal phases at 5 different time points, each 1 week apart, progressing towards the onset of an exacerbation,

An iterative LCGA using the BIC and BLRT p-value was conducted to determine the pilot number of sub-population clusters (Table 4.18) for COPD patients' salivary CRP levels. Although the BLRT favoured increasing the number of clusters from 3 to 4, there was only a minimal improvement in the BIC beyond 3 clusters; the posterior probability matrix (Table 4.19) also demonstrated a high level of homogeneity between the clusters. Each cluster formed three discrete COPD sub-populations with an inter-cluster overlap of less than 1% for clusters 1 and 2 and no overlap for cluster 3. 3 sub-population clusters within the exacerbator group were chosen to interrogate salivary CRP levels across the stable-prodromal phase: Cluster 1 = 41; Cluster 2 = 4; and Cluster 3 = 4 patients. The membership of each COPD patient in their respective cluster is shown in Table 4.20. Each sub-population cluster was defined as "low", "mid" and "high" range based on the relative levels of salivary CRP to each other. Thus sub-population cluster 1 was defined as "low-range"; cluster 2 as "mid-range" and cluster 3 as "high-range". Overall these sub-population clusters exhibited three unique salivary CRP level trajectories across the stable-prodromal phases (Figure 4.17).

Table 4.18: LCGA Goodness of fit tests for stable-prodromal salivary CRP levels.

LCGA	BIC	BLRT (p-values)
1 cluster	1401	n/a
2 cluster	1240	**p<0.001**
3 cluster	1217	**p<0.001**
4 cluster	1217	**p<0.001**

The BIC value represents the goodness-of-fit; basically the lower value; the better the model. The p-value represents whether there is a significant improvement in the model with "n" clusters compared to "n-1". Of importance is the finding of minimal improvement in the BIC from 3 to 4 clusters.

Table 4.19: Homogeneity of COPD patients cluster membership for stable-prodromal salivary CRP levels.

Cluster	1 (n = 41)	2 (n = 4)	3 (n = 4)
1	97%	3%	0%
2	0%	100%	0%
3	0%	0%	100%

Table 4.20: Exacerbator COPD patient cluster membership for stable-prodromal salivary CRP levels.

COPD Patient	Cluster Membership
1	3
2	1
3	1
4	1
5	1
6	1
7	1
8	1
9	1
10	1
11	1
12	1
13	1
14	1
15	1
16	1
17	3
18	1
19	1
20	1
21	1
22	1
23	1
24	3
25	1
26	1
27	1
28	1
29	1
30	1
31	2
32	3
33	1
34	2
35	1
36	1
37	2
38	1
39	1
40	2
41	1
42	1
43	1
44	1
45	1
46	**1**
47	**1**
48	**1**
49	**1**

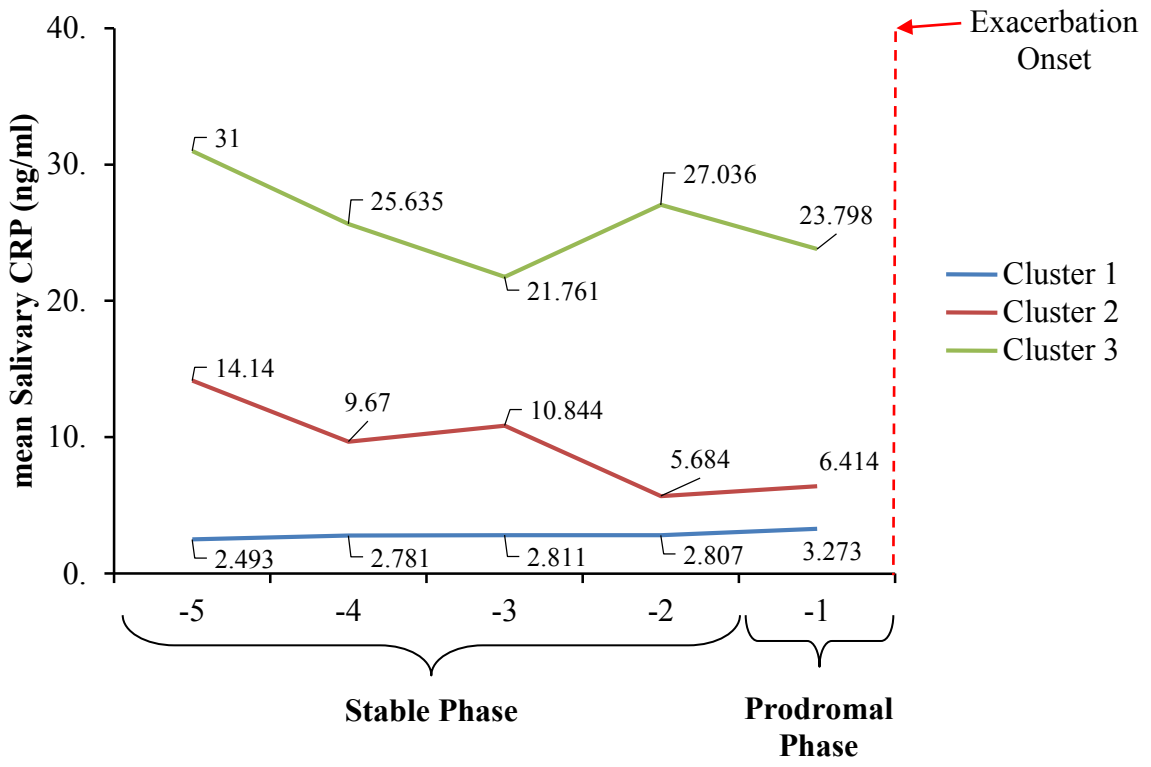

Figure 4.17: Salivary CRP clusters across the stable-prodromal phases.

This line chart represents the LCGA for the mean salivary CRP levels for COPD patients who experienced at least one acute exacerbation of COPD (n = 49) in the stable and prodromal phases. This period is defined as 5 weeks prior to the onset of an exacerbation phase and each interval represents 1 week. Cluster 1 = 41, Cluster 2 = 4, Cluster 3 = 4 COPD patients. The values represent mean salivary CRP levels at each time-point for the respective clusters.

4.4.3.1.2. Baseline characteristics associated with cluster membership

A separate sub-analysis was conducted to explore whether there were significant differences between the 3 clusters for a panel of known COPD co-variates (age, gender, BMI, total co-morbidities, FEV_1 and FVC). Multinomial analysis between the 3 clusters cross compared with each other demonstrated no significant difference in the 6 tested co-variates between each sub-population cluster (Table 4.21, Page 325). Following on, a further sub-analysis was performed between the patient exacerbator individual clusters and the non-exacerbator control cohort (n = 6) to determine if there was a significant difference between each sub-population cluster and the control cohort (Table 4.22, Page 326). Overall there was no significant difference between the 3 exacerbator sub-population clusters and the control non-exacerbator cohort for all co-variates: age (p=0.653), gender (p=0.188), BMI (p=0.308), total co-morbidities (p=0.540), FEV_1 (p=0.298) and FVC (p=0.345 by one-way ANOVA).

Sub-population cluster 1 “low-range” salivary CRP consists of COPD patients (n = 41) with a lower age and total co-morbidities and much better lung function compared to sub-population clusters 2 and 3. This cluster demonstrated a subtle increased trajectory in salivary CRP levels towards the onset of an exacerbation (Figure 4.17). Sub-population cluster 2 “medium-range” salivary CRP (n = 4) had the lowest FEV_1 and FVC, reflecting a more severe disease status cohort despite the levels of salivary CRP in a “mid-range”. The trajectory for this cluster appeared to be more variable than cluster 1 (Figure 4.17). Sub-population cluster 3 “high-range” salivary CRP (n = 4) had the highest total number of recorded co-morbidities and comparatively mid-range FEV_1 and FVC values. This sub-population cluster demographic is interesting as patients within this cluster had the- highest stable-prodromal phase salivary CRP and co-morbid burden despite having an FEV_1 similar to cluster 1. These observations could reflect the presence of a cohort of COPD patients with an accelerated disease progression

driven by an enhanced systemic inflammatory status consequent on a higher co-morbidity burden. Cluster 3 also demonstrated a variable trajectory similar to cluster 2. The control non-exacerbator cohort was not significantly different than the 3 sub-population clusters for any of the tested covariates

In summary, salivary CRP levels appear to rise somewhere up to 7 days prior to exacerbation onset. LCGA reveals 3 distinct sub-population clusters of COPD exacerbator patients based on the trajectory of stable-prodromal phase salivary CRP levels over the preceding 5 weeks to an exacerbation onset, with subtle differences existing between the different clusters. However these differences were not statistically significant to each other or the control cohort (Table 4.21; Table 4.22). These clusters will be used in the corollary analysis (Section 4.4.4, Page 350) to form a multidimensional classification score of COPD patients encompassing symptoms, spirometry and salivary biomarkers.

Table 4.21: Multinomial regression of covariates between the 3 exacerbator sub-population clusters for salivary CRP.

Baseline Covariates	COPD Exacerbator Sub-Population Clusters for Salivary CRP (n = 49)		
	*Cluster 2 (n = 4)	*Cluster 3 (n = 4)	**Cluster 3
Age, years	p=0.468	p=0.811	p=0.614
Gender Male, (Female)	p=0.342	p=0.466	p=0.583
BMI, kg/m^2	p=0.117	p=0.586	p=0.208
Total Co-Morbidities, n	p=0.145	p=0.700	p=0.187
FEV_1 (% predicted)	p=0.996	p=0.818	p=0.913
FVC (% predicted)	p=0.423	p=0.630	p=0.615

*p-values compared to cluster 1 (n = 41), **p-values compared to cluster 2.

Table 4.22: Covariates comparisons between the 3 patient exacerbator clusters and non-exacerbator control cohort for salivary CRP.

Baseline Covariates	COPD Exacerbator Clusters for sCRP (n = 49)			COPD Control Non-Exacerbator Cohort	*p-value
	1 (n = 41)	2 (n = 4)	3 (n = 4)	(n = 6)	
Age, years	67.7 ± 8.1	68.7 ± 7.8	72.3 ± 6.7	70.33 ± 6.4	p=0.653
Gender Male, (Female)	20, 21	2, 2	3, 1	2, 4	p=0.188
BMI, (kg/m^2)	27.8 ± 5.7	24.3 ± 5.7	25.4 ± 2.7	24.40 ± 4.2	p=0.308
Total Co-Morbidities, n	1.22 ± 0.99	1.25 ± 0.96	1.75 ± 0.95	0.83 ± 0.8	p=0.540
FEV_1 (% predicted)	51.7 ± 18.6	39.3 ± 12.7	48.8 ± 24.3	63.44 ± 27.9	p=0.298
FVC (% predicted)	77.9 ± 15.0	67.8 ± 10.6	76.5 ± 5.0	84.95 ± 16.9	p=0.345

Data are presented as mean ± SD. *p–value indicate the difference between the 3 patient exacerbator sub-population clusters (n =49) and the control non-exacerbator patient cohort (n = 6). sCRP = salivary CRP levels.

4.4.3.2. Salivary Procalcitonin (PCT)

Analysis across the 4 defined phases in the COPD exacerbator patient cohort (n = 49) demonstrated a statistically significant change in salivary PCT levels ($p<0.001$). Post-hoc analysis further showed that salivary PCT levels were only significantly increased at exacerbation onset (0.12, 0.11 ng/ml) compared to the stable phase (0.09, 0.09 ng/ml, $p<0.001$($)) (Table 4.23). Prodromal phase salivary PCT levels were not statistically significantly higher than stable phase salivary PCT ($p=0.07$), nor was there significant difference to post-exacerbation-recovery phase salivary PCT ($p=0.453$) (Figure 4.18). However, within the exacerbator cohort salivary PCT was increased during the prodromal (mean sample collection time: 5.4 ± 1.6 days) prior to the onset of an exacerbation) and exacerbation phases compared to stable phase in 30 of 49 COPD patients (Figure 4.19). Cross-analysis between the exacerbators (n = 49) and control non-exacerbators (n = 6) cohorts demonstrates no statistically significant difference in stable phase salivary PCT levels ($p=0.484$, by Mann-Whitney U Test).

Table 4.23: Salivary PCT levels across the whole COPD study cohort.

	Salivary PCT (ng/ml)			
COPD patients	**Stable Phase**	**Prodromal Phase**	**Exacerbation Phase**	**Post-Exacerbation-Recovery Phase**
Exacerbators (n = 49)	*0.09, 0.01	*0.09, 0.09	0.12, 0.11	*0.09, 0.50
Non-Exacerbators (n = 6)	*0.09, 0.00	n/a	n/a	n/a

Data presented as median, IQR. *Values below the lower limit of assay quantification assigned as 0.09ng/ml for statistical analysis

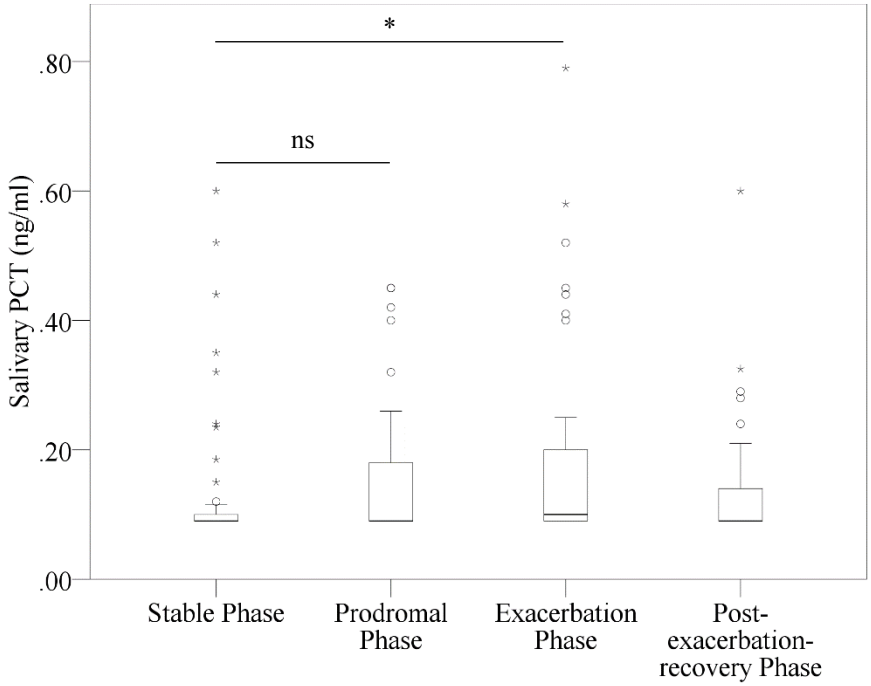

Figure 4.18: Salivary Procalcitonin (PCT) levels across the 4 defined COPD disease phases in the whole exacerbator group.

Box and whisker plots for salivary CRP levels across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery for the whole exacerbator group (n = 49). Outliers are identified by º (1.5 x the interquartile range) and * (3 x interquartile range). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days post treatment completion. There was a significant statistical difference across the 4 phases ($p<0.001$ by Freidman's Two-way ANOVA). Post-hoc analysis showed salivary PCT was only significantly increased in the exacerbation phase compared to stable phase (*$p<0.001$($) by Wilcoxon signed rank test). Prodromal phase salivary PCT was not significantly higher than stable phase salivary PCT (ns: $p=0.07$); no significant difference was demonstrated between post-exacerbation-recovery phase and stable salivary PCT ($p=0.453$)

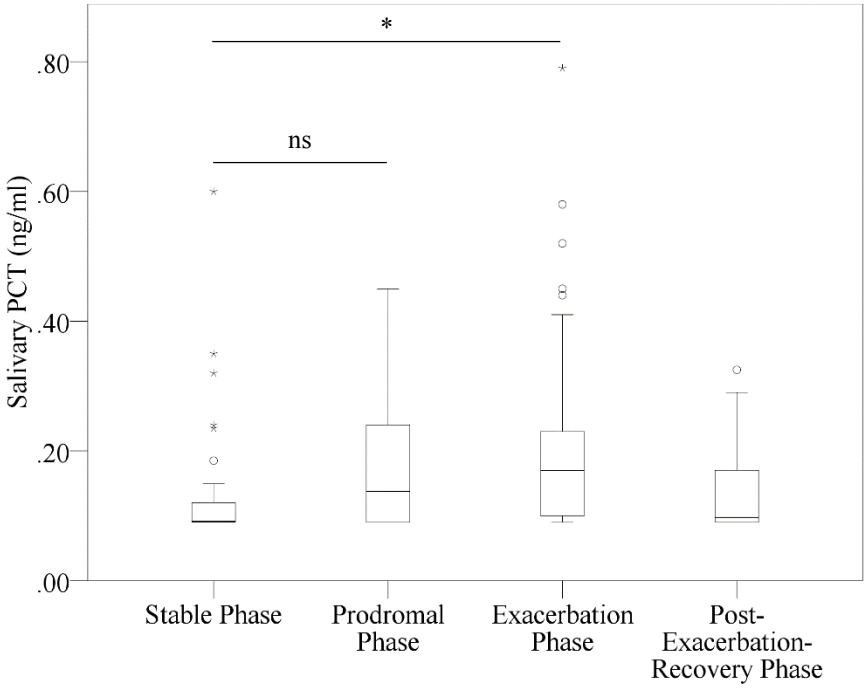

Figure 4.19: Salivary Procalcitonin (PCT) levels across the 4 defined COPD phases in patients who experienced raised analyte levels.

Box and whisker plots for salivary CRP levels across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery are shown only in those COPD patients who experienced a rise in PCT in either the prodromal and/or exacerbation phase (n = 30). Outliers are identified by º (1.5 x the interquartile range) and * (3 x interquartile range). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days post treatment completion. The statistical analysis for this data set is identical to whole patient exacerbator group (Figure 4.18). There was a significant statistical difference across the 4 phases ($p<0.001$ by Freidman's Two-way ANOVA). Post-hoc analysis showed salivary PCT was only significantly increased in the exacerbation phase compared to stable phase (*$p<0.001$($) by Wilcoxon signed rank test). Prodromal phase salivary PCT was not significantly higher than stable phase salivary PCT (ns: $p=0.07$); no significant difference was demonstrated between post-exacerbation-recovery phase and stable salivary PCT ($p=0.453$)

To understand whether a cut-off could be attributed to exacerbation phase levels of salivary PCT, a further sub-analysis was conducted utilising AUC analysis with ROC curves. Overall there was significant AUC = 0.63, $p<0.03$, 95% CI (0.52 to 0.74) (Figure 4.20). Sensitivity and specificity at 2 levels of salivary PCT for detecting the onset of acute exacerbation is shown in Table 4.24. These points were chosen as they represented optimal salivary PCT values for sensitivity, specificity and both parameters combined.

Table 4.24: Sensitivity and specificity of exacerbation phase salivary PCT levels.

Salivary PCT (ng/ml)	Sensitivity	Specificity
0.11	57%	67%
0.23	16%	86%

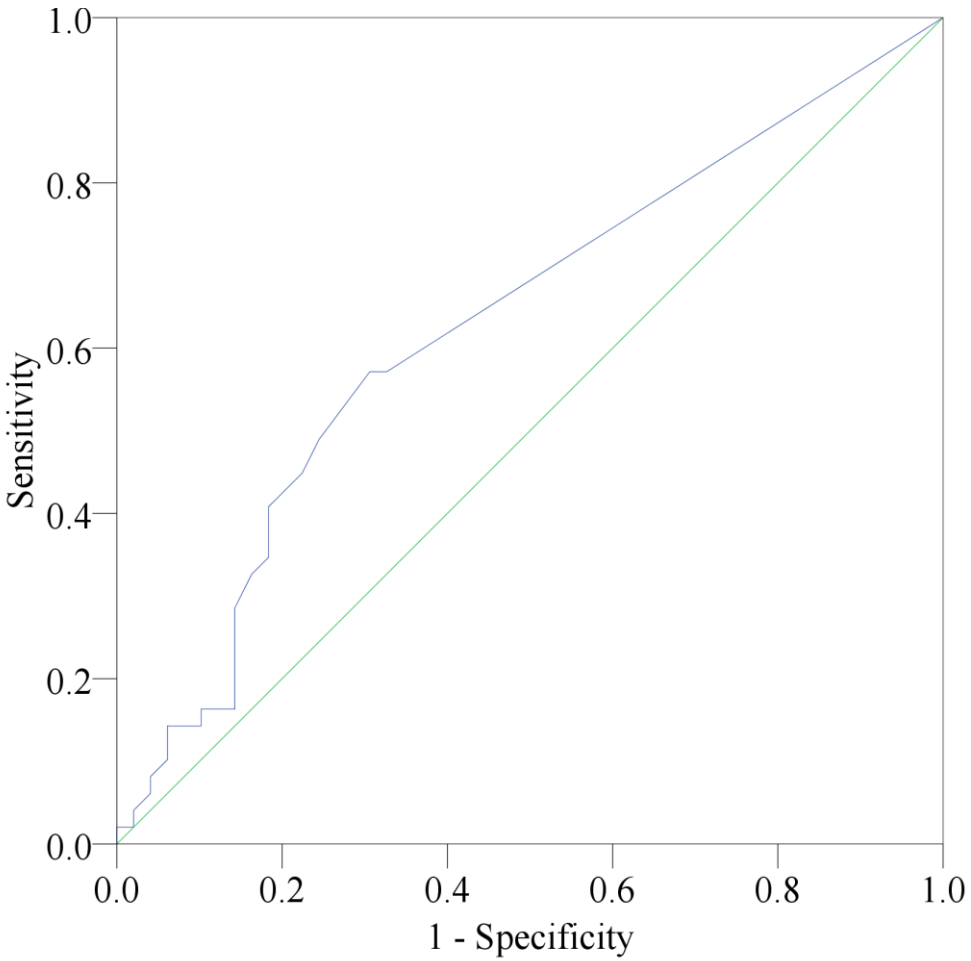

Figure 4.20: ROC curve of salivary PCT levels.

This ROC curve highlights the sensitivity and specificity of salivary PCT in distinguishing an acute exacerbation of COPD. Overall there is a significant AUC = 0.63, p<0.03, 95% CI (0.52 to 0.74) This highlights that salivary PCT levels at the onset of an exacerbation can be used with moderate diagnostic accuracy.

4.4.3.2.1. Latent class growth analysis

Latent class growth analysis (LCGA) was performed on salivary PCT levels from the exacerbator cohort (n = 49) across the stable-prodromal period at 5 different time points, each 1 week apart progressing towards an acute exacerbation of COPD.

As previously, an iterative LCGA was conducted using the BIC and BLRT p-value to determine the pilot number of sub-population clusters (Table 4.25) for COPD patients' salivary PCT levels. From this result, the posterior probabilities for cluster membership (Table 4.26) were assessed and it was determined whether increasing the cluster number improved or worsened the homogeneity of the created sub-populations. Overall sub-population cluster membership was shown to be the most discrete at 2 clusters: Cluster 1 = 41 and Cluster 2 = 8 COPD patients. The membership of each COPD patient to their respective cluster is shown in Table 4.27. Overall each cluster formed two completely discrete sub-populations of COPD exacerbator patients with no inter-cluster overlap. Each sub-population cluster was defined as "low" or "high" range based on the relative levels of salivary PCT to each other, with cluster 1 assigned as "low" and cluster 2 as "high" range. These sub-population clusters exhibited 2 unique salivary PCT trajectories over the stable-prodromal (Figure 4.21).

Table 4.25: LCGA Goodness of fit tests for stable-prodromal salivary PCT levels.

LCGA	BIC	BLRT (p-values)
1 cluster	-146.563	n/a
2 cluster	-290.948	**p<0.001**
3 cluster	-267.597	p=1.000

The p-value represents whether there is a significant improvement in the model with "n" clusters compared to "n-1".

Table 4.26: Homogeneity of COPD patients cluster membership for stable-prodromal salivary PCT levels.

Cluster	1 (n = 41)	2 (n = 8)
1	100%	0%
2	0%	100%

Table 4.27: COPD patients cluster membership for stable-prodromal salivary PCT levels.

COPD Patient	Cluster Membership
1	1
2	1
3	1
4	1
5	1
6	1
7	1
8	1
9	1
10	2
11	1
12	1
13	1
14	1
15	1
16	2
17	2
18	1
19	1
20	1
21	1
22	2
23	1
24	2
25	1
26	1
27	1
28	1
29	1
30	1
31	1
32	1
33	1
34	2
35	1
36	1
37	1
38	1
39	1
40	2
41	1
42	2
43	1
44	1
45	1
46	1
47	1
48	1
49	1

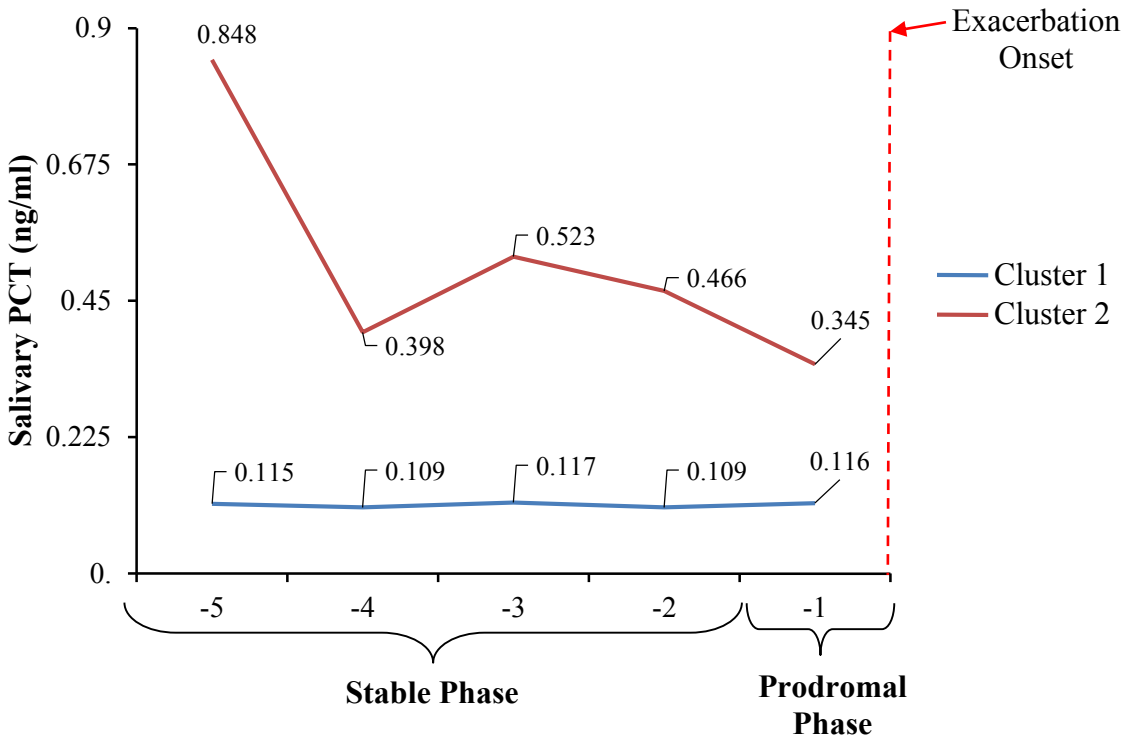

Figure 4.21: Salivary PCT clusters across the stable-prodromal phases.

This line chart represents the LCGA for the mean salivary PCT levels for COPD patients). who experienced at least one acute exacerbation of COPD (n = 49) in the stable and prodromal phases. This period is defined as 5 weeks prior to the onset of an exacerbation phase and each interval represents 1 week. Cluster 1 = 41 and Cluster 2 = 8 COPD patients. The values represent mean salivary PCT levels at each time-point for the respective clusters.

4.4.3.2.2. Baseline characteristics associated with cluster membership

A further separate sub-analysis was conducted to investigate if there were significant differences in co-variates (age, gender, BMI, total co-morbidities, FEV_1 and FVC) between the 2 sub-population clusters and also between these exacerbator clusters and the control non-exacerbator cohort. There was no significant difference in covariates between the 2 exacerbator clusters: age (p=0.702), gender (p=0.352), BMI (p=0.419), total co-morbidities (p=0.301), FEV_1 (p=0.801) and FVC (p<0.690 by Binomial analysis respectively) (Table 4.28). Also no significant differences were found between the individual clusters and the control non-exacerbator cohort (n = 6) for all covariates: age (p=0.414), gender (p=0.570), BMI (p=0.343), total co-morbidities (p=0.442), FEV_1 (p=0.071) and FVC (p=0.145 respectively) (Table 4.28).

Sub-population cluster 1 "low-range" salivary PCT had comparatively the lowest age, total number of co-morbidities and better lung function compared to cluster 2 "high-range" salivary PCT (Table 4.28). In contrast to cluster 1, the trajectory of cluster 2 declined towards the onset of an acute exacerbation; however salivary PCT levels still remained significantly elevated compared to cluster 1 (Figure 4.21). Although this analysis was conducted on an exacerbator cohort it may perhaps not be expected that elevated PCT levels would occur several weeks prior to an exacerbation. This cluster could possibly reflect a sub-population of patients with a high systemic inflammatory response or chronic bacterial airway colonisation. These two identified clusters for salivary PCT will also be used in the corollary analysis (Section 4.4.4, Page 350) to form a multidimensional score of COPD patients encompassing symptoms, spirometry and salivary biomarkers.

Table 4.28: Covariates comparison between the 2 patient exacerbator clusters and non-exacerbator control cohorts for salivary PCT.

Baseline Covariates	COPD Sub-Population Clusters for sPCT Exacerbation Cohort (n = 49)		*p-value	COPD Control Non-Exacerbation Cohort	**p-value
	1 (n = 41)	2 (n = 8)		(n = 6)	
Age, years	67.6 ± 8.3	71.1 ± 5.1	p=0.702	70.3 ± 6.4	p=0.414
Gender male, (female)	20, 21	5,3	p=0.352	2, 4	p=0.570
BMI, kg/m^2	27.6 ± 5.6	26.0 ± 5.1	p=0.419	24.4 ± 4.2	p=0.343
Total Co-Morbidities	1.2 ± 1.0	1.5 ± 1.1	p=0.301	0.8 ± 0.8	p=0.442
FEV_1 (% predicted)	52.6 ± 18.7	39.4 ± 14.7	p=0.801	63.4 ± 27.9	p=0.071
FVC (% predicted)	78.4 ± 14.6	69.8 ± 10.5	p=0.690	85.0 ± 16.9	p=0.145

Data presented as mean ± SD unless stated. * P-value represents the difference between the 2 patient exacerbator sub-population clusters; **p-value represents the difference between the exacerbator sub-population clusters and the non-control control cohort. sPCT = salivary PCT levels.

4.4.3.3. Salivary Neutrophil Elastase (NE)

Analysis across the 4 defined phases in the COPD exacerbator patient cohort who experienced at least one acute exacerbation (n = 49) showed revealed a statistically significant change in salivary NE levels ($p<0.011$). Post-hoc analysis demonstrated that salivary NE levels were increased during an exacerbation (745, 1260 ng/ml) compared to the stable phase (381, 1336 ng/ml, $p<0.012(\$)$). Prodromal phase salivary NE levels (900, 1904 ng/ml) were also significantly higher than stable phase ($p<0.007(\$)$) but no significant difference between post-exacerbation-recovery phase and stable phase salivary NE levels ($p=0.322$) (Table 4.29, Figure 4.22). Salivary NE in the exacerbator cohort was increased in the prodromal phase (mean saliva sample time: 5.5 ± 1.7 days) prior to the onset of an exacerbation) and exacerbation phases from baseline in 35 out of 49 COPD patients (Figure 4.23). Cross-analysis between the exacerbator (n = 49) and non-exacerbator (n = 6) cohorts demonstrated no statistically significant difference in stable phase salivary NE levels ($p=0.265$, by Mann-Whitney U Test).

Table 4.29: Salivary NE levels across the whole COPD study cohort.

	Salivary NE (ng/ml)			
COPD patients	**Stable Phase**	**Prodromal Phase**	**Exacerbation Phase**	**Post-Exacerbation-Recovery Phase**
Exacerbators (n = 49)	381, 1336	900, 1904	745, 1260	270, 1412
Non-Exacerbators (n = 6)	71, 164	n/a	n/a	n/a

Data presented as median, IQR.

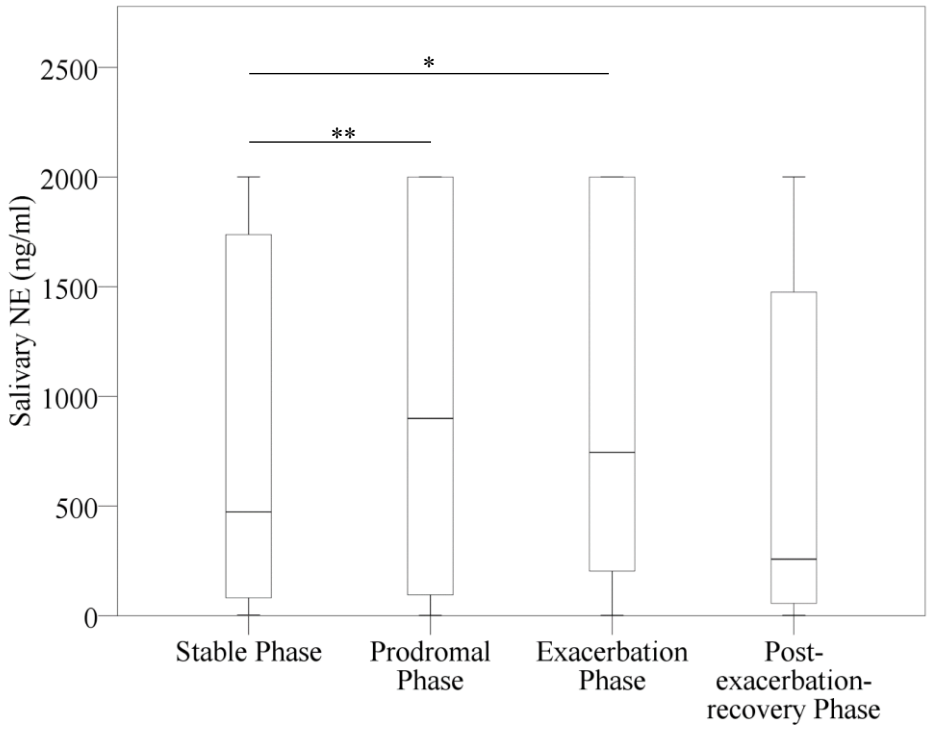

Figure 4.22: Salivary Neutrophil Elastase (NE) levels across the 4 defined COPD phases in the whole COPD exacerbator cohort.

Box and whisker plots for salivary CRP levels across the 4 disease phases: (1) Stable, (2) Prodromal, (3) Exacerbation, (4) Post-exacerbation-recovery (n = 49). Box and whisker plots for salivary CRP levels across the 4 disease phases: (1) Stable, (2) Prodromal, (3) Exacerbation, (4) Post-exacerbation-recovery (n = 49). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days post treatment completion. There was a significant statistical difference across the 4 phases ($p<0.011$ by Freidman's Two-way ANOVA). Post-hoc analysis showed that salivary NE levels were significantly increased during an exacerbation compared to the stable phase (*$p<0.012$($)) by Wilcoxon signed rank test). Prodromal phase salivary NE levels were also significantly higher than stable phase (**$p<0.007$($)) with no significant difference demonstrated between post-exacerbation-recovery and stable NE phase levels ($p=0.322$).

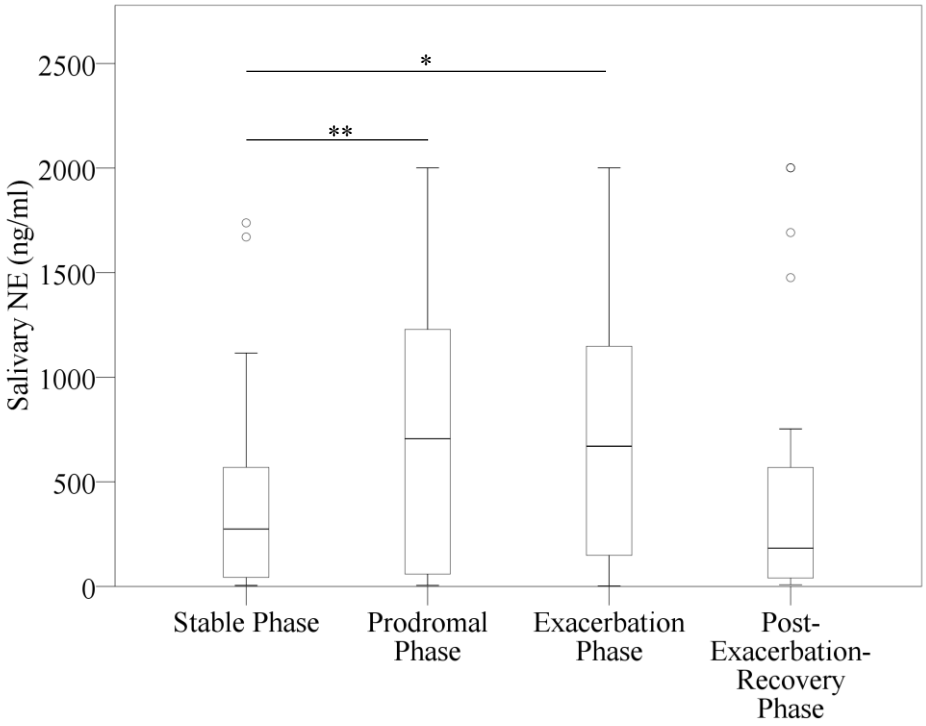

Figure 4.23: Salivary Neutrophil Elastase (NE) levels across the 4 defined COPD phases in patients who experienced raised analyte levels.

Box and whisker plots for salivary NE levels across the 4 disease phases: (1) Stable; (2) Prodromal; (3) Exacerbation; (4) Post-exacerbation-recovery in COPD patients who experienced a rise in either prodromal or exacerbation phase (n = 35). Outliers are identified by ° (1.5 x the interquartile range). The stable phase is defined as between 35 to 8 days prior to a patient-defined onset of an exacerbation. The prodromal phase is defined as between 7 to 1 days leading to an exacerbation onset. The exacerbation phase is defined as the onset of the acute episode and start of treatment (Day 0) to treatment completion and return to baseline (Day 7 post exacerbation). The post-exacerbation-recovery phase is defined as the immediate 14 days post treatment completion. The statistical analysis for this data set is identical to whole patient exacerbator group (Figure 4.22). There was a significant statistical difference across the 4 phases ($p<0.011$ by Freidman's Two-way ANOVA). Post-hoc analysis showed that salivary NE levels were significantly increased during an exacerbation compared to the stable phase (*$p<0.012$($)) by Wilcoxon signed rank test). Prodromal phase salivary NE levels were also significantly higher than stable phase (**$p<0.007$($)) with no significant difference demonstrated between post-exacerbation-recovery and stable NE phase levels ($p=0.322$).

To understand whether a cut-off could be attributed to exacerbation phase salivary NE levels, a further sub-analysis was conducted utilising AUC analysis with ROC curves. Overall there was no statistical significance in AUC (0.57, p=0.210, 95% CI (0.52 to 0.74)). This was an interestingly result and suggests that in isolation, salivary NE is not able to give absolute cut-offs for an acute exacerbation and thus stable-prodromal phase levels need to be established for each individual COPD patient.

4.4.3.3.1. Latent class growth analysis

Latent class growth analysis (LCGA) was performed on salivary NE levels from the exacerbator cohort (n=49) across the stable-prodromal period at 5 different time points, each 1 week apart, progressing towards an acute exacerbation.

As previously, an iterative LCGA was conducted using the BIC and BLRT p-value to determine the pilot number of sub-population clusters (Table 4.30) for COPD patients' salivary NE. From this result the posterior probabilities for cluster membership (Table 4.31) were assessed and it was determined whether increasing the cluster number improved or worsened the homogeneity of the created sub-populations. Each cluster formed 3 discrete COPD patient sub-populations; however, unlike similar analyses on salivary CRP and PCT levels, the NE inter-cluster overlap was higher. Cluster 1 demonstrated a 98% membership rate and cluster 2 approximately 93%; and cluster 3 was reduced at 94% with a small overlap into cluster 2 only. These overlaps are small and so the clusters are considered to be essentially discrete. Thus the 3 sub-population clusters were used to interrogate salivary NE across the stable-prodromal period of the exacerbator cohort. Cluster 1 = 24, Cluster 2 = 4 and Cluster 3 = 21 patients. I defined each sub-population cluster as "low", "medium" or "high" range based on the relative levels of salivary NE to each other (Figure 4.24). Accordingly, sub-population cluster 1 was defined as "low-range", sub-population cluster 2 as "medium-range" and cluster 3 as "high-

range". The membership of each COPD patient in their respective cluster is shown in Table 4.32.

Table 4.30: LCGA Goodness of fit tests for stable-prodromal salivary NE levels.

LCGA	BIC	BLRT (p-values)
1 cluster	3375.884	n/a
2 cluster	3235.409	**p<0.001**
3 cluster	3147.219	**p<0.001**
4 clusters	3128.913	p=0.249

The p-value represents whether there is a significant improvement in the LCGA model with "n" clusters compared to "n-1".

Table 4.31: Homogeneity of COPD patients cluster membership for stable-prodromal salivary NE levels.

Cluster	1 (n = 24)	2 (n = 4)	3 (n = 21)
1	98%	0%	2%
2	1%	5%	94%
3	0%	93%	7%

Table 4.32: COPD patients cluster membership for stable-prodromal salivary NE levels.

COPD patient	Cluster Membership
1	3
2	3
3	1
4	1
5	1
6	3
7	3
8	2
9	3
10	3
11	1
12	1
13	1
14	2
15	1
16	3
17	3
18	3
19	1
20	1
21	1
22	3
23	3
24	3
25	1
26	2
27	3
28	1
29	1
30	3
31	1
32	1
33	3
34	3
35	3
36	3
37	3
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41	2
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44	1
45	1
46	1
47	3
48	1
49	1

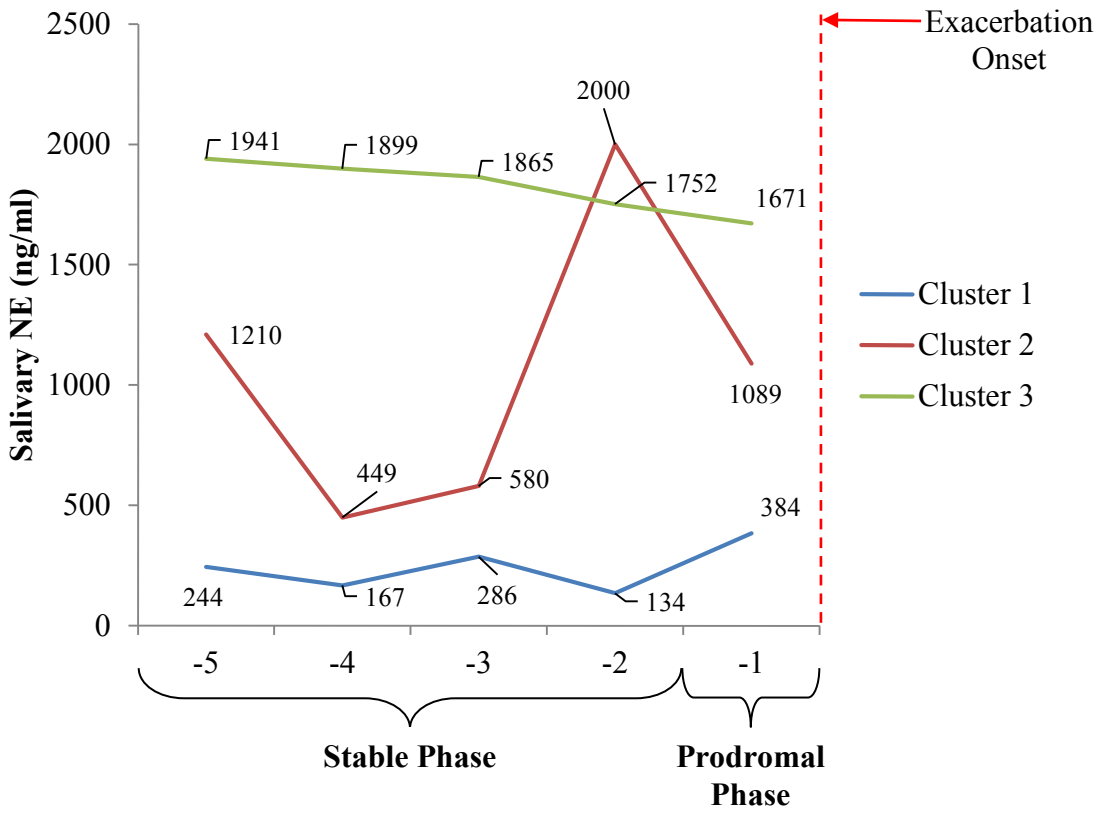

Figure 4.24: Salivary NE clusters across the stable-prodromal phases.

This line chart represents the LCGA for the mean salivary NE levels for COPD patients who experienced at least one acute exacerbation of COPD (n = 49) in the stable and prodromal phases. This period is defined as 5 weeks prior to the onset of an exacerbation phase and each interval represents 1 week. Cluster 1 = 24, Cluster 2 = 4, Cluster 3 = 21 COPD patients. The values represent mean salivary NE levels at each time-point.

4.4.3.3.2: Baseline characteristics associated with cluster membership

Further separate sub-analysis was conducted to investigate if there were significant difference in covariates (age, gender, BMI, total co-morbidities, FEV_1 and FVC) across the 3 exacerbator sub-population clusters and between theses clusters and the non-exacerbator control cohort. There was a significant statistical difference in total co-morbidities between sub-population clusters 1 and 3 ($p<0.001$ by multinomial analysis) and cluster 2 compared to 3 ($p<0.001$) (Table 4.33). Comparison between the individual exacerbator clusters and the non-exacerbator control cohort ($n = 6$) also demonstrated a significant difference in co-morbidities between the 2 groups ($p<0.001$) (Table 4.34).

Sub-population cluster 1 "low-range" salivary NE had the highest FEV_1, age and total co-morbidities compared to clusters 2 and 3 (Table 4.34). This was different to both salivary CRP and PCT data where the low-range clusters were associated with a low co-morbidity burden.

Cluster 3 "high-range" has the lowest FEV_1 consistent with the increased severity of COPD (Table 4.34). This demographic is consistent with that observed in both the high-range inflammation profiles for both salivary CRP and PCT sub-population clusters. Cluster 2 "mid-range" is of interest, with a significant degree of oscillation in salivary NE levels this variability is also displayed in the sub-population cluster 2 albeit that the patients therein did not have any co-morbidities (Figure 4.24); such variability in analyte levels was also observed in the sub-population cluster 2 for salivary CRP (Figure 4.17, Page 322) although not to the same magnitude. There was also a significant difference in co-morbid burden between the 3 exacerbator sub-population clusters and the non-exacerbator control cohort ($p<0.008$). The 3 salivary NE clusters for the exacerbator cohort will also be used in the corollary analysis

(Section 4.4.4, Page 350) to form a multidimensional score of COPD patients encompassing symptoms, spirometry and salivary biomarkers.

Table 4.33: Multinomial regression of covariates between the 3 exacerbator sub-population clusters for salivary NE.

Baseline Covariates	*Cluster 3 (n = 21)	*Cluster 2 (n = 4)	**Cluster 3 (n = 21)
Age, years	p=0.357	p=0.152	p=0.299
Gender male, (female)	p=0.991	p=0.438	p=0.426
BMI, kg/m^2	p=0.586	p=0.321	p=0.255
Total Co-Morbidities	p=0.134	**p<0.001**	**p<0.001**
FEV_1 (% predicted)	p=0.176	p=0.739	p=0.456
FVC (% predicted)	p=0.361	p=0.705	p=0.498

*P-values compared to cluster 1 (n = 24); **P-values compared to cluster 2.

Table 4.34: Covariate comparison between the 3 patient exacerbator clusters for salivary NE and the non-exacerbator control cohort.

Baseline Covariates	COPD Sub-Population Clusters Exacerbation Cohort (n = 49)			COPD Control Non-Exacerbator Cohort	
	1 (n = 24)	**2 (n = 4)**	**3 (n = 21)**	**(n = 6)**	***p-value**
Age, years	69.3 ± 8.1	62.8 ± 11.4	67.9 ± 8.5	70.3 ± 6.4	p=0.421
Gender male, (female)	12, 12	2, 2	2, 4	2, 4	p=0.882
BMI, kg/m²	28.5 ± 5.2	22.6 ± 3.7	26.9 ± 5.8	24.4 ± 4.2	p=0.117
Total Co-Morbidities	1.6 ± 1.0	0.0 ± 0.0	1.1 ± 0.9	0.8 ± 0.8	**p<0.008**
FEV_1 (% predicted)	54.5 ± 19.4	47.6 ± 9.9	46.2 ± 18.6	63.4 ± 27.9	p=0.233
FVC (% predicted)	77.1 ± 15.2	73.5 ± 9.9	77.5 ± 14.3	85.0 ± 16.9	p=0.616

Data are presented as mean ± SD. *P–value represents the difference between the exacerbator sub-population clusters and the non-exacerbator control cohort. sNE = salivary neutrophil elastase.

4.4.3.4. COPD sub-population re-exacerbators

Within the exacerbation cohort (n = 49) there were 15 COPD patients who re-exacerbated within 14 days. Stable phase salivary levels were compared for the target biomarkers with the index exacerbation and post-exacerbation-recovery equivalent levels between the single exacerbator (n = 34) and re-exacerbator (n = 15) groups (Table 4.35). Overall only index exacerbation phase salivary CRP levels were found to be significantly higher in the sub-population who re-exacerbated compared to those who remained stable following recovery from their first index episode ($p<0.04$ by Mann Whitney U Test). Additionally, the presence of 2 or more raised salivary biomarkers at an index exacerbation was also higher in the re-exacerbation group ($p<0.016$) There was no significant difference in exacerbation and post-exacerbation-recovery phase salivary CRP levels between the 2 sub-populations ($p=0.572$ and $p=0.680$ respectively).

For the sub-population clusters identified using LCGA, CRP was not significantly different between the single and re-exacerbation groups ($p=0.674$ by Kruskal-Wallis test); nor was PCT ($p=0.719$ by Mann Whitney U test) or NE ($p=0.596$ by Kruskal-Wallis test).

Table 4.35: Comparison of salivary biomarker levels between single and re-exacerbator patient cohorts.

	COPD Patients (n = 49)		
Salivary biomarkers (ng/ml)	**Single Exacerbators (n = 34)**	**Re-Exacerbators (n = 15)**	**p-value**
Stable phase: CRP	2.43, 2.19	2.70, 1.20	p=0.572
Exacerbation phase: CRP	3.38, 6.48	7.46, 6.69	**p<0.043**
Post-Exacerbation-Recovery phase CRP	2.51, 3.29	2.50, 1.39	p=0.680
Stable phase PCT	0.09, 0.09	0.09, 0.01	p=0.411
Exacerbation phase PCT	0.10, 0.10	0.12, 0.11	p=0.556
Post-Exacerbation-Recovery phase PCT	0.09, 0.07	0.09, 0.01	p=0.300
Stable phase NE	513, 1955	449, 651	p=0.930
Exacerbation phase NE	755, 1817	739, 749	p=0.843
Post-Exacerbation-Recovery phase NE	258, 1952	311, 700	p=0.797
Total Number of Raised Biomarker	1.5 ± 1.0	2.3 ± 0.8	**p<0.016**

Salivary biomarkers are expressed as median, IQR and total number of raised biomarkers as mean ± SD.

4.4.4. Stable COPD individual-specific multi-dimensional composite score

Utilising the whole patient exacerbator real-life dataset and the generated sub-population clusters within for each COPD patient for each parameter studies (stable-prodromal phase wellbeing, FEV_1 and salivary biomarkers), an overall composite score was produced for each individual. This score was split into 4 distinct groups each one integer greater than each other to ensure the allocations were even:

- Group 1 = composite score greater than or equal to 3 but less than 4;
- Group 2 = score greater than or equal to 4 but less than 5;
- Group 3 = score greater than or equal to 5 but less than 6;
- Group 4 = score greater than or equal to 6.

Group membership of: Group 1: 11; Group 2: 20; Group 3: 15 and Group 4: 3 patients (Table 4.36). Correlations of each group's composite scores were then performed to COPD disease severity and MRC score as well a sub-analysis for across-group differences in age, BMI, total co-morbidities, re-exacerbation and annual exacerbation frequency.

Overall there was a statistically significant correlation between the composite score and COPD disease severity ($r = -0.400$, $p<0.005$ by Spearman's Rank correlation co-efficient) but not with MRC score ($r = 0.251$, $p=0.081$), although a clear trend was noted. Sub-analysis did not reveal a statistically significant difference in re-exacerbation ($p=0.439$), BMI ($p=0.215$), annual exacerbation frequency ($p=0.832$), total co-morbidities ($p=0.583$) and age ($p=0.836$ by Kruskal-Wallis test respectively) across the 4 groups.

Table 4.36: Multidimensional composite score for each COPD exacerbator patient.

COPD Patient	Salivary CRP	Salivary PCT	Salivary NE	Mean Biomarker cluster	Symptom Cluster	FEV_1 Cluster	Composite Score	Multifactor Group Allocation
1	3	1	3	2.33	1	2	6.33	**4**
2	1	1	3	1.67	1	2	4.67	**3**
3	1	1	1	1.00	1	2	4.00	**3**
4	1	1	1	1.00	1	1	3.00	**1**
5	1	1	1	1.00	1	2	4.00	**3**
6	1	1	3	1.67	1	2	4.67	**3**
7	1	1	3	1.67	1	1	3.67	**1**
8	1	1	2	1.33	1	2	4.33	**3**
9	1	1	3	1.67	1	2	4.67	**3**
10	1	2	3	2.00	2	2	6.00	**4**
11	1	1	1	1.00	1	1	3.00	**1**
12	1	1	1	1.00	1	2	4.00	**3**
13	1	1	1	1.00	2	1	4.00	**3**
14	1	1	2	1.33	2	2	5.33	**2**
15	1	1	1	1.00	1	1	3.00	**1**
16	1	2	3	2.00	1	2	5.00	**3**
17	3	2	3	2.67	1	2	5.67	**3**
18	1	1	3	1.67	1	1	3.67	**1**
19	1	1	1	1.00	1	2	4.00	**2**
20	1	1	1	1.00	1	1	3.00	**1**
21	1	1	1	1.00	1	2	4.00	**2**
22	1	2	3	2.00	1	1	4.00	**2**
23	1	1	3	1.67	1	2	4.67	**2**
24	2	2	3	2.33	1	2	5.33	**3**
25	1	1	1	1.00	1	2	4.00	**2**
26	1	1	2	1.33	1	2	4.33	**2**
27	1	1	3	1.67	1	2	4.67	**2**
28	1	1	1	1.00	1	1	3.00	**1**
29	1	1	1	1.00	1	2	4.00	**2**
30	1	1	3	1.67	1	2	4.67	**2**
31	1	1	1	1.00	1	2	4.00	**2**
32	1	1	1	1.00	1	1	3.00	**1**
33	1	1	3	1.67	2	2	5.67	**3**
34	2	2	3	2.33	2	2	6.33	**4**
35	3	1	3	2.33	1	1	4.33	**2**
36	1	1	3	1.67	1	2	4.67	**2**
37	1	1	3	1.67	1	2	4.67	**2**
38	1	1	1	1.00	1	2	4.00	**2**
39	1	1	1	1.00	2	2	5.00	**3**
40	2	2	1	1.67	1	2	4.67	**2**
41	1	1	2	1.33	1	2	4.33	**2**
42	1	2	3	2.00	1	2	5.00	**3**
43	1	1	1	1.00	1	1	3.00	**1**
44	1	1	1	1.00	1	1	3.00	**1**
45	1	1	1	1.00	2	2	5.00	**3**
46	1	1	1	1.00	1	2	4.00	**2**
47	1	1	3	1.67	1	2	4.67	**2**
48	1	1	1	1.00	1	2	4.00	**2**
49	1	1	1	1.00	1	1	3.00	**1**

4.4.5. Exacerbation phase phenotypes

To determine the presence of any distinct phenotypes in the exacerbation phase of the tested COPD metrics an exploratory factor analysis was conducted. All exacerbators (n = 49) data was utilised on day 1 of an exacerbation alongside other demographics. Overall factor analysis demonstrated 3 distinct phenotypes in COPD patients at the onset of an exacerbation: Phenotype 1 components (FEV_1, FVC), Phenotype 2 (total co-morbidities, BMI, MRC score) and Phenotype 3 components (salivary CRP, salivary PCT and salivary NE) (Figure 4.25). Interestingly age, total number of exacerbations and co-morbidities did not improve the homogeneity of the respective phenotypes. This result could reflect that the variables in each of these phenotypes groups may be combined into a single multidimensional outcome factor, so that COPD exacerbations can be classified or characterised into groups based on the particular profile mix of PROs, spirometric status and salivary biomarker levels.

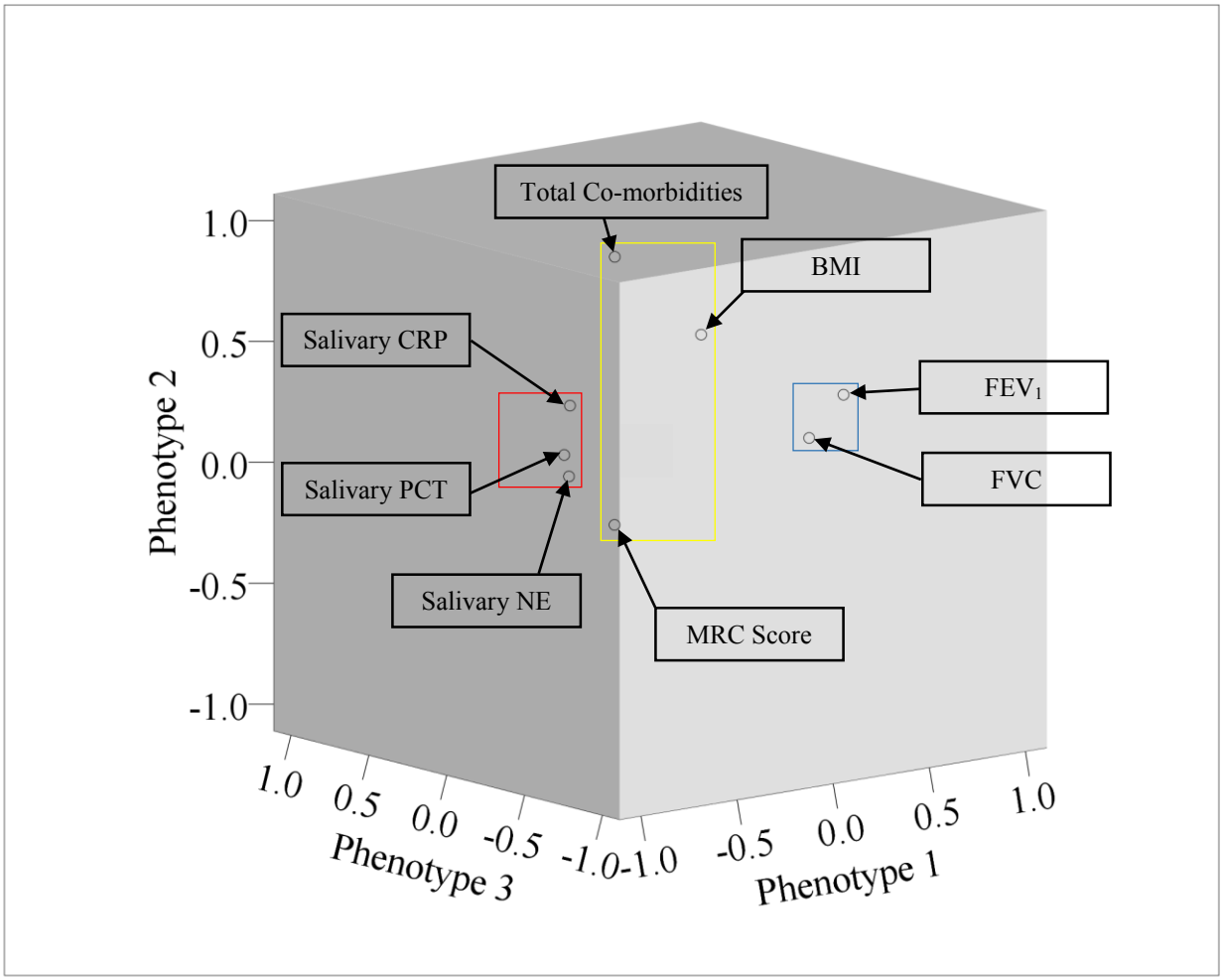

Figure 4.25: Factor plot of the 3 exacerbation phenotypes.

This 3D factor plot displays the relationship between the exacerbator group (n = 49) data for each identified phenotype. Blue box = Phenotype 1; Yellow box = Phenotype 2, Red box = Phenotype 3

4.5 Discussion

This study is the first in COPD patients to investigate daily PROs and weekly salivary biomarker levels alongside spirometry, with correlation to HROs. The electronic Wellbeing and Self-Assessment diary allowed for accurate daily symptom monitoring with a compliance rate of approximately 98%. This is greater than reported in most studies (62% to 92%) in COPD patients who have used electronic diaries for daily symptom capture (Cummings et al., 2010, Kulich et al., 2015, Sund et al., 2009, Walters et al., 2012). Diary compliance in my study was also far in excess of the 80% considered "high" compliance for electronic diary assessments (Morren et al., 2009).

Analysis of the COPD Wellbeing score data utilised several novel approaches. Firstly, a baseline symptom burden was calculated and used to standardise the COPD Wellbeing Score across all COPD patients. This approach dispenses with the need to establish a cut-off value for clinically significant scores which was required in other symptom score studies (de Torres et al., 2014). Data exploration then utilised CPA which is capable of detecting multiple and subtle changes in longitudinal sets of data. This test provides a confidence level indicating the likelihood that a change has occurred and a confidence interval indicating when the change occurred (Taylor, 2014). These analytical approaches are more sensitive than moving-averages (Gavit et al., 2009) which have been employed in other COPD studies involving daily diary data (Alahmari et al., 2014). Using sensitive CPA, wellbeing scores in the stable-prodromal period showed no significant variation until around 4 days prior to the onset an exacerbation. This demonstrates COPD Wellbeing score reproducibility over the observation period, a key component of valid symptom assessment (Leidy et al., 2014).

The enhanced symptom burden from 4 days before exacerbation onset is also consistent with previous observations (Aaron et al., 2012). The gradient of symptom improvement was linear across the 7-day exacerbation phase when COPD patients were receiving treatment with antibiotics and steroids. This, coupled with the observation that all salivary biomarker levels had returned towards baseline levels post-treatment highlights that recovery on the treatment initiation was consistent for the whole COPD exacerbator cohort. This may provide a possible explanation for why short treatment courses for acute exacerbations of COPD appear to be as effective as longer treatments (El Moussaoui et al., 2008). Interestingly, analysis of the post-exacerbation-recovery phase demonstrated a worsening in COPD Wellbeing score trajectory at day 10. However, analysis of the scores on that day did not reveal a significant change. This further supports the argument that snap-shot data is not enough to predict and monitor wellbeing in COPD. Additionally, it confirms that capture of symptoms via daily self-assessment diaries requires a far higher compliance than has currently been acceptable in the literature. It has been previously demonstrated that median time to a re-exacerbation event is between 29 to 43.5 days (Leuppi et al., 2013); this is much later than my observations but could be related to my COPD patient cohort having a frequent exacerbator phenotype.

The results for spirometry revealed a significant reduction in both FEV_1 and FVC during an acute exacerbation of COPD. However, neither assessment declined significantly in the prodromal phase. The LCGA data was interesting and demonstrated that there was no variability in the change of FVC across the stable-prodromal. FEV_1 demonstrated 2 clusters with different trajectories. This is consistent with the known variability of FEV_1 decline over time in the COPD population (Casanova et al., 2014). Importantly the LCGA of spirometry to generate the sub-population clusters does so without using an arbitrary cut-off; thus there is no *a priori* assumption about the rate of FEV_1 change prior to analysis (Herpel et al., 2006). The

result for FVC is interesting as it appears to demonstrate that the rate of change over time is consistent irrespective of underlying disease severity.

The COPD patients who re-exacerbated within this study (n = 15) had more severe disease (as judged by their FEV_1); this has previously been observed in other published studies which have demonstrated an increased risk of re-exacerbation in COPD patients with worse lung function re-exacerbating within 90 days of an index exacerbation (Liu et al., 2015, Liu et al., 2007).

All 3 target salivary biomarkers demonstrated a significant increase during an acute exacerbation of COPD. These results are consistent with the study findings in Chapter 3, Page 239. Both salivary CRP and NE levels increased in the prodromal phase. Although PCT was significantly higher in the whole COPD exacerbator group (n = 49) at exacerbation, this was not reflected in the prodromal phase. As discussed in Chapter 1, Page 69. PCT is highly selective for the presence of bacterial infections (Jaresova et al., 1999). It is plausible that a rise may occur in the prodromal phase of a bacterial-driven acute exacerbation, with the magnitude of the rise being dependent on bacterial load and severity of the infective episode. However, as bacteria account for 50 to 69% of COPD exacerbations (Chapter 1, Page 37) this may provide a partial explanation for why a rise in prodromal phase PCT levels was only observed in approximately 40% of the exacerbator cohort. Unfortunately, sputum microbiological profiles were nor examined in the study.

A review of the literature (web of science: search term: Exacerbation prediction COPD), revealed no studies to date that have investigated the predictive value over time of an early rise in physiological metrics or biomarkers of inflammation in COPD. All 3 salivary biomarkers returned to stable phase levels once the exacerbation has been treated. Interestingly the post-

exacerbation-recovery phase levels of all 3 biomarkers do not appear to predict a re-exacerbation event. However, the index exacerbation phase salivary CRP levels and total number of raised biomarkers were observed to be higher in patients who re-exacerbated. In general, my results are in agreement with studies that have shown elevated levels of multiple biomarkers in stable COPD to be associated with an increased risk of exacerbation (Thomsen et al., 2013). This result might suggest that the index exacerbation was perhaps more severe; however, normalisation of all 3 salivary biomarkers occurred at the end of the exacerbation/treatment phase, thereby implying that the medication given had been effective. It is possible that within the complexity of an exacerbation, all components may not have been considered, for example eosinophilic inflammation shows a high responsiveness to steroids and has been demonstrated to be a distinct phenotype in acute exacerbations of COPD (Bafadhel et al., 2011b). Therefore, the re-exacerbation group in my study would have had a return to baseline for the salivary biomarkers tested following the 7-day course of high dose steroids but relapsed at around day 10 in the post-exacerbation-recovery phase due to a sustained eosinophilic-driven process possibly requiring a longer steroid course; peripheral eosinophil counts were not examined in my study

The LCGA approach highlighted several clusters for all 3 target salivary biomarkers based on their levels and trajectory over the stable-prodromal phases. These clusters highlight that even in a small cohort of patients there is variability in salivary biomarker trajectories although these clusters were not associated with re-exacerbation risk. Multinomial analysis however revealed that the total number of co-morbidities is a significant discriminatory component of the salivary NE cluster. The prevalence of co-morbidities has been shown in a recent systematic review to be a consistent factor in the phenotyping of COPD subjects (Pinto et al., 2015). The 3 baseline salivary clusters identified in this study will require an iterative validation process before their

relevance to clinical outcome is determined. The further validation of these clusters within much larger patient cohorts could lead to targeted and personalised drug therapy for these patient sub-populations. Unfortunately, the literature demonstrates that current studies invariably attempt to cluster individuals with COPD based on "a snapshot in time" view of objective and subjective metrics; therefore, confusion exists as to whether subjects remain in the same cluster over time (Han et al., 2010a). Thus for COPD patient cluster studies to be meaningful and accurate there is a need for them to incorporate a temporal design and analysis of longitudinal real-life data to reflect the variability of COPD-relevant metrics over time.

This study has also attempted to establish a novel composite individual score based on a profile of PROs, spirometry and salivary biomarkers. This multidimensional composite score showed significant correlation with COPD disease severity and a trend with MRC score. Further work needs to be undertaken to understand the role of this multidimensional scoring system and its relevance to COPD outcomes and management.

The control cohort (n = 6) who did not experience an acute exacerbation served as a control cohort. They were classified as frequent exacerbators prior to enrolment into the community-based longitudinal study; however their baseline annual exacerbation frequency was overall lower at 2 episodes per year compared to that of the exacerbator cohort at 4 episodes per year. This is interesting and perhaps highlights the need to split the frequent exacerbator phenotype into a further "frequent-frequent" sub-population based on an annual exacerbation frequency of greater than 4 acute episodes.

In acknowledgement that there exist limitations to the study and these need to be considered. Cluster membership appears to be discrete; however the study was exploratory with

comparatively small patient numbers. The next step would be to utilise the defined clusters in a much larger cohort of patients and monitor said patients over a longer time frame. Although 3 exacerbation phenotypes were identified, the specific aetiology of the exacerbations was not characterised. Although this would add further classification information to my results, it could also be considered d as an advantage as clustering was completely blinded from aetiology. The principle components of these phenotypes could again be followed up longitudinally to understand any variations between them and their relationship to the stable phase clusters.

An arbitrary, albeit structured process has been utilised, for assigning individual scores in the multidimensional composite score; for example, COPD patients with higher levels of stable-prodromal phase inflammation were assigned into a higher score and indeed this may be an incorrect presumption. There is therefore a need to monitor these sub-population clusters to clarify whether higher inflammatory levels are related to a worse long-term outcome. Finally, the COPD population in this study are a distinct cluster in their own right (frequent exacerbators was a study inclusion criterion). Longitudinal monitoring needs to be applied to non-frequent exacerbators to understand whether the same trajectories in PRO, spirometry and salivary biomarkers exist and their relationship to health status monitoring.

Results from this study provide the foundation for a COPD health status monitoring algorithm for earlier exacerbation recognition and re-exacerbation risk. Additionally, multidimensional stable phase clusters have been identified for both the stable-prodromal and exacerbation phase data which lend themselves to further testing. Importantly, as none of the exacerbators required hospital admission for their acute episodes it could be argued that the study methods represent a successful self-management protocol. Within the framework of this thesis, the patient experience in respect of such monitoring [electronic wellbeing self-assessment and self-testing

of saliva] would be a valuable step towards further development of a viable bio-clinical intervention for point-of-care prediction of COPD exacerbations. This will be further investigated in dedicated workshops in the next two chapters (Chapter 5, Page 361 and Chapter 6, Page 384).

4.6. Conclusion

All 3 target salivary biomarkers can identify an exacerbation of COPD from the stable phase and both CRP and NE appear to identify a prodromal phase. The CRP level at exacerbation and the disease severity as measured by FEV_1 appear to be the best predictors of the likelihood of a re-exacerbation within 2 weeks of an index exacerbation. COPD patients at their baseline stable phase appear to have 3 unique cluster trajectories: low-range, medium-range and high-range inflammation. These clusters are apparent for both salivary CRP and NE, whilst salivary PCT appears to exhibit just low-range and high-range inflammatory clusters. Salivary NE, appears to have a significant association with co-morbidity burden.

Chapter 5:

Exploration of Patient Experiences on the Use of an Electronic Wellbeing and Self-Assessment Diary

5.1 Introduction

PROs are increasingly advocated within the wider healthcare and clinical research. They are seen to provide invaluable subjective information particularly when assessing the effects of a proactive intervention on patients and also in screening specific health disorders (Li et al., 2007). They have been shown to be important in COPD management and numerous PROs exist in the wider literature (Chapter 1, Page 27). In this thesis a novel PRO score was developed encompassing clinically-relevant COPD metrics (COPD Wellbeing score) (Chapter 2, Page 210). This was incorporated into a purposeful paper-based diary (Wellbeing and Self-Assessment diary, (Chapter 2, Figure 2.29, Page 212) and tested in a community-based cohort study (Chapter 3, Page 213). The results of the community-based cohort study demonstrated that components of the COPD Wellbeing Score correlated with MRC score and salivary biomarkers (Chapter 3, Page 233). Based on the results for the paper-based Wellbeing and Self-Assessment diary and a literature review on the limitations of paper-based symptom diary (Chapter 4, Page 254) an electronic Wellbeing and Self-Assessment diary (Chapter 4, Page 258) was designed. This electronic Wellbeing and Self-Assessment diary installed on an iPad (Apple, USA) was provided to COPD patients in a pilot community-based longitudinal study to understand COPD patients' symptom self-management and the correlation of these symptoms to health status and salivary biomarkers (Chapter 4, Page 270).

5.1.1. The concept of COPD self-management

In recent times work on patient self-management has demonstrated the efficacy of involving and empowering patients in their own care (Trappenburg et al., 2013). Specifically, for COPD patients, the most recent update of the *Cochrane Database of Systematic Reviews* demonstrated that self-management programmes reduce the probability of COPD-related hospital admissions and also increase patient wellbeing (Zwerink et al., 2014). To date, the diversity in content and

duration of the self-management programmes reviewed, have elicited no clear recommendations regarding the most effective form and content of self-management in COPD (Zwerink et al., 2014). However, a major feature common to all the self-management programmes reviewed is some form of patient self-assessment of their condition.

Arguably, self-monitoring demands a significant shift in patients' behaviour; a change which has been proposed could be attained only if patients' self-efficacy is such that they have enough confidence in their ability to successfully respond to certain events (Bischoff et al., 2012). Self-efficacy is defined as ''the belief in one's capabilities to organise and execute the courses of action required to produce given attainments" (Bandura, 1995). This cognitive construct is different from simply believing in a positive outcome; rather, it refers to a person's judgement about their ability and skills required to carry out a specific behaviour. Self-efficacy beliefs held by individuals are considered to be a central and persuasive factor in determining the course of action required, the degree of effort given and a person's perseverance to continue in the face of challenges and difficulties (Bandura, 1989).

Most patient self-management programmes, based in the theory of self-management, (Ryan and Sawin, 2009) are founded on Bandura's work on self-efficacy (Bandura, 1989, Bandura, 1995, Bandura, 1997). Within the context of COPD patients, effective patient self-management including self-monitoring of symptoms might involve seeking treatment for a potential acute exacerbation of COPD before it becomes a full-blown event with associated consequences on respiratory reserve; thus avoiding the necessity for A&E attendance and hospital admission. Currently, this level of well-designed and guided patient self-management seems to be limited and acute COPD exacerbations remain a leading cause of unscheduled GP visits and the most common cause of emergency hospital admissions (Wedzicha and Vestbo, 2012). Yet it has

been shown that patients use online applications regularly when healthcare interaction is available (In 't Veen et al., 2012).

The implementation of two or more of the following factors result in a fall in COPD exacerbations and reduced healthcare utilisation: (1) an extensive self-management programme with an individualised action plan (Sedeno et al., 2009). This educates patients in the management of their condition which empowers them to recognise the signs of a clinical deterioration and initiate prompt treatment; (2) "advanced" access to care, which includes a knowledgeable healthcare provider (Fromer, 2011). This ensures that patients are monitored and assessed by healthcare providers with expertise in COPD; (3) guideline-based therapy to ensure up-to-date evidence-based treatment for COPD is being provided (NICE, 2010); (4) a clinical registry system containing population information. This provides a database for healthcare professionals to ensure that all patients are receiving standardised care (Adams et al., 2007).

5.1.1.1. Telehealthcare

Advances in telehealthcare technologies including medical Apps, computer programmes, diagnostic algorithms; all of which utilise different interfaces (e.g. tablet computer, laptop, mobile phone, video conference technology) to interact with patients, seem to offer a mechanism to help meet these criteria cost-effectively (McLean et al., 2013). However, the current systems do not offer an analytical or decision-making structure; nor have they been widely embraced by all. Research across a range of conditions demonstrates mixed responses to different mobile health technologies in terms of both patient satisfaction and also their use as part of effective interventions, in terms of both real economic efficiency and clinical efficacy (Abrams and Geier, 2006, Barlow et al., 2007, Car et al., 2012, Parker and Hawley, 2013).

To date, telehealthcare in COPD can be divided into two systems; the first is one in which patients monitor their symptoms at home and log them electronically and the second is where patients are asked to report their symptoms via, for example, a telephone and/or computer (Polisena et al., 2010). Both systems have been demonstrated to reduce hospital attendance and exacerbation frequency (Alrajab et al., 2012, Polisena et al., 2010, McLean et al., 2012). PROs however, do not seem to improve with telehealthcare although the evidence for this is conflicting. The variety of measurement instruments for these PROs within studies hinders direct comparison (Polisena et al., 2010). Relevant effects have been observed in a successful self-manager sub-group, representing about 40% of COPD patients (Bucknall et al., 2012). Overall it remains unclear whether improvements in these PROs are driven primarily by objective improvements in physical health or by subjective improvements in perceptions of agency or control. Additionally, in most interventions, transfer was shown to be reliant on automated algorithms for interpretation. Care providers can recognise important changes in essential measurements, but delays occur as the systems are only active during office hours.

The purpose of the electronic Wellbeing and Self-Assessment diary was to utilise mobile health technology to provide both a platform to improve COPD patients' self-management and also, a direct link to a COPD specialist care team; thus implementing two of the factors outlined above, as critical for a fall in acute exacerbations and reduced health care utilisation. All COPD patients recruited into the community-based longitudinal study (Chapter 4, Page 270) had been previously self-managed their disease. This self-management consisted of initiating treatment for acute exacerbations of COPD with "rescue" medications (antibiotics and steroids) prescribed by their general practitioner (GP) and kept in reserve at the patients' own home in line with NICE COPD guidance (NICE, 2010). The COPD patients were not formally

monitored and they relied on their own judgement as to whether they needed to commence treatment.

A qualitative study was conducted with the objective (Chapter 1, Page 78) of understanding whether the self-management ecosystem provided in the community-based longitudinal study (Chapter 4, Page 270), centered upon the electronic Wellbeing and Self-Assessment diary (Chapter 4, Page 258) supported by myself and the research team of COPD specialist nurses, could improve patients' self-management by increasing their self-efficacy: Specifically, their judgement and confidence in their ability and skills required to determine appropriate self-care (Ryan and Sawin, 2009).

5.2. Methods

All participating COPD patients were asked to provide their written feedback during the community-based longitudinal study (Chapter 4, Page 271) on the use of the electronic Wellbeing and Self-Assessment diary in the free-text section of the diary, and on completion of the study their thoughts were recorded on the electronic diary in the HRO section. The community-based longitudinal study design is described in Chapter 4, Page 270.

Briefly, the electronic Wellbeing and Self-Assessment Diary installed on an iPad (Apple, USA) was provided to COPD patients to be completed daily in conjunction with weekly visits by myself and a respiratory specialist research nurse to assess health status. All COPD patients were taught and shown how to use the iPad (Apple, USA) and electronic Wellbeing and Self-Assessment diary on their first visit by myself. They were encouraged to contact the research team (me or the named clinical research nurse (Ms Pauline Jones/ Ms Vicki Adamson)) if any problems arose and informed that help would be provided if required. COPD patients were aware that their diary entries would be monitored remotely by myself and the clinical research nurses with any deterioration in symptoms triggering contact by either myself or the research nurses. In addition, COPD patients were also aware that on commencement of treatment for an acute exacerbation of COPD either myself or the research nurses would visit as soon as possible and no later than 12 hours after contact been initiated. This level of support was available 7 days a week (7.30am to 7.30pm). A paper-based version of the electronic Wellbeing and Self-Assessment diary (Appendix 1, Page 485) was also available if required or preferred; though none of the COPD patients chose to use this method. The average duration of each COPD patient in the study was 9.5 weeks with 98% diary entry compliance. Overall all COPD patients documented that the electronic Wellbeing and Self-Assessment diary was end-user friendly,

encompassed symptoms relevant to their condition of COPD and helped with the management of their disease.

To elucidate these concepts further, COPD patients' attitudes and experiences were explored by setting up semi-structured workshops at eight months after the start of the community-based longitudinal study (Chapter 4, Page 270). COPD patients who had completed or were still active within the community-based longitudinal study were invited to take part (Figure 5.1). As such, they formed a purposive sample (Ritchie, 2014).

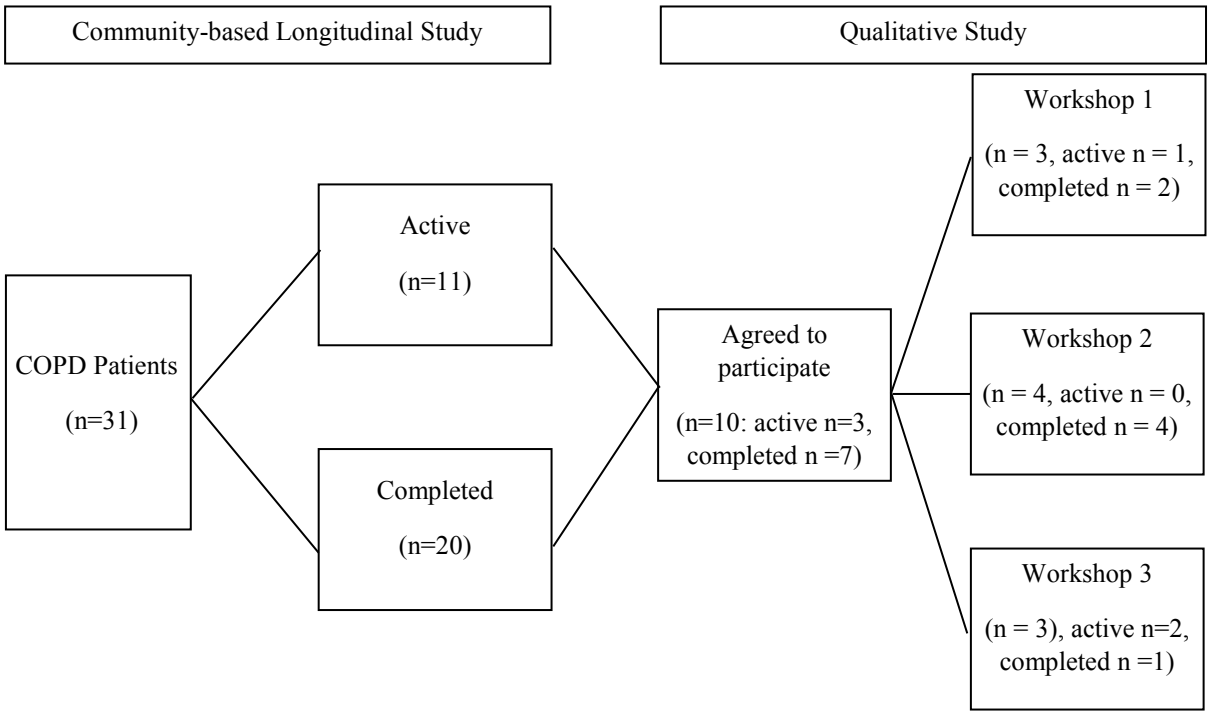

Figure 5.1: Population and workshop recruitment.

This figure demonstrates COPD patient recruitment into the focus groups from the community based longitudinal study (Chapter 4, Page 270). Active represents patients who were still active in the longitudinal study whilst completed represents patients who have finished the study.

5.2.1. Workshop demographics

Fifteen COPD patients responded that they wished to be involved in the workshops; unfortunately, 5 COPD patients had an acute exacerbation and could not attend on the set dates. The COPD patient demographics are described in Table 5.1.

Table 5.1: Workshop demographics.

	Male (n = 7)	**Female (n = 3)**
Age, years	72.15 ± 10.52	63.55 ± 9.81
BMI, (kg/m^2)	22.78 ± 2.45	24.68 ± 6.85
Co-morbidities, n	1.65 ± 0.65	1.78 ± 0.68
FEV_1, (% predicted)	49.25 ± 5.62	48.78 ± 6.82
FVC, (% predicted)	72.25 ± 8.29	74.25 ± 9.25

Data presented as mean ± standard deviation unless stated.

Due to the debilitating nature of COPD, specifically severe breathlessness which often limits fluent speech, the number of participants in each workshop was deliberately kept small. This then facilitated much time and opportunity for each COPD patient to fully engage in the discussion.

The main purpose of this workshop approach was to facilitate the generation of data by interaction between the COPD patients, who were able to present their own views and experiences but also to hear others' contributions. In this way they were then enabled to reflect further upon their own viewpoint and contributions and make additional comments. COPD

patients were able to ask questions of each other, which prompted further additional spontaneous comments from others: "A workshop is therefore, not a collection of individual interviews with comments directed solely through the researcher" (Finch, 2014). Rather, it is synergistic (Stewart, 2007) with the interaction of the group members used to generate data and insights (Berg, 2012).

5.2.2. Data collection

In preparation for the workshops, due consideration was given to a venue, information that I wanted to elicit, and the structure of the groups. The venue chosen was the location known and frequented by all COPD patients for their exercise-based treatment (pulmonary rehabilitation). A series of questions was constructed with the help of a clinical psychologist (Dr. Kathryn Kinmond, Manchester Metropolitan University) and refined (Table 5.2) to seek a better understanding of the following factors: how far the COPD patients engaged with and embraced the use of the electronic Wellbeing and Self-Assessment Diary and iPad (Apple, USA) ; the impact of daily monitoring of their symptoms and weekly visits from the clinical research team (myself and the COPD research nurses) in terms of monitoring their condition; how they felt about this approach to self-management of their COPD; and how far the relationship between the patients and the clinical research team impacted on their self-monitoring of their condition.

Table 5.2: Workshop questions.

1. How did you learn about the study?
2. What did you think when you first saw the *iPad diary?
    a. How helpful/useful was it, to have the *iPad Diary demonstrated to you?
    b. How could it have been better?
    c. Are there any extra bit that we have missed or you would like to see added?
3. How long did it take you to feel ok using the *iPad Diary?
    a. What do you think about the design?
        i. For example, the colours, layout, brightness, buttons?
    b. Were you able to tell us about all your symptoms easily?
    c. The free space that you could enter a message was it a good thing?
    d. Did you find it a pain to use?
4. What was it like to have a weekly visit from Polly/Vicki?
    a. Did you find it helpful?
    b. Did it make you feel more in control of your COPD?
    c. Did you like the fact that someone was looking at your diary every day and your progress?
        i. What was good?
        ii. What was bad?
5. In what way do you think your life was different by being in the study using the *iPad Diary?
6. Have we missed anything?

*Throughout the longitudinal community study COPD patients referred to the electronic Wellbeing and Self-Assessment diary as the iPad Diary for simplicity.

All three workshops were purposefully facilitated by the clinical research nurse (moderator). The rationale for this was that the nurse was from the same geographical area as the COPD patients and thus, shared a common use of regional dialect; additionally, patients were familiar with her and the aim of the workshops was to facilitate the COPD patients to engage in open discussion. Arguably, the different roles of COPD patients and researcher became more blurred as the discussion and interactions evolved such that at times during the workshops the COPD patients took over some of the 'interviewing' of each other and the researcher became an active listener (Egan, 2013). No audio-recording took place so the conversations were transcribed in "real-time" by myself and the clinical psychologist for each focus group. Every session commenced with a general introduction, a demonstration of the electronic Wellbeing and Self-Assessment dairy to act as an *aide de mémoire* and then the main discussion. During the workshops all COPD patients were encouraged to talk freely about their experiences and at the end of the session asked to raise anything that had not already been covered. Each workshop lasted two hours. All COPD patients received a copy of the questions (Table 5.2) to be discussed one week prior to the workshop. In this way it was aimed to offer the COPD patients an opportunity to give informed consent to take part, as they were as fully briefed as possible, including being made aware of the questions that were going to be asked of them. All COPD patients gave consent to their conversation being transcribed and quotes being used.

5.3. Data analysis

Data transcription and analysis proceeded simultaneously. The "substantive" approach (Spencer, 2014) to data analysis; specifically, the Framework Method (Gale et al., 2013) was used to analyse the workshop transcripts. The Framework Method sits within a broad family of thematic analysis methods (Braun and Clarke, 2006) which identify commonalities and differences in qualitative data, before focusing on relationships between different parts of the data, "thereby seeking to draw descriptive and/or explanatory conclusions clustered around themes" (Gale et al., 2013a). It is being used increasingly in health research, particularly when there is a multidisciplinary research team engaged in analysis. The Framework Method is a highly systematic method of catergorising and organising what may seem like unmanageable qualitative data. It is not aligned to a particular epistemological, philosophical, or theoretical approach. Rather, like thematic analysis (Braun and Clarke, 2006) it is a flexible tool that can be used with many qualitative approaches that aim to generate themes. In the analysis phase the gathered data is sifted, charted and sorted in accordance with key issues and themes. This involves a 5 step process (Gale et al., 2013):

1. Familiarisation where the researcher(s) become familiarised with the transcripts of the data collected.
2. Identifying a thematic framework where emerging themes are recognised and documented.
3. Indexing where one identifies portions or sections of data that correspond to a particular theme.
4. Charting where specific pieces of data that were indexed in the previous stage are now arranged into charts of the themes
5. Mapping and interpretation which is the final stage involves the analysis of key characteristics from the charts and interpretation of these characteristics.

The Framework Method further embraces involvement in analysis by all members of the research team, regardless of their level of research experience (Gale et al., 2013). This approach then seemed wholly appropriate to our workshop team (myself as the clinician, Dr Kinmond as clinical psychologist and Ms Jones as the clinical research nurse). As someone with limited experience in qualitative research, by using this method approach I was able to engage in both a multi-disciplinary and multi-experience-level approach to the analysis of the workshop data. After each workshop there was a team de-brief to discuss the key themes. Following which, myself and the clinical psychologist prepared individual reports containing a transcript of the conversation and key themes to have emerged from the session; reports were then exchanged and mapped. Finally, after all three workshops were completed, a face-to-face team meeting was held to discuss the overall results and final refinement of the concluding report. The Framework Method has no allegiance either to inductive, deductive or combined thematic analysis (Gale et al., 2013). For this study, a combined approach was deemed appropriate as the study had some specific issues to explore (evidenced in the workshop questions), but also aimed to consider other unexpected aspects of the participants' experience and the way they assigned meaning to phenomena.

As any form of qualitative or quantitative analysis is not a purely technical process, but influenced by the researchers' characteristics, experiences and disciplinary paradigms (Ritchie, 2014), critical reflection across the research team member occurred throughout the process.

5.4. Results

The data was grouped into four major themes. "Technology" covered the aspects in relation to the electronic Wellbeing and Self-Assessment diary; this included the impact of its use on patients' self-efficacy (Bandura, 1995) and self- management (Ryan and Sawin, 2009) of their condition. "Humanity" covered COPD patients' experiences in relation to the clinical research team. A third theme of "Interactions with the GP" was clear in the stories of all COPD patients. The final theme "All alone" illustrates the very real isolation and fear of the COPD patients as they face life and managing their condition without the electronic Wellbeing and Self-Assessment diary and the clinical research team.

5.4.1. Technology

It was initially felt that the demographics of the study population might mean that in the community-based longitudinal study (Chapter 4, Table 4.2, Page 280) there would be a small but significant use of the paper-based version (Appendix 1, Page 485) of the electronic Wellbeing and Self-Assessment diary, which had been offered as an alternative to the electronic diaries. Clearly there was initial apprehension from some COPD patients about using the electronic Wellbeing and Self-Assessment diary. As one stated "It was scary; I was frightened about making mistakes; I didn't want to break it; If you do x and y you might put in a wrong answer". Although this was not universal as another COPD patient exclaimed "At last! I've got an iPad; Bees knees!" Such mixed responses resonate with published research which report mixed responses by patients across a range of conditions, to different technologies in healthcare (Abrams and Geier, 2006, Barlow et al., 2007, Car et al., 2012, Parker and Hawley, 2013). Unexpectedly one COPD patient felt "differently" empowered because of the technology and the impact of this on his physical disabilities: "I have an essential tremor so I wouldn't do a written diary. I had no problems using it". This perhaps reminds of the need to

consider the 'whole person' when offering intervention (Crossley, 2000) as one approach may not be relevant to all.

Despite the feelings of apprehension, no paper-based versions of the electronic Wellbeing and Self-Assessment diaries were used either at enrolment or during the community-based longitudinal study (Chapter 4, Page 270) by switching from the electronic Wellbeing and Self-Assessment to paper-based diary. This level of uptake may have been attributable to the fact that the participants were aware that the paper-based version of the electronic Wellbeing and Self-Assessment diary entries would be collected only weekly. Thus, remote symptom monitoring would not be possible.

All COPD patients felt that the electronic Wellbeing and Self-Assessment diary and iPad (Apple, USA) provided were simple to use and there were no problems with the colours or fonts. The majority of COPD patients also provided suggestions on how the electronic Wellbeing and Self-Assessment Diary could be refined further. The bulk of these suggestions focused on how the COPD patients felt that their symptoms could be portrayed more accurately or that there were other symptoms of relevance. As one COPD patient stated, "There's not enough space for writing comments". Some COPD patients even wanted the ability to enter the electronic Wellbeing and Self-Assessment Diary more than once a day. COPD patients were clearly feeling empowered and there was evidence of their self-efficacy (Bandura, 1995) as they felt able to contribute to further refinement of the electronic Wellbeing and Self-Assessment Diary.

The electronic Wellbeing and Self-Assessment dairy helped to provide a strong sense of support. Comments included "Can keep an eye on you"; "Feel better using the App"; "Feel it

is a must"; "Be snookered without the App"; "Cut off without this"; "Provides security like pulmonary rehab"; "Feels secure"; "Someone knows what is happening". This last comment was echoed both verbally (with a resounding; "mm"; or "yes!") and non-verbally by participant nods. It was perhaps best summed up by one participant who exclaimed "It's like a lifeline".

Other telehealth systems do not seem to have offered patients quite the same level of support and wellbeing (Car et al., 2012). So perhaps it was the combination of the electronic Wellbeing and Self-Assessment Diary together with the other features of the care provided that made the COPD patients feel supported. This would link into the work reviewed above (Section 5.1.1.1, Page 364), which showed that a fall in exacerbations and reduced healthcare utilisation demanded application of two or more variables which the community-based longitudinal study in Chapter 4, Page 251 implemented; such reduced illness and healthcare utilisation surely indicating a psychosocial impact of effective telehealthcare which links directly to our COPD patients' comments.

The electronic Wellbeing and Symptom Diary also fulfilled an educational role which empowered the COPD patients. One COPD patient stated "Before I didn't even look at the colour of my sputum or think about how I feel but I do now". Another one commented "You get that used to filling it in that it makes you more aware and think about how you are today. I'm more confident about my self-management than before starting the trial". This led to an improved confidence and possibly better self-management as the COPD patients were made aware of clinically relevant parameters in their disease. These comments suggest an increase in patients' self-efficacy (Bandura, 1995) and find resonance in the effective self-management (Ryan & Sawin, 2009) championed by self-management programmes (Zwerink et al., 2014).

5.4.2. Humanity

The research team conducted routine weekly visits to assess health status. They were also available either in person or via the phone if COPD patients noticed a clinical deterioration indicated on the electronic Wellbeing and Self-Assessment Dairy. If the patients decided to commence treatment for an exacerbation this would also trigger a visit from either myself or the nurse. Thus, the research nurse team helped to provide a safety net not only during exacerbations but also when COPD patients were well. One COPD patient commented, "Off day; unwell the nurses will ring up which is good". Another COPD patient commented proudly, "The nurse came out when I started treatment which is fantastic". Some COPD patients felt that the clinical research team provided a bridge between existing healthcare provision and home self-management, particularly as there seemed to be a reluctance to contact the GP. Again, this resonates with the points discussed previously (Section 5.1.1.1, Page 364) which highlights the need for a holistic approach and that there are different components to implementing an intervention so that it can be received as efficaciously.

This was further illustrated by COPD patients' comments and regret that their condition meant that they would be frequent users of the healthcare system. Many were not comfortable with this. As one COPD patient commented "You don't want to go to your GP every day". This individual clearly wanted to engage with self-management (Ryan & Sawin, 2009) and the electronic Wellbeing and Self-Assessment diary together with our visits, supported this.

All COPD patients found the electronic Wellbeing and Self-Assessment diary useful with many stressing the communication/human contact features of the system (Egan, 2013). One COPD patient stated, "It's good because you have contact with someone. If you're having a good day, then it doesn't matter.". However, "If someone is there and replies when you need it then that's

fine". This contact was "virtual" contact with a clinician. However, importantly, our visits seemed also to be a vital part of the system; this perhaps raises questions about the true efficacy of the electronic monitoring alone as effective patient self-management (Ryan & Sawin, 2009).

As one COPD patient commented "Nice that nurse comes in and says you are unwell go to the hospital"; and another commented unsurely, "better you make that judgement than me". Perhaps with increased experience of the accuracy and effectiveness of their own decision making, this apparent uncertainty might be alleviated. *Or*, maybe, given the isolating nature of many chronic conditions, including COPD the "humanity" and human contact offered by a clinical team is an essential feature of effective technological intervention. As one commented appreciatively, "I loved you coming". The human contact was clearly very important and COPD patients felt valued and cared for, "This wasn't just an extra level of care, but a personal level of care for me". The security offered by the clinical research team's visits was clearly key to the success of the remote monitoring diary in patients' self-monitoring.

5.4.3. Interactions with the GP

There was a strong feeling of frustration expressed towards current healthcare structure especially towards the GP. One COPD patient stated: "I rang the GP practice and I could only see the nurse as the GP I wanted to see is popular and thus has a 2 week waiting list. The nurse called back and said to attend A&E. I commented: You're joking. I'll look like an idiot going to A&E. The things you have to go through". This very angry outburst from one male elderly participant perhaps presents the opposite face of published work (Effing et al., 2007) which merely measures the number of admissions to A&E without delving more deeply into how far patients have attempted to manage their own condition – with support from Primary Care.

COPD patients were not complimentary about their care by GP or Community Nursing staff. Some COPD patients felt that there was not enough focus on them. "If you see your GP it's for 5 minutes and they don't even ask how you feel". However, some COPD patients lacked confidence in self-management of their condition and were thus apprehensive: "When I ring my GP and I say that I think I have an infection the receptionist tells me to take my rescue medication; Sometimes you just want to see the Doctor and not treat myself". It is possible that this type of COPD patient in the community is less likely to start treatment at an appropriate time which in turn could lead to an increase in exacerbation severity and additional hospital admission (Effing et al., 2007).

5.4.4. All alone

The final theme identified is "All alone". During the workshops and the subsequent analysis of the reports it became clear that the COPD patients were deriving a strong sense of support from both the electronic Wellbeing and Self-Assessment diary and the clinical research team. However, this theme of support was likely to be enhanced by a fear of being "all alone". The COPD patients felt that they had been left to "just get on" with self-management of their condition and this caused feelings of isolation, apprehension and fear. One COPD patient commented "When I finished the trial I felt: "Oh no I don't want to finish"; Another one stated sadly, "makes you feel without this there is nothing apart from inhalers and medication". Such responses raise major concerns in a context of the focus on patient well-being reducing the probability of COPD-related hospital admissions (Zwerink et al., 2014) and the need to avert disability, hospitalisation and even death (Garcia-Aymerich et al., 2011).

5.5. Discussion

In this qualitative study bespoke workshops (with the help of a clinical psychologist and the research nurse) were conducted for patients living with COPD, who were part of a pilot community-based longitudinal study investigating COPD and health status (Chapter 4, Page 251). Following thematic analysis within a Framework Method (Gale et al., 2013) conducted by a multi-disciplinary team approach the COPD patient comments were positioned within four themes: *technology, humanity, interactions with the GP* and *all alone*. The results of this qualitative study demonstrate a successful model of care to deliver effective self-management for patients with COPD. Using the theory of self-management (Ryan & Sawin, 2009) which is based in self-efficacy theory (Bandura, 1995) COPD patients were engaged into an intervention involving an electronic Wellbeing and Self-Assessment Diary accessed via an iPad (Apple, USA), and regular contact with a dedicated clinical team. The electronic Wellbeing and Self-Assessment diary was well received with an excellent compliance rate for completion of the daily diary (98%). This was echoed in the community-based longitudinal study (Chapter 4, Page 251) free-text feedback received for all enrolled patients; specifically, comments indicated that the COPD patients found the electronic Wellbeing and Self-Assessment Diary to be helpful in assisting them in their daily self-management and as a platform to interact with myself and a named nurse. The recording of readings/self-assessments in COPD can provide a sense of security by offering an ability to explain symptoms and widen the possibility of taking action (Huniche et al., 2013). The electronic Wellbeing and Self-Assessment Diary helped to facilitate this by providing a platform to record symptoms and allow healthcare professionals to take action based on this information. The recording of daily symptoms helped to increase patients' awareness of their own disease and arguably, this has the potential to improve self-management for patients living with COPD in the future. The use of education in self-management has been shown to reduce urgent healthcare utilisation in COPD (Dickens et

al., 2014). The research team helped provide support by creating a safety net for the COPD patients.

It could be argued that the key to the electronic Wellbeing and Self-Assessment diary's success was the real time support provided by the team. In addition to the regular visits and remote surveillance of symptoms the intervention by the team when the COPD patients were unwell helped to add to the sense of support. This intervention was not restricted to "office hours" and thus meant that an additional level of healthcare input was available "out of hours". Thus COPD patients did not have to rely on traditional modalities that are unfamiliar or time consuming. Overall COPD patient confidence in self-management of their condition was improved resulting in none of the enrolled study patients Chapter 4, Page 251 requiring hospital-based care for an acute exacerbation. Consequently, this had a major (positive) impact on NHS resources; besides having a beneficial impact in the wellbeing of the COPD patients. Thus, it can be argued that the provision of a well-designed and appropriately supported electronic PRO instrument for COPD patients has the potential to allow for a more cost-effective way of managing these patients, crucially in the comfort of the patients' home.

This study demonstrates that COPD patients will engage in a process they are initially apprehensive about if there is an appropriate level of specialist support. The study helped to improve patient self-efficacy reduce hospital admissions and enhance COPD patient wellbeing. It can be argued that all key patient-related elements should be considered when designing self-management tools for patients with COPD (Wortz et al., 2012).

Workshops were conducted as they provided advantages. They do not discriminate against people who cannot read or write, and they encourage people who would find it uncomfortable

to be interviewed on their own and/or people who feel they have little to say (Ritchie, 2014). The location of the workshops was in a place where the COPD patients held their weekly exercise classes (pulmonary rehabilitation) which they all enjoyed attending. This may have created a positive feeling going into each workshop; however during the workshops it was discovered that these classes were ending which dismayed all the COPD patients. The moderator for the workshops was the research nurse who had also visited the COPD patients. This might have made some COPD patients reluctant to criticise the community-based longitudinal study (Chapter 4, Page 251) especially those that were still active within it. There were no audio recordings which may have led to incomplete transcription, although two transcribers (myself and a clinical psychologist) were used and the generated reports were consistent in data capture. The multi-disciplinary team all engaged with data analysis.

In conclusion the results from this chapter have demonstrated that COPD patients are willing to embrace technology to improve the management of their chronic disease. However, one modality alone cannot be comprehensive enough to manage the disease. An interactive platform between the patients and their healthcare team needs to actively exist. Telehealthcare is increasingly being promoted as this platform but an appropriate support system needs to be in place for it to be effective and sustainable. A faceless communication technology or technologies that cannot be responded to promptly may end up alienating the end-user. This could lead to telehealthcare modalities being deemed ineffective and the opportunity to enhance chronic disease management being missed/abandoned.

Chapter 6:

Exploration of Patient Experiences on Saliva Sampling: Development of a Bespoke Saliva Collector Prototype

6.1. Introduction

The saliva sampling container (collector) selected for individuals in this thesis was a pre-marked 15ml centrifuge tube (Nunc, Denmark), (Chapter 2, Page 84). Informal feedback from COPD patients involved in the first community-based cohort study (Chapter 3, Page 221) highlighted that although this collector was functional it could be end-user refined. Taking this into account a process was begun, within the timeframe of this thesis, to develop a prototype saliva collector. The design of this collector is intended to be patient-driven but also incorporating internal engineering solutions that could substitute pre-analysis saliva sample processing such as centrifugation (Chapter 2, Page 92) that presently requires laboratory-based instruments.

To understand COPD patients' needs and preferences in addition to the informal individual patient feedback received (Chapter 3, Page 221), a qualitative study utilising workshops was conducted; with COPD patients who were either participating or had completed the community-based longitudinal study (Chapter 4, Page 251). The methodology for these workshops was based on the successful workshop model conducted in Chapter 5, Page 361.

The purpose of these workshops was to explore in detail COPD patients' experiences of providing saliva, its collection into a container and the usefulness of the provided collection protocol (Chapter 2, Figure 2.28, Page 209). These workshops also discussed key features that patients wished to see incorporated into an "ideal" saliva collector fit for purpose and ease of use. Workshop discussions enabled me to produce design concepts of a first saliva collector (with integrated filter system) and 3-dimension (3D)-printed proof-of-principle physical prototypes for further evaluation in workshops and laboratory testing. This approach provided the necessary information for further refinements to be made to the prototype saliva collector.

Overall this chapter reflects the start of an iterative research-patient driven interactive process, the data from which will eventually lead to the manufacture of a bespoke saliva collector for commercial diagnostic application. Thus the objective of this chapter is to further understand the practicalities for development of a POC saliva monitoring tool (Chapter 1, Page 78).

6.2. Patient experiences on saliva sampling and method of collection

COPD patients' attitudes and experiences were explored by setting semi-structured workshops 12 months after the start of the community-based longitudinal study (Chapter 4, Page 251). COPD patients who had completed or were still active within the community-based longitudinal study were invited to take part (Figure 6.1). As such, they formed a purposive sample (Ritchie, 2014).

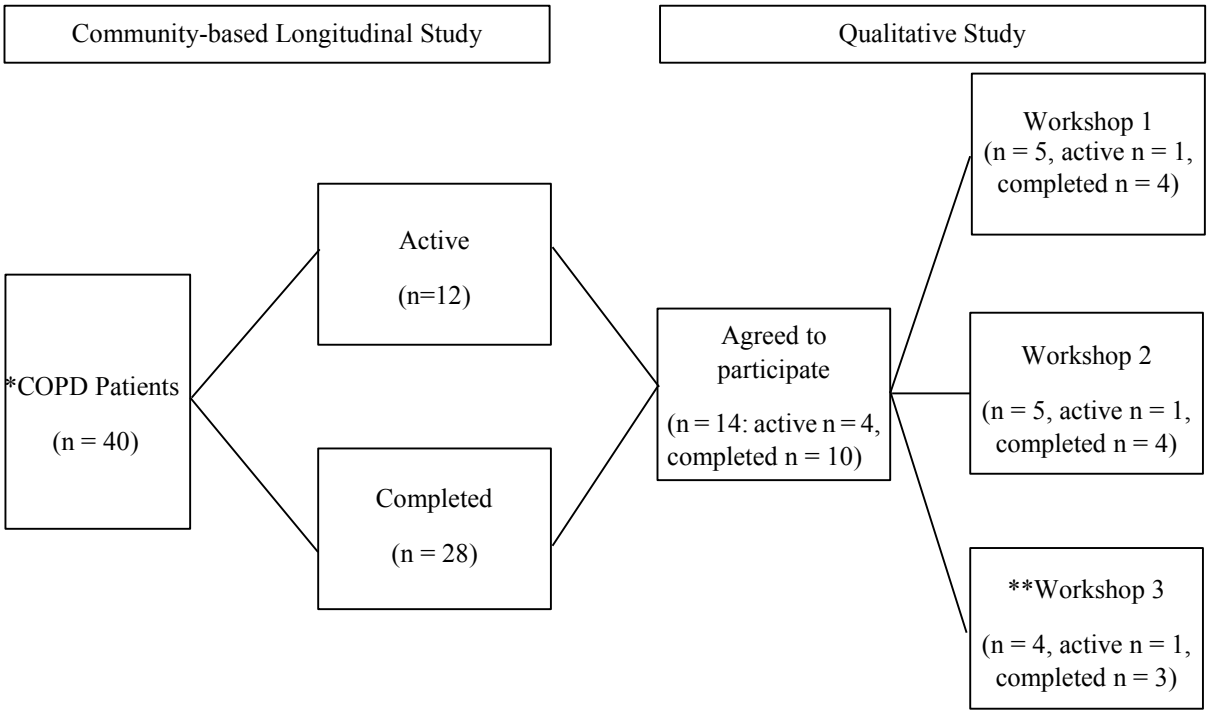

Figure 6.1: Workshop recruitment

*COPD patients (n = 31): at this time-point in the community-based longitudinal study only 31 COPD patients had been recruited. **Workshop 3 was conducted with the 3D-printed physical saliva collector prototype.

6.2.1 Workshop demographics

Fourteen COPD patients accepted invitations to the workshops; however, 4 patients developed exacerbations at the time of the scheduled workshop so only 10 patients attended (Figure 6.1, Table 6.1). Workshops 1 and 2 were conducted using just concept discussions; workshop 3 demonstrated the 3D-printed prototype collector and filters.

Table 6.1: Workshop demographics.

	COPD Patient (n = 14)	
	Male	**Female**
Gender	10	4
Age, years	72.25 ± 4.56	63.15 ± 2.25
BMI, (kg/m^2)	26.58 ± 2.45	27.84 ± 3.45
Total Co-morbidities, [a]n	1.56 ± 0.45	1.55 ± 0.25
FEV_1, (% predicted)	59.15 ± 4.55	58.17 ± 4.88
FVC, (% predicted)	72.15 ± 2.88	74.89 ± 5.45

Data presented as mean ± SD unless stated.

6.2.2. Data collection

In preparation for the workshops, due consideration was given to the structure of the groups, the venue and the required information to elicit. The chosen venue was the location known and frequented by all patients for the exercise-based treatment (pulmonary rehabilitation). A series of questions was constructed with the help of a clinical psychologist (Dr. Kathyrn Kinmond, Manchester Metropolitan University) and refined (Table 6.2) to seek a better understanding of the following factors: patient perception of saliva sampling and their views on how it compared with venepuncture sampling for their blood tests; the saliva sampling protocol (Chapter 2, Figure 2.28, Page 209) and whether the language used was appropriate; their views on an "ideal" saliva collector – its shape, size and feel, its markings if any, texture (opaque or transparent); and in workshop 3 only their perceptions of the produced physical prototype

.

Table 6.2: Workshop questions.

1. How did you initially feel about the idea of giving a sample of saliva?
2. You were given a set of instructions to follow before giving a saliva sample.
    a. Were the instructions clear?
    b. Were they difficult to stick to?
    c. Do you feel the instructions should be changed in any way?
3. Was it easy to produce and give a saliva sample?
    a. Could anything have made it easier?
    b. Did anything make it difficult?
4. Do you think the current tubes are ok for collection the saliva?
    a. Was it easy or difficult to use the tubes?
5. What would you change about the collection tube?
6. Usually you are asked to give a blood sample for teste; we are trying to see whether saliva can be used as an alternative.
    a. Do you think that this is better than having a blood sample taken?
    b. Would you be happy in the future giving saliva instead of blood?

Demonstrate the new proto-type saliva collection device and then pass it around the group.

7. *What are your likes/dislikes of our new saliva catcher?
8. Are there any other comments you would like to share with us?

*This question was only included in workshop 3 to obtain feedback of the manufactured prototype saliva collector.

All 3 workshops were purposefully facilitated by the clinical research nurse (moderator). The rationale for this has been explained in Chapter 5, Page 372. No audio-recording took place so the conversations were transcribed in "real-time" by myself and the clinical psychologist for each workshop. Every session commenced with a general introduction, and then the main discussion. During the workshops all patients were encouraged to talk freely about their experiences and at the end of the session asked to raise anything that had not already been covered. Each workshop lasted two hours. All patients received a copy of the questions (Table 6.2) to be discussed one week prior to the workshop. In this way it was aimed to offer the patients an opportunity to give informed consent to take part, as they were as fully briefed as possible, including being made aware of the questions that were going to be asked of them. All COPD patients gave consent to their conversation being transcribed and quotes being used.

6.2.3. Data analysis

Data transcription and analysis proceeded simultaneously utilising the Framework Method (Gale et al., 2013). After each workshop there was a team de-brief to discuss the key themes. Following which, the clinical psychologist and I prepared individual reports containing a transcript of the conversation and key themes to have emerged from the session; reports were then exchanged and mapped. Finally, after all three workshops were completed, a face-to-face team meeting was held to discuss the overall results and final refinement of the concluding report.

6.2.4 Results

Analysis of the transcripts from the workshops produced two main themes on patients' perceptions and feelings about saliva sample collection.

6.2.4.1. Apprehension and embarrassment

COPD patients described the process of producing and depositing a saliva sample into the provided conventional tube collector that was used during the clinical study. They felt the collector length was "comfortable" to hold in their hand but had initially felt "anxious" that it would be difficult to drool the sample into the tube due to its "narrow" opening (aperture diameter: 15mm) without spilling outside of the tube and soiling. This was echoed by most of the COPD patients in particular one stated "hit or miss; sometimes hit, sometimes miss" although this was not universal as another COPD patient proclaimed "no problem; bigger bottle would be better". Patients initially felt embarrassed on providing the saliva sample at the first visit, especially in front of the research nurse, "Strange at first; old fashioned didn't like spitting in front of a lady even though they are professionals; now I have got over that; at first I felt a little embarrassed then fine". This was explained by feelings that they were "going against the grain" having been brought up not to spit (this term was used synonymously with drool), particularly in front of someone. They also felt slightly "under pressure" to fill the pre-set volume of saliva (2mls) as requested; this was related to the perceived "time" they felt it would take to achieve the goal. They expressed that having to provide a saliva sample over a time frame longer than 10 minutes would heighten apprehension and lead to COPD patients not embracing saliva for bio-sampling in the future. Their preference for saliva collection was for individuals to be given the collectors with a saliva sampling protocol, and then to be left to provide the sample in private; they were happy to safeguard the samples as guided until

collection. However, they felt that these initial feelings settled over the study period as they got used to the technique and concept, as one COPD patient remarked "I found it easier with time."

6.2.4.2. Empowerment

All COPD patients stated that they would prefer to provide saliva samples rather than having to have venepuncture for blood tests: "Doesn't bother me; Experiment in saliva better than blood, no needles; prefer saliva much easier. They also felt that being able to test their saliva at home was also advantageous compared to travelling to the GP surgery, a community clinic or hospital central pathology to have their samples taken. They would be happy to monitor their saliva as part of a self-management protocol if it meant they could monitor their disease better and would feel "more in control" if they could "spot" their acute exacerbations earlier. As several COPD patients neatly summarised, "Shall I or shan't I; Wait until tomorrow and tomorrow comes I think I should have taken them yesterday; When I give blood it takes 1 week for the results; when you have a chest infection you give blood then you wait 1 week I'm 100 times worse; need something there and then that says you have a bad infection take treatment".

6.2.4.3. Feedback on the provided saliva collection protocol

The saliva collection protocol (Chapter 2, Figure 2.28, Page 209) asked for fasting for at least 30 minutes, abstinence from alcohol for 12 hours and tooth brushing avoidance for 1 hour. Patients felt that the saliva collection protocol provided for the studies was non-cumbersome. They did not feel that the 30-minute fast time was excessive or that abstinence from alcohol for 12 hours would make them less likely to embrace saliva-based testing. They felt the protocol was clear and easy to follow. They did however suggest replacing the word "dribbling" with "drool".

6.2.4.4. Feedback on designs of a bespoke saliva collector.

Workshop participants provided useful ideas on features they wished to see incorporated in a future saliva collection device. These included:

- The length and diameter of a collector. They felt the grip on the provided conventional tube was adequate but that the opening should be wider and that the length could be improved upon to a maximum of 60mm without compromising the grip.
- The collector to incorporate an ergonomic lip in order to increase comfort, facilitate ease of use and provide reassurance when providing a sample.
- The collector to be made of a clear material for example: polycarbonate plastic, and to have a minimum saliva volume level indicator to inform when an adequate sample had been deposited.
- The indication of saliva passage through any filters incorporated in the collector was not seen as important.
- Patients had no concerns about handling the saliva collector and felt that a lid would not be essential.
- There had to be a clear set of instructions provided with the sampler on how many times it could be used as well as its disposal. They suggested that the collector should be single-use only packaged in a re-sealable plastic bag.
- They had no objection to the saliva collector being connected to an assay container (cartridge) so that analysis of biomarkers could be performed on the saliva. Patients expressed that if possible the collector should be part of the assay cartridge. If separate, there would need to be a clear auditory indication when the collector attached securely to the cartridge; the locking needed to be simple: no twisting and “slide and click” would be preferred.

6.3. Development of a bespoke saliva collector

Within the time-frame of this thesis, and in response to the COPD patient feedback in the above workshops designs were produced for a new saliva collector device. The driving objective for the design was to improve and enhance the end-user's experience with respects to patient providing and collecting saliva samples production and their storage. To augment the functionality of the collector, designs purposefully included ideas on filtration systems in order to produce a clarified sample for protein analysis and to avoid the need for centrifugation in a laboratory (Chapter 2, Page 92). This would be an essential requirement for the future POC testing. Design concepts and specifications were used to create compute aided design (CAD) files (provided in STEP format) in collaboration with Design Reality (St Asaph Business Park, Denbighshire). This allowed 3D-printing of physical prototype models for: (1) a series of preliminary experiments to test the functionality of individual components; and (2) feedback from COPD patients within a further workshop.

6.3.1. Key factors considered in the design process

Specific design factors considered included the length and width of the collector, engagement of the collector with an end-user's lip, internal air displacement to allow for salivary flow and total time for saliva to flow through the device. The design elements also addressed issues of (1) the inevitable foam (both macroscopic and microscopic) that occurs when saliva samples are drooled and (2) the *in-situ* filtration of saliva to produce a debris-free filtrate suitable for target analyte testing.

6.3.1.1. Ergonomic lip feature

A key problem with the use of conventional tube containers, noted both by us and the patients during the community-based studies (Chapter 3, Page 213; Chapter 4, Page 251), and the workshops, was the lack of lip engagement and risk of spillage. Thus whilst designs of the new saliva collector retained its cylindrical shape a profiled area was constructed around the aperture at one end, to rest just below the users' lower lip; this ergonomic lip feature would increase user comfort during saliva production and prevent spillage (Figure. 6.2). The diameter of the aperture was also increased to 20mm to help saliva capture and flow.

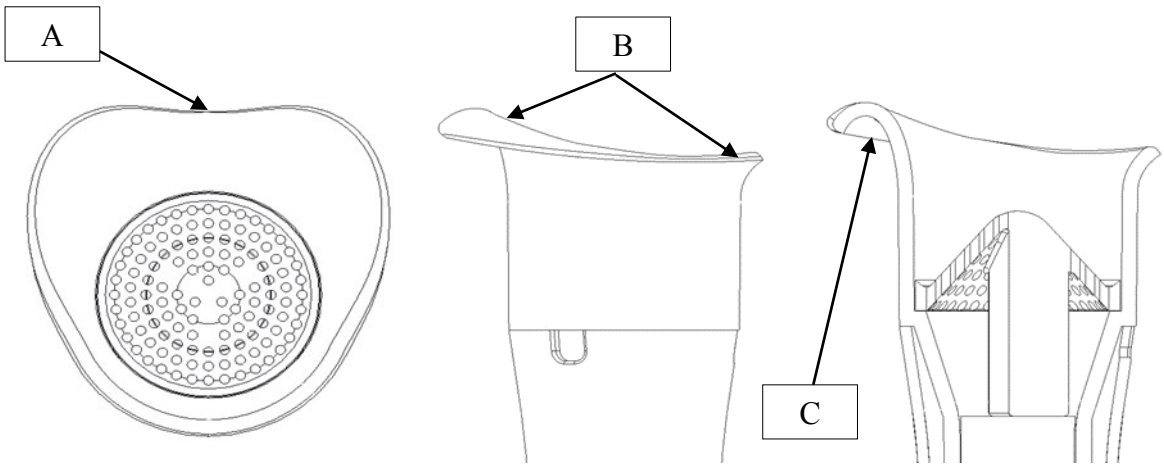

Figure 6.2: Ergonomic lip feature.

This figure illustrates the ergonomic lip feature in a number of different vantage points to highlight its key novel concepts: A = concave geometry and rolled lip feature; B = elevated datum side compared to the opposing side resulting in a height differential, which further defined the lip-engaging feature. Additionally, the ergonomic lip feature is designed to guide the end-user visually by shape, and physically by touch, on the correct orientation and position for the collector during saliva sampling; C = rolled lip feature.

6.3.1.2. Body of the collector

During the workshops (Section 6.2.4, Page 390) the COPD patients did not report a problem with grip of the conventional tube collector so a diameter of 15mm was used for the body of the prototype saliva collector. However, its length was shortened as in the workshops patients had reported, that albeit subconsciously they felt pressurised to fill the container when given the sample tube (apprehension theme) (Section 6.2.4.1, Page 391). The workshops revealed that 60mm was a maximum acceptable length for a saliva collector that would avoid apprehension and maintain a comfortable grip for patients. The set dimensions of a prototype saliva collector (diameter: 15mm, length: 60mm) would maximally accommodate 10mls of saliva. Although this volume is far in excess of the saliva quantity that would be required for target analyte testing, these dimensions would allow for a filter system to be integrated into the collector.

6.3.1.3. Saliva sample filtration

Biomarker analysis requires a "pure" and macroscopically clear sample prior to undertaking testing. The reason for this is that any particulate debris, for example, food (Chiappin et al., 2007) in the sample can interfere with immunoassays (Wong et al., 2008). In this thesis centrifugation was employed to clarify saliva (Chapter 2, Page 92); however for POC testing this is not practical. Thus for the prototype collector to meet the needs of POC saliva diagnostics it would require an internal filtration system to avoid having to send samples to a laboratory for sample centrifugation and processing (Chapter 2, Page 92). The filtration system within the collector would need to be able to: (1) de-foam, (2) remove macroscopic and (3) microscopic debris. A series of designs were produced for a dual filtration system as a possible solution. The first filter (conic) (Figure 6.3) was designed to de-foam saliva and remove macroscopic debris. The key concepts of the first filter were a conic surface, which allowed for

an increase in overall surface area and a stem to retain the second filter. The conic filter incorporated a total of 127 pores each with a diameter of 0.80mm arranged in concentric circular groups covering 16% of the total surface area. These pores acted to de-foam and macroscopically filter a sample of saliva.

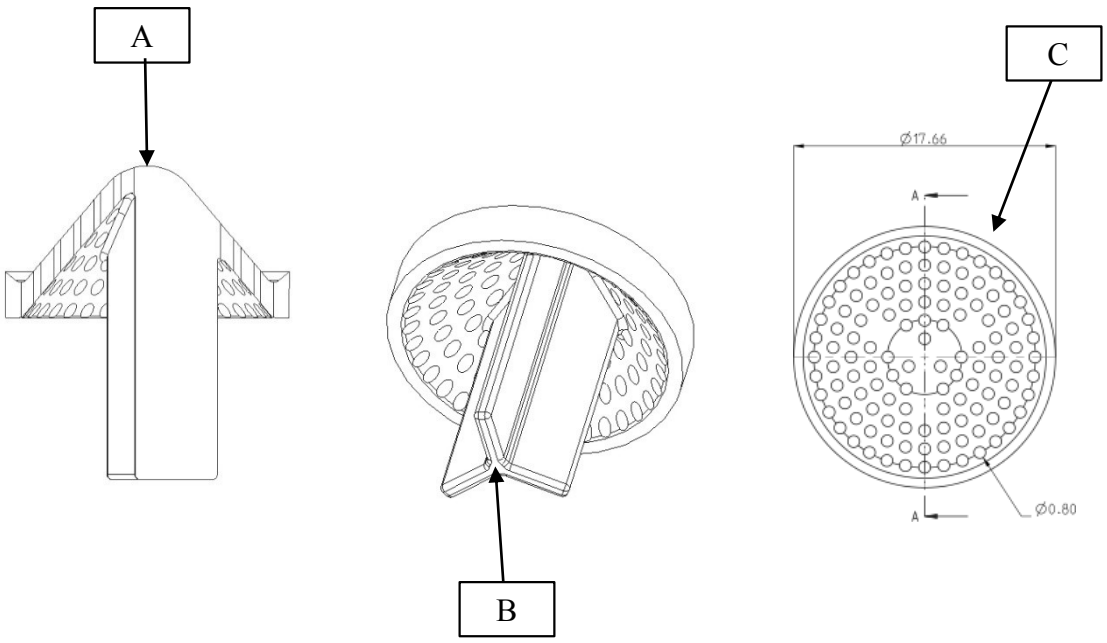

Figure 6.3: First (Conic) Filter.

This figure illustrates the first (conic) filter at 3 different vantage points demonstrating the key components: A = Conic surface; B = Stem to retain a second (cellulose acetate) filter; C = concentric pore arrangement for de-foaming and macroscopic filtration.

The second filter was for additional micro-filtration of saliva; cellulose acetate (Swan, UK) (Figure 6.4) was chosen as the composition of the second 'micro' filter. The key concept of the second (cellulose acetate) filter was to remove any residual debris (macroscopic and microscopic) that had not been removed by the first (conic) filter. Cellulose acetate (Swan, UK), chosen for its inert qualities, has been shown not to affect the level of certain proteins in

saliva (Chapter 1, Page 53) and is also used in commercially available saliva kits, for example, in oral swabs to collect saliva via passive absorbance (Saliva Bio Oral swab; Salimetrics, USA). Although the exact minimum pore diameter on the cellulose acetate filter manufactured by Swan, UK is not available; it has been demonstrated in one study of cellulose acetate filters manufactured by 5 different companies that the most abundant pore diameter is in the range (0.013 to 0.016µm) (Sameer, 2010). The mean molecular radius of CRP (the largest of the three biomarkers) is 48.8 ANGSTROM (Kushner and Somerville, 1970) which would be trapped in a filter with a minimum pore size of less than 0.005µm.

Figure 6.4: Cellulose acetate filter

This figure demonstrates a box of cellulose acetate filters (Swan, UK) which contains 120 individual cellulose acetate filters organised into cylindrical bundles of 6.

6.4. Bespoke saliva collector

Transferring above concepts and designs incorporated into CAD files enabled 3D-printed saliva collector prototypes; these were built on an Object Connex (Stratasys, USA) using a VeroClear resin (Objet Inc, USA). The prototype material used was an acrylic monomer, which was built in layers, each ultra-violet cured before moving onto the next layer (Figure 6.5). This prototype and its individual components were then used in a series of simple experiments designed to explore: (1) transit time of saliva through the prototype saliva collector; (2) sample clarity post-filtration; (3) levels of the target saliva biomarkers levels in the filtered saliva.

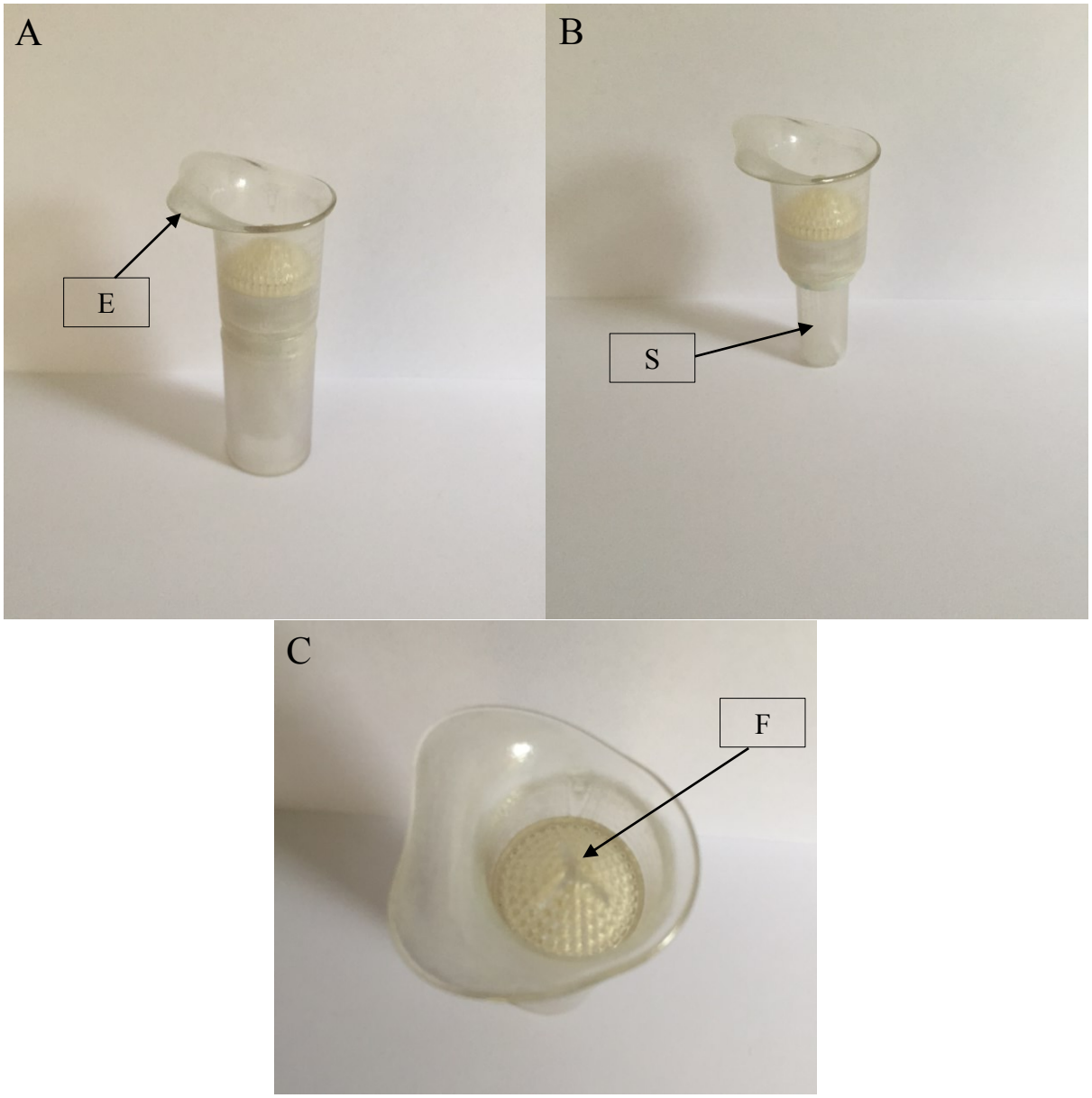

Figure 6.5: **3D-printed prototype saliva collector.**

These figures display the prototype saliva collector built on an Object Connex (Stratasys, USA) using a VeroClear resin (Objet Inc, USA): A = complete prototype saliva collector; B = prototype saliva collector with outer casing removed to highlight the location of the second (cellulose acetate filter); C = "bird's-eye" view of the prototype collector showing the first (conic) filter; E = ergonomic lip feature; F = first (conic) filter; S = second (cellulose acetate) filter "house".

6.4.1. Salivary flow through the prototype saliva collector

The overall purpose was to determine the transit time of saliva through the bespoke prototype saliva collector under specific conditions:

1. Conic filter *in-situ* only.
2. Conic and cellulose acetate filter (length: 14mm) *in-situ*.
3. Conic and cellulose acetate filter (length: 7mm) *in-situ*.

6.4.1.1. Materials and methods

Unstimulated whole saliva (7mls) from a healthy non-smoker was collected via passive drool into a marked centrifuge tube (Nunc, Denmark). The healthy non-smoker subject (aged: 32 years, BMI: 22.79kg/m^2, no known co-morbidities and no regular oral medications) was selected from the Directorate of Respiratory Medicine's research and outpatient clinic database (Chapter 2, Page 88). This subject gave informed written consent and adhered to the saliva sampling protocol created in Chapter 2 (Figure 2.28, Page 209) prior to providing the saliva sample. To assess transit time through the prototype saliva collector, a set volume (2mls) of the above saliva was deposited manually at the top aperture of the prototype collector using a pastette (alpha labs, UK).

Transit times were separately determined with the first (conic) filter *in-situ* and also with both first (conic) and second (cellulose acetate) filters *in-situ*. The second (cellulose acetate) filter was tested at two lengths: 14mm and 7mm.

Within the said prototype collector, the cellulose acetate filter is "housed" in a plastic sheath (Figure 6.5 (S)) with an open aperture at the apex and an aperture at the base which has three

struts to provide a bed. This set-up provides a firm but non-compressed grip to hold the cellulose acetate in place. This "housing" for the second (cellulose acetate) filter has a diameter of 10mm. Thus three cellulose acetate filters were concentrically fixed together using pressure-sensitive tape (3M, USA) and placed into the second filter (cellulose acetate) "housing" in the prototype collector. The rationale for using two filter lengths was to understand effects on saliva clarity and transit time through the prototype collector. It was hypothesised that the reduction in filter length (14mm to 7mm) would result in an improved salivary transit time. The volume of saliva to transit and emerge through the dual filtration process was documented at a pre-set time of 10 minutes. Transit time was quantified using the stopwatch function on an iPhone (Apple, USA). This time was chosen as the workshop participants had expressed that total saliva sampling time should take no longer than 10 minutes as longer times could precipitate anxiety and non-compliance (Section 6.2.4.1, Page 391). Throughout these experiments clarity was defined by a direct visual comparison to a standard laboratory-centrifuged sample of saliva (Figure 6.6D).

6.4.1.2. Results

The transit time for 2mls of saliva through the prototype collector with the first (conic) filter only *in-situ* was 6 minutes and 32 seconds. This saliva sample was completely de-foamed although visually it contained debris (Figure 6.6A). The next experiment involved assessment of filtered saliva clarity and transit time through the prototype collector with both the first (conic) and second (cellulose acetate) filters (length: 14mm) *in-situ*. After ten minutes no saliva had emerged through the dual filtration system and thus the experiment continued to be observed. At 55 minutes, 500ul of visually clarified filtered saliva (Figure 6.6B) had emerged, by 60 minutes no further filtered saliva emerged. The final experiment assessed transit time and filtered saliva clarity through the prototype collector with the first (conic) and second

(cellulose acetate) filter (length: 7mm) *in-situ*. After 10 minutes 100ul of visually clarified filtered saliva was produced (Figure 6.6C).

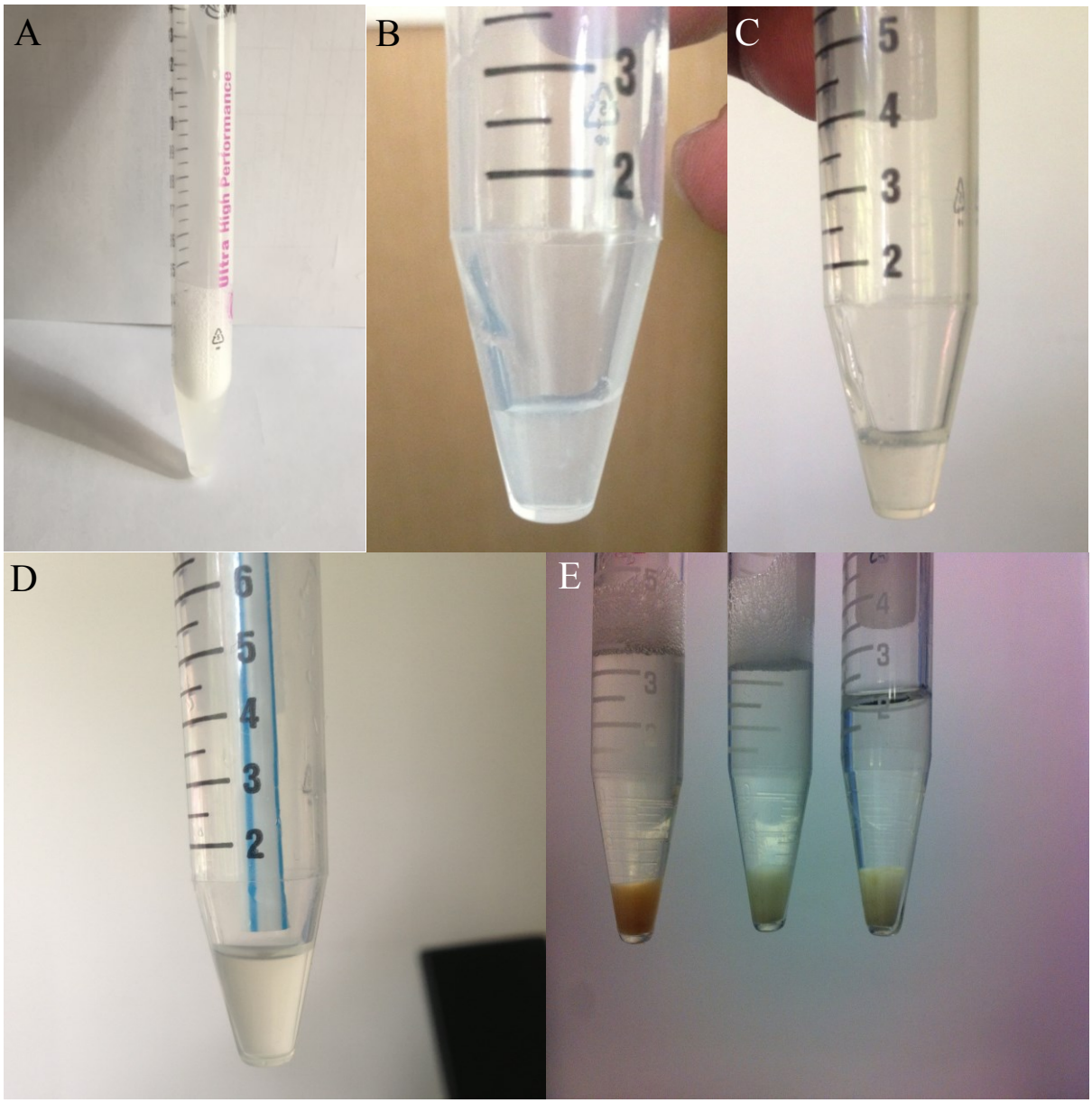

Figure 6.6: Saliva post transit through the prototype collector.

These figures illustrated the clarity of saliva after transit through the prototype collector: A = fresh saliva sample with macroscopic debris and a foam head; B = first (conic) filter only; C = first (conic) filter and second (cellulose acetate) filter (length: 14mm); D = first (conic) filter and second (cellulose acetate) filter (length: 7mm). Figure E illustrates 3 separate saliva samples post-centrifugation at 3000rpm for 15 minutes as a visual comparison to the prototype filtered samples. It can also be observed that the dual filtered saliva samples (C and D) contain non visible cell debris.

6.4.1.3. Conclusion

The developed prototype saliva collector incorporating the dual filtration system (conic and cellulose acetate (Swan, UK)) produced a de-foamed, visually clear (comparable to centrifugation) sample of saliva that can be tested for target analyte quantification. However, the flow of saliva through these filters would be too long for POC testing and thus further experiments were conducted in an attempt to reduce this time.

6.4.2. Measures to improve transit time of saliva

Following on the above results there was a need to improve the transit time for saliva through the prototype collector to meet POC expectations. In addition, the workshops (Section 6.2.4.1, Page 391) had revealed that a sample transit of greater than 10 minutes was likely to result in apprehension and non-compliance, with the majority of users not wanting to provide further follow-up samples. The above experiments suggested that the delay in saliva transit was occurring as a result of the second (cellulose acetate) filter. This filter however was required in order to further "cleanse" the saliva. As a solution to increase saliva transit through this system, the possibility of applying a pressure gradient across the cellulose acetate was explored. This was based on Darcy's Law which states that if there is a pressure gradient applied across a porous material flow will occur from high pressure towards low pressure (Gray and Miller, 2004).

6.4.2.1. Materials and methods

Unstimulated whole saliva (2mls) from two healthy non-smokers collected via passive drool in a marked collector was obtained. The healthy subjects were selected from the Directorate of Respiratory Medicine's research and outpatient clinic database (Chapter 2, Page 88), (Table 6.3); gave informed written consent and adhered to the saliva sampling protocol created in Chapter 2 (Figure 2.28, Page 209) prior to providing a sample of saliva. Each saliva sample was tested in duplicate.

Table 6.3: Subject demographics

	Healthy Subjects
Demographics	**Non-smokers (n = 2)**
Age, years	33.15 ± 2.20
Gender, Male, (Female), n	1 (1)
BMI, (kg/m^2)	21.65 ± 1.61
Total Co–morbidities, n	0
Total Number of Oral Medications, n	0

Data presented as mean ± SD, unless stated.

To test the concept of a pressure gradient, a modified second (cellulose acetate) filter was built, utilising a 1.5ml eppendorf (length: 32mm, diameter 10mm) (alpha labs, UK) as "housing". Firstly, three cellulose acetate filters 7mm in length were fixed, using pressure-sensitive tape (3M, USA), around a hollow soft flexible plastic tube. The hollow plastic tube provided a channel for air displacement. The overall length and diameter of this construct was 7mm by 15mm (Figure 6.7). Up to 3mm in length of this construct was then inserted into a 1.5ml eppendorf with the other 4mm remaining external (Figure 6.8). The diameter of the eppendorf (100mm) was smaller than the modified second (cellulose acetate) filter construct and thus the 3mm portion housed inside the eppendorf was externally compressed relative to the 4mm portion of the modified second (cellulose acetate) filter construct outside the eppendorf. This difference in external compression resulted in a pressure gradient (high to low) across the modified filter. A volume of saliva (1ml) was first passed through the prototype saliva collector with just the first (conic) filter in place; the obtained filtrate was then aliquoted using a pastette (alpha labs, UK) through the modified second (cellulose acetate) filter.

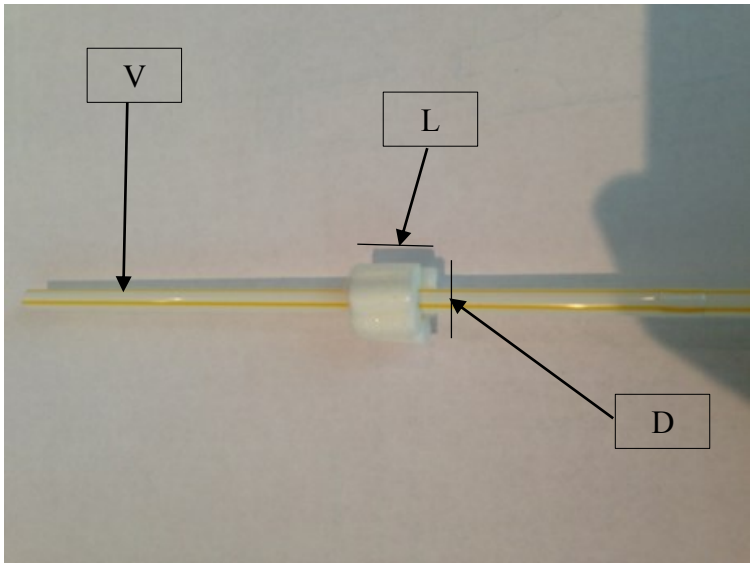

Figure 6.7: Cellulose acetate filter construct.

Three cellulose acetate filters were fixed using a pressure sensitive adhesive around a hollow plastic tube (V) with dimensions of: length (L) 7mm and diameter (D) 15mm. This drawing is not to scale.

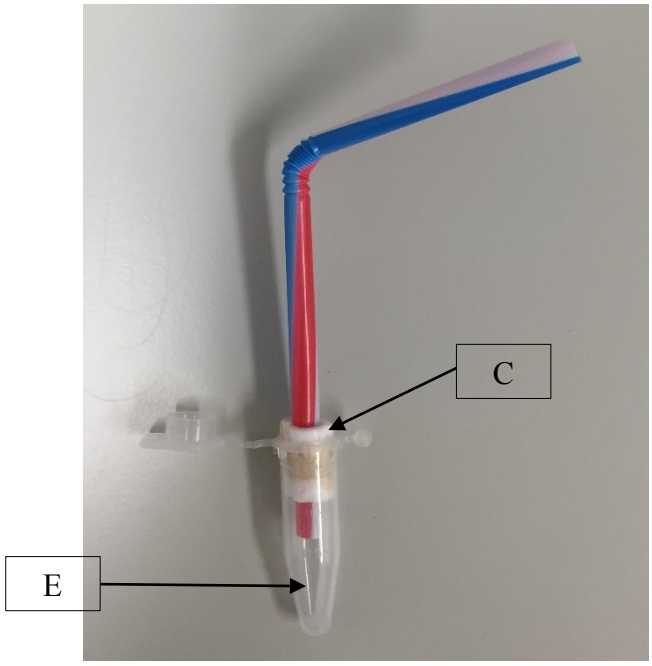

Figure 6.8: Modified second (cellulose acetate) filter.

The cellulose acetate filter construct (C), (length 7mm, diameter 15mm) is inserted (4mm) into a 1.5ml eppendorf (E), (length 15mm, diameter 10mm). The discrepancy in diameter between the two results in a pressure gradient across the cellulose acetate filter construct.

6.4.2.2. Results

Using the above filtration approach, on two different 1ml saliva samples tested in duplicate, volume of 500ul of filtered saliva was retrieved for all 4 tests (50% saliva sample loss). Crucially the transit time of saliva through this modified second (cellulose acetate) filter set-up was only 30 seconds. The experiment continued to be observed for up to 5 minutes; no further saliva was obtained perhaps due to saliva saturation of the filter membrane.

6.4.2.3. Conclusion

This experiment supports the creation of a pressure gradient across the second (cellulose acetate) filter as a possible solution to enhancing saliva sample transit time, whilst providing an adequate volume of sample recovery for subsequent salivary biomarker analysis.

6.4.3. Comparison of target biomarker levels between modified sampler filtration system and conventional tube sample centrifugation

The next set of experiments explored whether there would be method-dependent differences in the measured levels of CRP, PCT and NE in saliva retrieved following filtration using the modified method (Section 6.4.2, Page 406) and conventional tube centrifugation.

6.4.3.1. Materials and methods

Six healthy never-smokers provided 7mls of unstimulated whole saliva collected via passive drool into an ice-cooled marked centrifuge tube (Nunc, Denmark). The healthy subjects were selected from the Directorate of Respiratory Medicine's research and outpatient clinic database (Chapter 2, Page 88), (Table 6.4); gave informed written consent and adhered to the saliva sampling protocol created in Chapter 2 (Figure 2.28, Page 209). For each subject 2mls of the retrieved saliva were then aliquoted using a pastette into a separate conventional centrifuge tube (Nunc, Denmark) and centrifuged at 3000rpm for 15 minutes (Chapter 2, Page 92); whilst a separate 2mls were transferred onto the prototype saliva collector complete with filter 1 (conic) and then a modified second (cellulose acetate) filter 2. Saliva biomarker measurements for CRP, PCT and NE were conducted according to the methodology described in Chapter 2 (Section 2.4, Page 90). Measurements were conducted on 1 microtitre plate for CRP and NE and one kit for PCT. All saliva samples were analysed on the day of collection.

Briefly, CRP was measured in 15ul of saliva using a Salivary ELISA kit (Salimetrics Europe, UK) (Chapter 2, Page 90), which has a range of quantification of 0.90 to 30ng/ml; Salivary PCT and NE were measured using in-house modified commercial serum-based ELISAs. PCT was determined in 100ul of saliva diluted 1:2 in PBS-T using VIDAS BRAHMS PCT kit

(bioMérieux, France) (Chapter 2, Page 112) which has a range of quantification of 0.10 to 400ng/ml. NE was measured in 7.0ul of saliva diluted 1:200 in ELISA wash buffer using PMN-Elastase ELISA kit (Immundiagnostik, Germany), (Chapter 2, Page 149) which has a range of quantification of 2.2 to 2000ng/ml. Biomarker levels of salivary CRP utilised one Salivary ELISA kit (Salimetrics, USA), salivary PCT utilised one VIDAS® BRAHMS PCT kit (bioMérieux, France) and salivary NE utilised two PMN-Elastase ELISA kits (Immundiagnostik, Germany). The intra- and inter-assay CV was less than 8% and 12% respectively for all 3 assays.

Table 6.4: Subject demographics

	Healthy Subjects
Demographics	**Non-smokers (n = 6)**
Age, years	36.2 ± 12.6
Gender, Male, (Female), n	3 (3)
BMI, (kg/m^2)	21.9 ± 1.2
Total Co-morbidities	0
Total Number of Oral Medications, n	0

Data presented as mean ± SD unless stated.

6.4.3.2. Statistical analysis

The statistical tests employed in this chapter are discussed in Chapter 2, Page 86.

6.4.3.3. Results

6.4.3.3.1. Salivary CRP

The levels of salivary CRP in the saliva samples of healthy never-smokers were higher in the collector filtered samples (2.13, 0.30ng/ml) compared to equivalent centrifuged samples (1.94, 0.50ng/ml) although this difference was not statistically significant (p=0.36 by Wilcoxon Signed Rank Test) (Table 6.5). Interrogation of the individual subjects salivary CRP levels revealed higher levels in four out six of the collector filtered samples especially in subject 3 (Figure 6.9).

Table 6.5: Endogenous salivary CRP Levels: Tube centrifugation compared to modified saliva collector in the same subjects.

Healthy Never-smoker Subject	Tube Centrifuged Sample (ng/ml)	Collector Filtered Sample (ng/ml)
1	1.94	2.29
2	1.92	1.54
3	6.49	12.98
4	2.44	2.13
5	1.14	1.26
6	0.84	1.00
Median, IQR	**1.94, 0.50**	**2.13, 0.30**

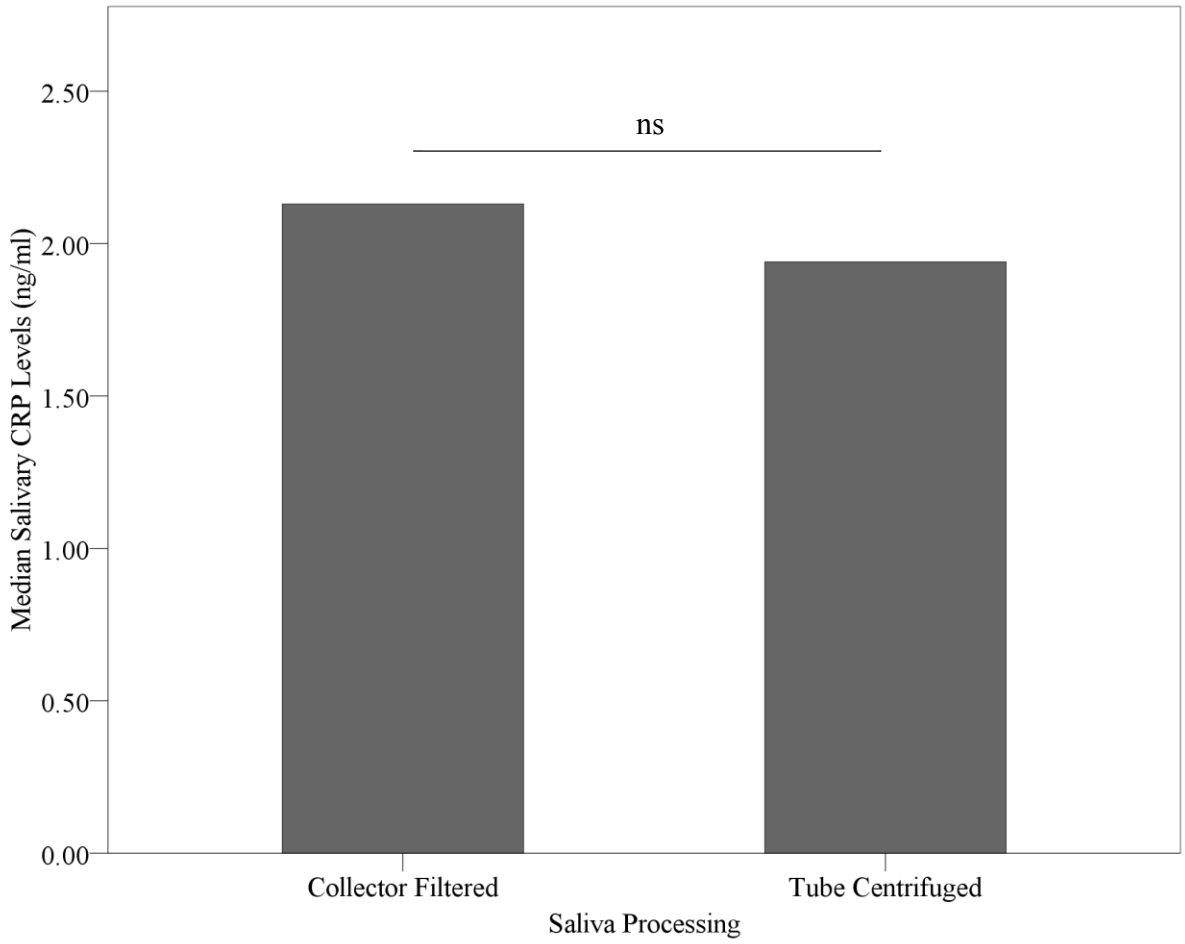

Figure 6.9: Salivary CRP levels across different saliva processing methods.

Bar charts representing the median salivary CRP levels between the collector filtered compared to equivalent tube centrifuged samples. There was no statistical significant between the two processing methods (ns; p=0.36).

6.4.3.3.2. Salivary PCT

As would be expected in samples from healthy subjects, initial analysis demonstrated no endogenous salivary PCT (all samples tested below the lower limit of assay quantification (0.10ng/ml) for the mini VIDAS (bioMérieux, France)), in both collector filtered and tube centrifuged samples of all subjects (n = 6). Thus all saliva samples were spiked with low-range PCT control provided inside the VIDAS BRAHMS PCT kit (bioMérieux, France) following the methodology described in Chapter 2 (Table 2.12, Page 118) to determine measurable effects if any between the two approaches. Overall there appeared to be a significant reduction in spiked salivary PCT levels for the collector filtered saliva (0.65, 0.19ng/ml) compared to equivalent tube centrifuged saliva (0.88, 0.24ng/ml) ($p<0.01$) (Table 6.6, Figure 6.10) The results were consistent across all six healthy never-smokers with all collector filtered samples demonstrating consistently reduced levels of spiked salivary PCT.

Table 6.6: Spiked salivary PCT levels: Tube centrifugation compared to modified saliva collector in all healthy subjects.

Healthy Never-smoker Subject	Tube Centrifuged Sample (ng/ml)	Collector Filtered Sample (ng/ml)
1	0.62	0.48
2	0.77	0.47
3	0.88	0.65
4	0.97	0.85
5	1.00	0.67
6	0.14	0.12
median, IQR	**0.88, 0.24**	**0.65, 0.19**

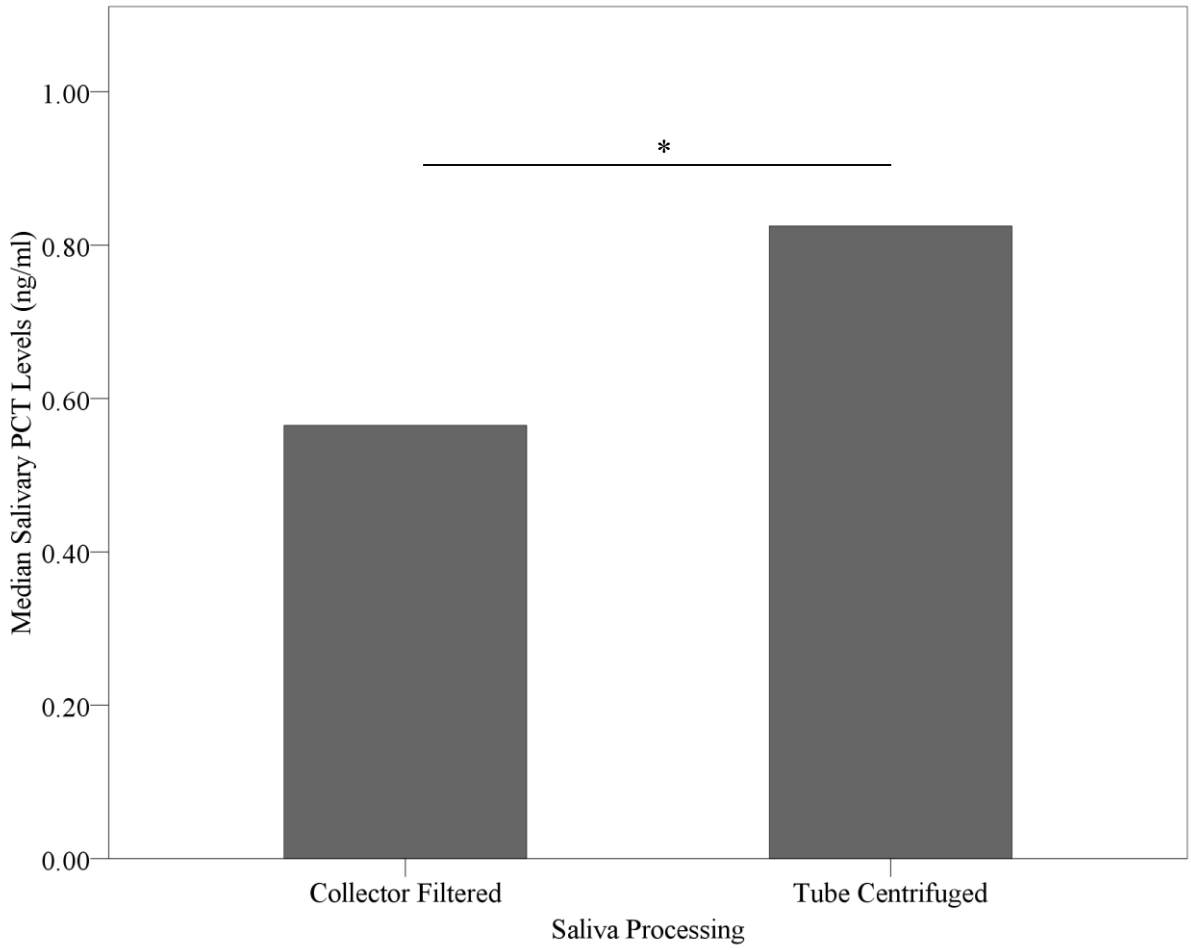

Figure 6.10: Salivary PCT levels across different saliva processing methods.

Bar charts representing the median salivary PCT levels between the collector filtered samples compared to equivalent tube centrifuged samples. There is statistical significant difference between the two processing methods (*$p<0.01$) with lower levels of salivary PCT observed in collector filtered saliva.

6.4.3.3.3. Salivary NE

The levels of salivary NE in the saliva samples of healthy never-smokers were lower in the collector filtered samples (125, 163ng/ml) compared to equivalent tube centrifuged samples (267, 471ng/ml); however, this difference was not statistically significant (p=0.07) (Table 6.7, Figure 6.11). Of the 3 biomarkers results for recovered salivary NE levels were the most variable. In particular, healthy subjects 2 and 5 demonstrated a large reduction in salivary NE levels for the collector filtered samples. Interestingly, a reduced level for both salivary CRP and PCT is also observed for subject 5, whilst subject 2 also demonstrates a reduced level of salivary PCT in collector filtered samples.

Table 6.7: Endogenous salivary NE Levels: Tube centrifugation compared to modified saliva collector in the same healthy subjects.

Healthy Never-Smoker Subject	Tube Centrifuged Sample (ng/ml)	Collector Filtered Sample (ng/ml)
1	167	76
2	751	323
3	69	93
4	159	155
5	718	95
6	373	290
Median, IQR	**269, 471**	**125, 163**

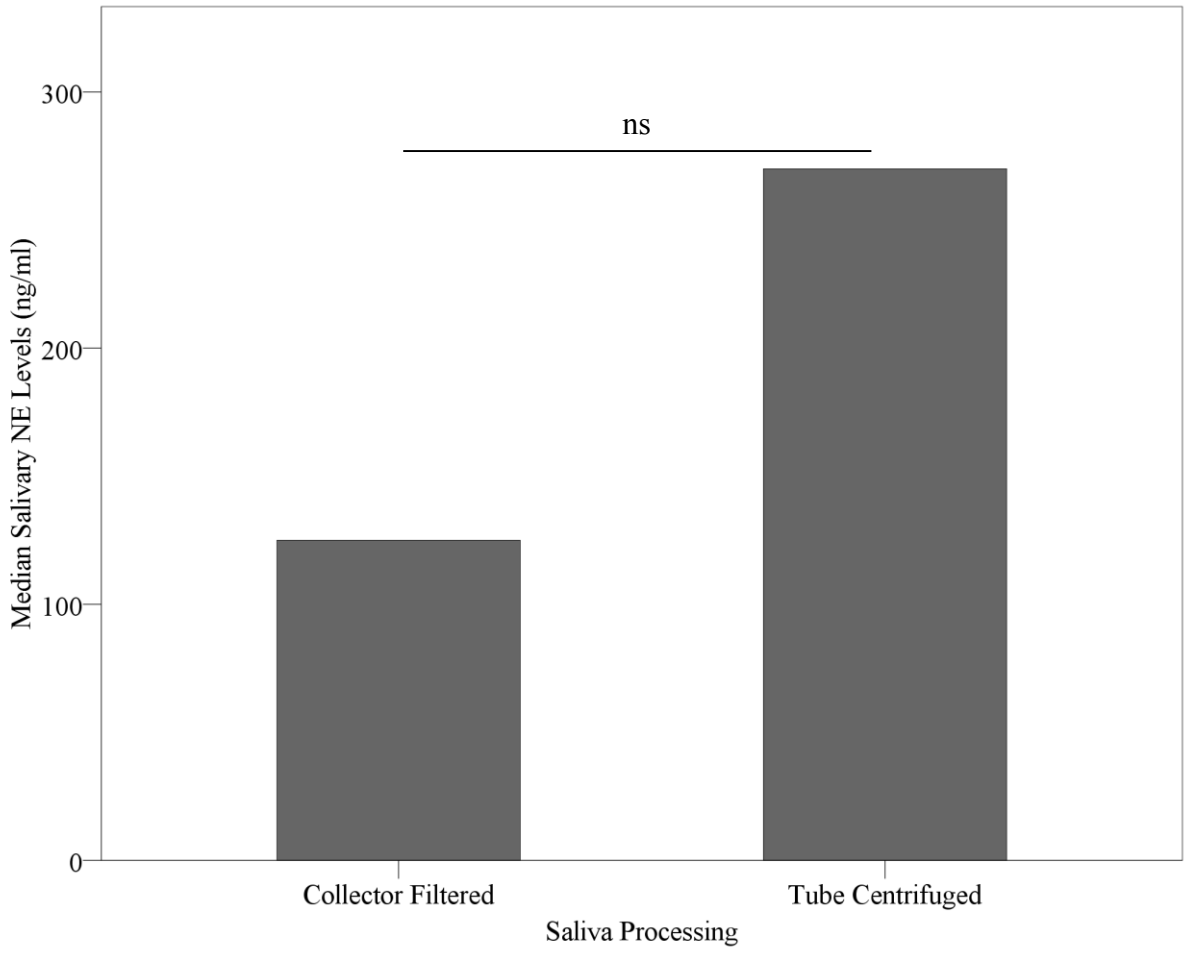

Figure 6.11: Salivary NE levels across different saliva processing methods.

Bar charts representing the median salivary NE levels between the collector filtered samples compared to equivalent tube centrifuged samples. Whilst there was no statistically significant difference between the two processing methods (ns: p=0.07), lower levels of NE were observed in the collector filtered saliva samples.

In an attempt to further investigate the observed variability in endogenous salivary NE levels between the collector filtered samples and tube centrifuged samples, a further experiment was conducted using fixed concentrations of NE that are supplied in the PMN Elastase ELISA kit (Immundiagnostik, Germany) (Chapter 2, Page 149) and used to generate the "curve-of-best-fit" (Chapter 2, Figure 2.18, Page 153).

6.4.4. Comparison of "standard" fixed concentration NE levels between modified collector filtration and conventional tube centrifugation

As the levels of endogenous NE demonstrated a variable difference between the collector filtered and tube centrifuged saliva samples, the effects of both sample processing methods were investigated on a fixed concentration of NE whilst eliminating the possible matrix effect of saliva. To achieve this the fixed concentration "standards" provided by the manufacturer in the PMN Elastase ELISA kit (Immundiagnostik, Germany) (Chapter 2, Page 149) were utilised.

6.4.4.1. Materials and methods

The PMN Elastase ELISA kit (Immundiagnostik, Germany) used in this thesis provides four sets of lyophilised "standard" at five fixed concentrations of NE: 0, 0.37, 1.1, 3.3 and 10ng/ml. Each fixed concentration standard is reconstituted in 500ul of dH_2O and the OD generated by these standards on the ELISA microtitre plate when read on a plate reader generates a standard "curve-of-best-fit" which permits calculation of the target sample NE levels (Chapter 2, Figure 2.18, Page 153).

To conduct the experiment, 4 sets of standards were reconstituted a total of 750ul of each standard concentration (0, 0.37, 1.1, 3.3, 10ng/ml) were aliquoted into five separate collectors for centrifugation (3000rpm for 15 minutes) and 750ul into the 5 separate collectors for filtration. The samples in the collectors were then aliquoted using an air-pipette onto the modified prototype saliva collector with a modified second (cellulose) acetate filter. The processed samples were then analysed in duplicate on one PMN Elastase ELISA kit (Immundiagnostik, Germany) using the methodology as described in Chapter 2, Page 149.

6.4.4.2. Statistical analysis

The statistical tests employed are discussed in Section 2.2, Page 86. Specifically, recovery of NE was calculated by assigning the manufacturer's documented concentrations of each reconstituted standard as 100% and then comparing the quantified NE levels processed either by tube centrifugation or modified collector filtration. The formula:

$$recovery\ percentage = 100\left(\frac{observed\ level\ of NE\ in\ standard\ processed\ by\ a}{expected\ level\ of\ NE\ in\ reconstituted\ standard\ b}\right)$$

where "a" is the method of saliva processing (filtration or centrifugation). The intra-assay CV was 4.7%.

6.4.4.3. Results

There was no significant difference in standard NE recovery between the collector filtered and tube centrifuged samples (p=0.897 by paired t-test) (Table 6.8) accounting for the small sample size. The NE recovery in the collector filtered samples demonstrated a high degree of variability with a "super" recovery in the 1.1 and 10ng/ml standards. This result is unusual, as one would not expect an enhanced recovery of NE from a fixed concentration standard. It is difficult to draw conclusions from these findings in the collector filtered samples; however, the tube centrifuged samples do demonstrate less variable recovery of NE with levels greater than 90% when compared to the unadulterated standard. Although the 3.3ng/ml standard demonstrates a "super" recovery, this is perhaps due to the inherent variability of the ELISA microtitre plate.

Table 6.8: Recovery of "standard" NE using two saliva sample processing methods.

Target NE Standard level (ng/ml)	***Unadulterated sample ng/ml (% recovery)**	**Tube Centrifuged Sample ng/ml (% recovery)**	**Collector Filtered Sample ng/ml (% recovery)**
10	9.99 (99.9%)	9.89 (98.9%)	16.07 (160%)
3.3	3.33 (101%)	3.83 (116%)	2.72 (82.4%)
1.1	1.04 (95%)	1.06 (96.4%)	1.70 (155%)
0.37	0.43 (116%)	0.36 (97.3%)	0.07 (18.9%)
0	0.00	0.01	0.00
Mean ± SD	**103 ± 9%**	**104 ± 13%**	**106 ± 71%**

Data presented as mean ± SD. *Unadulterated sample refers to the levels of the unadulterated reconstituted standard processed on a PMN Elastase ELISA kit (Immundiagnostik, Germany).

To better understand the results obtained with the collector filtered samples sub-analysis was performed to determine whether the actual levels in the collector filtered standard, as compared to the target standard level, would generate an appropriate standard curve-of-best-fit (Figure 6.12).

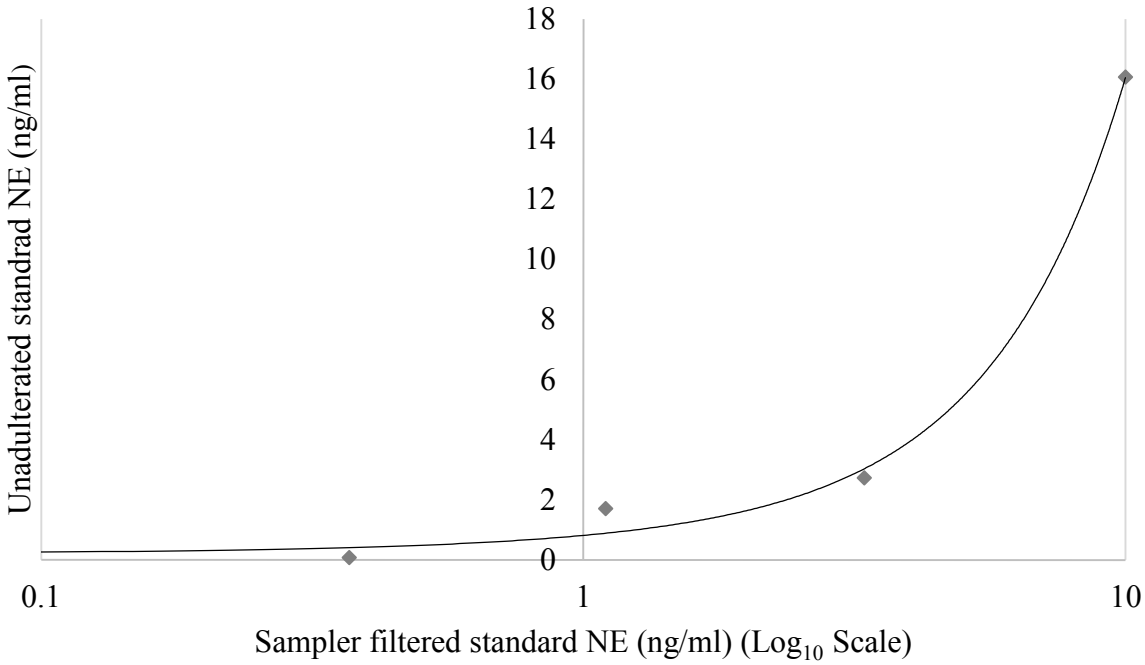

Figure 6.12: Unadulterated compared to collector filtered standard NE levels.

A scatter plot with "curve-of-best-fit" plotted on a Log_{10} abscissa (r^2=0.97) for the unadulterated standard NE and collector filtered standard NE quantified on the PMN Elastase ELISA kit (Immundiagnostik, Germany). The r^2 value indicates a 97% agreement between the curve-of-best fit and the observed NE levels.

6.5. End-user views on the developed saliva collector

As described in Section 6.2, Page 387 two workshops were conducted to understand COPD patients' views on saliva sampling, collection, sampling protocols and key features in a "perfect" saliva collector. The third workshop was conducted using the same approach as the previous two workshops with the inclusion of one extra question (Table 6.2, Page 389) and demonstration of the prototype saliva collector which incorporated design features discussed in the first two workshops.

COPD patients felt the bespoke prototype saliva collector provided an excellent grip and that its look, feel and dimensions would alleviate the feelings of apprehension noted with the bland conventional tube. They particularly embraced the ergonomic lip feature and felt this would aid collection and provide "comfort" whilst drooling and reduce any anxiety/embarrassment about spilling/miss-directing the sample. They felt that the new collector was a "step-in-the-right-direction" and would be an important component of any saliva based POC testing system.

6.6. Discussion

Currently there are numerous manufacturers of saliva-based testing kits and unstimulated whole saliva collection aids (Chapter 2, Page 81); however, they all require a saliva sample to be transported back to the particular manufacturer's laboratory for processing. Thus the saliva collectors within these kits merely function as sample retrieval and storage devices. The aim, within the permitted time-frame of this thesis, was to commence the design and development of a saliva collector prototype that would function as both a device for collection and filtration of the saliva, thereby avoiding the need for laboratory-based processing and centrifugation. This would be a first step in enhancing near-patient salivary diagnostic testing.

In this chapter it has been established that COPD patients would welcome saliva-based home testing, which provides a minimally invasive alternative to serum or plasma testing, provided that the required volume of saliva is small and that processing would occur in as short period as possible. Patient feedback on the basic design requirements of a bespoke collector for saliva proved invaluable, most notably the construction of a lip-engaging feature to make saliva retrieval more comfortable. The introduction of a pressure gradient within the collector appeared to address the need for enhanced saliva passage through the system reducing sample transit times of the saliva to an end user acceptable level.

To by-pass the need for saliva samples to be sent to a central laboratory for processing, the ability of the collector system needed to incorporate capability to de-foam and purify a sample of saliva ready for biomarker testing. This was addressed through the insertion of a combinatorial filter system composed of a multi-pored conic-surfaced first filter and a cellulose acetate-based second filter. This arrangement provided macroscopically clear saliva. Results from initial experiments exploring the recovery of target biomarkers in samples processed

through this newly developed filtration system, as compared to conventional laboratory centrifugation, demonstrated no statistically significant difference in the endogenous levels of salivary CRP and NE, although CRP levels following collector filtration were higher. Interestingly salivary CRP has been found to be reduced in centrifuged saliva compared to unprocessed saliva samples (Mohamed et al., 2012). Saliva spiked with PCT (as no endogenous PCT was quantified in the healthy saliva samples) was significantly reduced in the collector filtered samples compared to tube centrifuged samples by a magnitude of 25%. This was consistent for all tested samples and will need to be investigated further so as to avoid losing important subtle changes in this biomarker. PCT (13kDa) has the lowest molecular weight of the 3 proteins in this thesis compared to CRP (110kDa) and NE (29.5 kilodaltons (kDa)). The minimum pore size of the filters is such that it should not trap any of the 3 target proteins (Section 6.3.1.3, Page 396); however, it is possible that PCT forms protein complexes with other larger salivary proteins and thus is retained in the filter. The same effect has been observed for myoglobin, a protein with a similar molecular weight (16.7kDa) to PCT (Lipps, 2008).

The quantification of endogenous NE was higher in the tube centrifuged samples compared to collector filtered samples except for subject 3; both subjects 2 and 5 there was a large difference in endogenous NE levels between collector filtered and tube centrifuged samples, with the latter having higher levels (Table 6.7, Page 416). A possible explanation for this may have been the effect of centrifugation on saliva neutrophils causing increased NE release from the azophilic granules due to trauma or possibly the intrinsic breakdown of NE during centrifugation. Indeed the used rate of 3000prm equates to approximately 1500g; it has been hypothesised that a centrifuge speed above 600g may generate sufficient sheer stress to damage the interior structure of the neutrophil and thus release NE (Fukuda and Schmid-Schönbein,

2002). It is unclear however whether these sheer forces are sufficient to disrupt the exterior structure (cell membrane). Indeed, another study has demonstrated that centrifugation at 1550g, albeit for only 3 minutes, did not result in liberation of elastase from neutrophils (Fischer et al., 1998).

The experiment in Section 6.4.4, Page 418 using reconstituted lyophilised manufacturer-provided NE "standards" demonstrated a consistent recovery in sample-centrifuged "standard" NE. This supports the hypothesis that centrifugation of a sample containing NE at 3000rpm for 15 minutes will not result in an intrinsic breakdown of exposed NE. The recovery of collector "standard" NE was variable with both the 10 and 1.1 ng/ml demonstrating a "super" recovery. An explanation for this is not immediately forthcoming. Overall there is no statistically significant difference in NE recovery between the 2 methods. It is also important to note that the actual NE levels across the 5 "standard" concentration ranges processed through the developed collector, as compared to the expected NE levels followed the curve-of-best-fit with a r^2 value of 0.97 (Figure 6.12, Page 421).

Within the limited time-frame of this thesis a bespoke end-user tailored saliva collector has been created as a proof-of-principle and tested. Importantly throughout the design-development process, COPD patients were able to provide ideas and refinements. This approach in diagnostics development brings invaluable details to improve the fit between the user, the particular technology and the organisation of care, which is key for the usability and acceptability of a new tool (van der Weegen et al., 2013)

Further detailed work is now required to optimise the filtration system and recovery times for all target biomarkers and to ensure close fit with a biomarker-specific assay consumable.

Chapter 7:

General Discussion and Future Directions

7.1. Introduction

COPD is a complex and heterogeneous disease, which is not accurately defined by current diagnostic and severity criteria based on post-bronchodilator FEV_1 (Vestbo et al., 2013). Progress is being made to identify phenotypes of the disease for targeted treatment and long-term disease trajectory (Pinto et al., 2015). Within the natural course of COPD, acute episodes of deterioration (exacerbations) arise which may lead to periods of extended disability, hospitalisation, reduced quality of life and even death (Toy et al., 2010). An increased frequency of exacerbations accelerates lung function decline; furthermore, the more severe the disease status the increased frequency of exacerbations (Seemungal and Wedzicha, 2014). Prevention, early exacerbation detection and prompt treatment would all have an important impact on clinical outcome and quality of life in patients (Wilkinson et al., 2004).

However, this requires practical yet sensitive tool/s for patient surveillance and/or self-management. COPD currently ranks 3rd on the global mortality statistics (Lozano et al., 2012). It is one of the costliest in-patient conditions treated by the NHS, accounting for approximately 12% of all hospital attendances and nearly 2% of total NHS capacity in the UK (Halpin and Miravitlles, 2006). The rate of mortality for COPD in the UK is one of the highest in the EU (Network, 2011). In an effort to address this, the Department of Health published a policy paper setting out six objectives, five of which are relevant to COPD; these need to be achieved to deliver on the Government's commitment to improve health outcomes and reduce inequalities in chronic long term conditions (Health, 2011). Therein key elements are highlighted that could improve overall life quality and outcomes for COPD patients:

1. Individual stratification; risk profiling,
2. Integrated care team approach moving away from reactive to proactive models of care,
3. Self-care that recognises the need for a partnership between healthcare professionals and patients.

The ideal is that management of COPD should actively [rather than just "lip-service"] involve the actual patients in their own care, using generic long-term conditions models, good practice in disease pathways and wherever possible incorporating non-intrusive assistive technologies. There still remains an unmet need to improve COPD self-management, by better understanding disease phenotypes and by defining the role of objective and subjective health measures in disease surveillance and self-care strategies. a series of objectives (Chapter 1, Page 76) were set out to provide a structured approach in this thesis to provide some solutions towards achieving these goals. This thesis has identified the "best" type of saliva (unstimulated whole collected via passive drool) and determined a group of factors required for near patient sampling including standardisation of pre-analysis protocols for saliva sampling (Chapter 2, Page 80). Preparatory work on saliva and modified non-saliva assays demonstrated precision, linearity and recovery of the target biomarkers although further work will need to be undertaken to ensure the assays are commercial market ready (Section 7.5, Page 443). A simple reproducible PRO score was created (Chapter 2, Page 209) and utilised in two clinical studies where its reproducibility and ease of completion were identified (Chapter 3, Page 213 and Chapter 4, Page 251). Further work is required to ensure the PRO is commercial market ready (Section 7.5, Page 443). The clinical studies (Chapter 3, Page 213 and Chapter 4, Page 251) demonstrated that

the saliva biomarkers could distinguish between health and COPD and importantly could be used to monitor disease status in COPD patients to provide the foundation for predictive health status change in synergy with PROs.

7.2. Saliva sampling: its role in clinical monitoring

The first facet of this thesis involved the evaluation of saliva as a feasible sample fluid for collection in COPD patients. Saliva is a body-fluid that can be non-invasively sampled and provides a window into the health of a patient (Wong, 2008, Denny et al., 2008). It requires minimal training for sample collection compared to phlebotomy (Koh and Koh, 2007) and therefore an individual could provide a self-directed sample at a time and location that is convenient to themselves. The primary challenges of saliva sampling that were addressed in this thesis were sample collection and processing protocols including effects of fasting and salivary flow, modification of commercially available non-saliva-based assays (PCT and NE) and blood contamination bias.

A non-intrusive method for was chosen for saliva collection (unstimulated whole via passive drool) that is arguably harder to standardise requiring a series of pre-sampling protocols, but is more practical, expeditious and simpler to provide (Nunes et al., 2015). Unstimulated whole saliva is a heterogeneous sample (Chapter 1, Page 49) and a sampling collection and processing protocol helps to ensure sample consistency (Henson and Wong, 2010). The first challenge was to design a bespoke COPD patient-orientated saliva sampling protocol to reduce the likelihood of sampling bias and to improve patient compliance (Kudielka et al., 2003). This required consideration of factors that could be refined to help the process of saliva production for the end-user (Chapter 2, Page 169). Outside a laboratory environment, ambulatory and near-patient saliva sampling for analyte measurements still require strict guidelines to follow and accurate timing of collections. However sampling protocols have been shown to have poor adherence by patients (Kudielka et al., 2003). Thus key to achieving reliable collections and compliance with set instructions, was my interaction with COPD patient workshops (Chapter 6, Page 382). This enabled the creation of a saliva collection protocol written in plain language

with a clear set of simple "do's and don'ts" (Chapter 2, Figure 2.28, Page 209) that was user-friendly. In addition, COPD patients particularly welcomed their involvement in establishing a method for repeated non-invasive testing over other modalities such as phlebotomy. These workshops also highlighted the importance of patient-research co-design when introducing potentially disruptive interventions into disease management pathways. COPD patients provided refinements and patient "know-how", bringing in valuable details to improve the fit between the user, the technology and the organisation of care, which is important for the usability and acceptability of a tool (van der Weegen et al., 2013). This process was not solely confined to the saliva sampling protocol but also used to develop a prototype saliva collector.

This thesis is the first to establish that COPD patients' salivary flow rate is comparable to healthy non-smoker subjects (Fenoll-Palomares et al., 2004) albeit in a small sample size. A crucial finding which reassured me that saliva sampling in COPD patients would be practically feasible and that patients would be able to effortlessly produce an adequate set volume for target analyte testing. Next to consider was the potential for sample contamination with blood (Chapter 2, Page 172). Presently within the literature the methodology for blood contamination detection is varied. The consensus is that significant blood contamination is rare and that samples contaminated with significant amounts of blood are visually discoloured and thus can be discarded without the need for formal testing (Kamodyova et al., 2015). Approximately a quarter of the pilot study samples tested positive for blood contamination; however there was no effect on target analyte quantification when accounting for co-variates such as gum disease. Despite this information, knowing that blood contamination could cause interference with immunoassays (Chiappin et al., 2007) random testing of saliva samples continued throughout the community-based studies to ensure the highest level of immunoassay accuracy and as a basis of quality control. This was important in view of the modified non-saliva based assays

for PCT and NE. Furthermore, saliva sampling instructions included the requirement for patients to avoid tooth brushing prior to collection with assessment of gum disease carried out regularly by the clinical team. Gum disease was important to account for as Kamodyova et al. (2015) observed that saliva contaminated with blood is more common in individuals with poor oral health and history of gingivitis or periodontitis. This thesis is the first to account for blood contamination in the quantification of salivary CRP, PCT and NE. The common use of a modified urine reagent stick to detect for presence of blood in saliva could be a potential limitation (Chapter 2, Page 200). However as discussed previously, this approach generated a high amount of false positives but was effective in identifying samples with nil contamination.

Presently, only CRP has a saliva-based immunoassay for target analyte quantification albeit labelled as a "for research use only". The quantification of PCT and NE required modification of "for diagnostic use" (Chapter 2, Page 91) serum-based immunoassays. As discussed saliva is a heterogeneous fluid and whole saliva sampling can introduce contaminants that affect immunoassay function. The sampling protocol addressed these issues however it is important to validate all 3 assays in the "hands of the user" to ensure that the levels of the target analytes are quantified accurately and reproducibly. My experiments demonstrated that modification of these said immunoassays for saliva resulted in reliable and reproducible results (Reed et al., 2002). This was also undoubtedly helped by having a clear sampling protocol. A potential limitation however was the modification of the serum-based PCT assay. PCT is undetectable in patients who are clinically stable (Chapter 1, Page 69) with the VIDAS B.R.A.H.M.S PCT (bioMérieux, France) lower limit of detection being set at 0.10ng/ml. This resulted, as would be expected, in a significant proportion of the saliva samples testing "negative" for PCT. A more sensitive assay that could quantify salivary PCT below this lower limit of assay quantification would perhaps provide additional data in stable phase COPD patients that could

be used for corollary analysis with the 2 other saliva biomarkers. Overall all 3 immunoassays reliably and reproducibly detect CRP, PCT and NE in the saliva of COPD patients and healthy subjects.

The range of quantification for an immunoassay however remains crucial for potential point-of-care applications. For example, salivary PCT using the BRAHMS VIDAS PCT kit (bioMérieux, France) on the mini-VIDAS instrument (bioMérieux, France) has a range of quantification: 0.10 to 400ng/ml. Thus samples of saliva tested for PCT using this instrument that are below the lower limit of assay quantification (0.10ng/ml) are quantified as "less than 0.10ng/ml". This has important implications for statistical analysis. These saliva samples that have tested below the lower limit of assay quantification (negative) need to be included in statistical analysis; however using the term "less than 0.10" is not possible as would be in a laboratory-generated report. A number needs to be chosen to represent these "negative" samples especially when the analyte is being tested in a bio-fluid known to have much lower concentrations of said biomarker than in blood/serum. This number can in this example for PCT range from 0 to 0.099ng/ml. The selection of this "negative" value is important. Saliva samples below the lower limit of assay quantification for PCT were assigned as 0.09ng/ml. This approach enhanced statistical robustness by maintaining the number difference between each quantifiable level of PCT above 0.10ng/ml i.e. the mini-VIDAS output for PCT is up to 2 decimal places. Accordingly, the same methodology as used for assigning numerical values to the "negative value" for salivary CRP and NE. Overall this approach is more stringent than using a value of zero or half the lower limit of quantification which would produce a downward bias on the data (Muir K, 2004) and thus increase the difference between negative and quantifiable concentrations of the target analytes. Published studies for these 3 biomarkers in serum and sputum of COPD patients do not always define the number of individuals who have

target biomarkers below the lower limit OD detection of the immunoassays used to detect the target analytes; and more importantly the subsequent mathematical approach to these subjects (de Torres et al., 2006, Ishikawa et al., 2015, Pinto-Plata et al., 2006) Other studies just revert to using a value of half the lower limit of assay quantification for analyte results that were below the lower limit of assay quantification (Dickens et al., 2011). This approach enhances the accuracy of the statistical analyses for target analyte salivary biomarker levels for all calculations in this thesis.

There is increasing evidence that saliva sampling of target analytes could be used as a mirror to reflect systemic profiles of an individual akin to blood sampling of the same target analytes (Lima et al., 2010). This thesis has established that saliva CRP and PCT reflect serum levels (Chapter 3, Page 242), and thus for these biomarkers saliva could be considered as providing an accurate substitute for serum testing. The correlations for saliva-serum CRP has been extensively investigated and established in the wider literature (Ouellet-Morin et al., 2011, Punyadeera et al., 2011). Presently the establishment that salivary PCT is a reflection of serum is a first. Salivary NE did not correlate with serum levels; a potential explanation for the discord may be the endogenous levels in the oral cavity, the counter-argument however is the observed significant correlations between salivary NE and both salivary CRP and PCT. This adds support that salivary NE is reflecting systemic, rather than an oral cavity, based processes. There also exists the possibility of rapid in-activation of NE *in-vivo* (Carter et al., 2013). It could be hypothesised that salivary NE testing is reflective of systemic inflammation that cannot be identified with serum-based NE testing. These results perhaps highlight an added benefit of saliva compared to serum testing for certain analytes. Overall the conclusion is that saliva-based testing of CRP and PCT is reflective of serum-equivalent findings and that salivary NE is also potentially reflective of serum-based indicators of inflammation. These

results support a saliva-based approach for the routine measurement of the 3 target analytes at point-of-care (POC) as a surrogate for serum-based testing.

Having established the correct saliva sampling protocol and validated the quantification of a panel of COPD-relevant biomarkers in saliva, there remains the challenge of having the correct collector that appeals to patients, reduces errors with sampling and does not interfere with analyte analysis. Interestingly throughout saliva sampling in the community-based studies it was observed that although the chosen saliva tube collector (Chapter 2, Page 84) was functional, it lacked engagement and appeared daunting to patients due to its length with a "pressure to fill" (Chapter 6, Page 391). Thus a bespoke prototype saliva collector was developed within the time-frame of this thesis.

Saliva has the potential to be used frequently in POC testing and by patients themselves in their homes. COPD patients as discussed earlier are willing to embrace saliva testing provided there is a clear instruction protocol and that collection times are less than 10 minutes. Accordingly, in designing the specifications of a near-patient saliva collector prototype, it was determined that it had to incorporate features to enhance patient comfort and compliance (Chapter 6, Page 393) with saliva sampling as well as components that would substitute the laborious laboratory-based sample purification procedures (Chapter 2, Page 92). A series of potential engineering solutions were evaluated, that would process and deliver a "filtered" sample of saliva which would not require first-step laboratory centrifugation for sample purification. Preliminary experiments confirmed the merits of the designed saliva collector; however there are limitations in the recovered levels of PCT and variability in NE levels. Further testing and end-user led design refinement of these concepts will be required in the future.

7.3. Salivary biomarkers and spirometry: their potential to predict COPD exacerbations

One of the ambitions of my thesis was to determine whether saliva could be used to detect health status change in COPD, and its potential role for the management of COPD exacerbations. To address this, 2 community-based studies were conducted (Chapter 3, Page 213 and Chapter 4, Page 251). Individual risk profiling in COPD patients was also explored, based on the trajectories of spirometry and these biomarkers over time (Chapter 4, Page 299 and 313). The driving concept was an attempt to characterise COPD patients based on the temporal variability of their disease and not just a "snap-shot" of status as is commonly found in the current risk profiles for example BODE (Chapter 1, Page 27). This approach appeared sensible in light of established findings that COPD patients have a non-uniform decline in objective measurables, for example FEV_1, over the course of their disease (Casanova et al., 2014).

The methodology of the first study (Chapter 3, Page 213) has been established as essential for comparison of all pulmonary biomarkers in patients with COPD (Barnes et al., 2006). Within that study stable phase level for all 3 biomarkers were established and reproducibility of these levels, which demonstrated a consistent agreement (low variability) in repeated saliva biomarker levels in the same COPD patients across the same status of their disease. This is important if saliva is to be utilised as a replacement for serum-based testing and indeed has been a major challenge for other novel monitoring approaches, for example electronic nose breath analysis (Chapter 1, Page 41).

All 3 target salivary biomarkers demonstrated a significant rise during a patient-defined exacerbation event in both clinical studies with the community-based longitudinal study

(Chapter 4, Page 251) statistically powered to establish this effect. The longitudinal study allowed for an understanding in exacerbation prediction with CRP and NE levels, both demonstrating early change (up to 7 days prior to exacerbation onset) within a defined prodromal phase (Table 4.1, Page 275). Additionally, the ability to identify COPD patients at risk of re-exacerbation within 2 weeks of completing an index exacerbation was demonstrated based on both spirometry (Chapter 4, Page 312) and salivary biomarker levels (Chapter 4, Pages 344). A potential limitation to these results is the significant but moderate ROC curve accuracy for exacerbation diagnosis for salivary CRP (Chapter 4, Page 318) and PCT (Chapter 4, Page 331) (Fischer et al., 2003). The result for salivary NE (Chapter 4, Page 341) was not significant perhaps highlighting that clinical cut-offs cannot be set for this variable but that instead comparison and establishment of an individual's baseline status is crucial. However, this would only be an issue if one is advocating that each salivary biomarker be used in isolation; rather the approach used in this thesis calls for a panel of biomarkers to be simultaneously tested for disease status monitoring (Agustí et al., 2012). Indeed, a rise in 2 or more salivary biomarkers during an index exacerbation (Chapter 4, Page 348) predicted the risk of re-exacerbation. It is proposed that these results could be incorporated in a diagnostic monitoring algorithm which might enable a disruptive shift change in COPD exacerbation management, away from a reactive towards a proactive model of care, adding power of effect by using in conjunction with PROs discussed later in this chapter (Section 7.4, Page 440).

LCGA was used to interrogate the study-generated real-life datasets to understand the temporal variability of both salivary biomarkers and spirometry and whether they, in conjunction with other established COPD metrics, could generate individual patient risk profiles (Chapter 4, Page 350). This analytical technique clusters patients into discrete sub-populations based on the variability of a target parameter over time. This analysis was not only conducted for salivary

biomarkers but also spirometry in an attempt to create a multidimensional composite score/cluster based on multiple metrics. This novel score will form part of my concepts for future work arising from this thesis (Section 7.5, Page 443). This approach is supported by the findings that COPD patients may manifest multiple phenotypes/clusters (Han et al., 2010a). Although discrete sub-population clusters were identified for each salivary biomarker there was no statistical significance in the COPD patient composition for each cluster and overall re-exacerbation risk, except for salivary NE. The role of these individual clusters at present remains elusive; the COPD patients who compose these individual clusters need to be followed-up over a longer time-frame to understand what additional information on COPD disease status can be provided. Salivary NE clusters however did show the importance of co-morbidity (Pinto et al., 2015) and interestingly for FEV_1 the separate sub-population clusters appeared to predict the risk of re-exacerbation (Chapter 4, Page 307). Overall, my thesis provided seminal data evaluations utilising LCGA to cluster sub-populations of COPD patients from observations derived in the stable-prodromal period of their disease; however limitations do need to be addressed. Although COPD patients were deemed to be clinically stable on enrolment into the longitudinal study, they could exacerbate at any time-point and, by virtue of the study design, exit from the study as soon as their post-exacerbation-recovery period was completed. As study participants were also frequent exacerbators, some had their first acute episode quite close to their point of entry into the study; thus reducing the length of stable-prodromal phase data to analyse (Table 4.4, Page 277). Importantly LCGA is robust to missing data sets as long as the data is missing at random (Chen et al., 2009).

This thesis perhaps for the first time also provides the ability to better define a COPD exacerbation based on a mixed model of subjective and objective markers of health. This is possible because saliva-based biomarkers can be frequently sampled and accurately quantified.

The aetiology of an exacerbation is not just simply pathogen-dependent but phenotypes based on low inflammatory and eosinophilic processes are now emerging (Bafadhel et al., 2011). Interestingly peripheral (blood) eosinophilia has been shown to be predictive of corticosteroid responsiveness during an acute exacerbation of COPD (Singh et al., 2014) and not just pathogen specific (Rohde et al., 2008). Recent studies have also begun to establish that a certain sub-group of COPD patients are at an increased risk of pneumonia when receiving inhaled corticosteroids as part of their maintenance treatment (Janson et al., 2013). This reinforces the argument that stable-prodromal phase and exacerbation phase clusters need to better defined and characterised; and that reliance on an exacerbation definition based solely on symptoms, whilst practical, needs to be improved to be more phenotypically focused which in turn could lead to better treatment stratification.

7.4. Patient-reported outcomes: their value in self-management protocols

A major component of my thesis work also involved PROs, which have been recognised as important in monitoring health status in COPD (Vestbo et al., 2013). Although a wide-array of PROs are available in the literature (Chapter 1, Page 27) none at present have been established as a reliable and practical monitor of COPD health status at point-of-care (Chapter 2, Page 210). Questionnaires such as SGRQ and CRQ are considered too long and complex to be used in routine everyday self-management protocols (Celli, 2003). A novel simple symptom score (COPD Wellbeing Score) was developed based on clinically-relevant metrics for COPD identified in the wider literature (Walters et al., 2012). Content-validity was established with correlations to MRC score, salivary biomarkers and changes during an acute exacerbation of COPD (Chapter 3, Page 232) (Jones et al., 2009), alongside COPD patient feedback in workshops (Chapter 5, Page 361) (Howard et al., 2012). Additionally, absence of significant changes in the trajectory of COPD Wellbeing Score during stable-prodromal phases adds to a high test re-test reliability (Chapter 4, Page 289). A further method of validation with respects to utilising the wellbeing score as a daily monitoring tool was to create a bespoke electronic diary (COPD Wellbeing and Self-Assessment diary). This was in recognition of the limitations of paper-based diaries and the arguments about the validity of daily symptom monitoring in studies using these diaries (Chapter 4, Page 254). This newly designed electronic diary also contained a HRO section to enable cross-reference to the PROs. The inclusion of HRO section takes this electronic diary one step further than currently available electronic diaries by capturing and correlating the two metrics with each other and providing a genuine interactive platform for patients and healthcare professionals alike.

The electronic Wellbeing and Self-Assessment diary demonstrated excellent compliance, usability and functionality which were confirmed in end-user led workshops (Chapter 5, Page

361). In conjunction with this, COPD patients felt the electronic Wellbeing and Self-Assessment diary enhanced education about their condition; patients provided feedback on how the diary was comprehensive but could be more encompassing for example: lack of appetite. This process as described above demonstrates content-validity for the COPD Wellbeing score and also provides patient-directed ideas for refinement, thus contributing to an overall enhanced level of participation and compliance (Morren et al., 2009). The electronic diary was seen to create an environment of partnership between the research team (healthcare professionals) and the patients, who remained central to all the decision-making. This was due to a proactive self-management care model with "real-time" COPD Wellbeing Score surveillance and proactive actions based on deteriorations of said score. It helped facilitate self-management by providing an enhanced feeling of security and safety. In effect, COPD patients were compelled to self-manage their disease with the knowledge that a care provider was monitoring, incentivising them into entering regular and accurate information as they were aware that without this they could not be remotely monitored. Such compliance is also important as it is recognised that COPD patients fail to report an acute exacerbation in a third of events (Langsetmo et al., 2008). It could be postulated that the methods in Chapter 4, Page 270 provided for a successful integrated self-management ecosystem, the framework of which still requires to be compared to current practices to better understand its overall benefit on disease outcome. A potential limitation that needs to be addressed was the single daily score entry which potentially misses intra-day fluctuations; however this feature can easily be accommodated in future software updates to the diary.

Analysis of the generated datasets of COPD Wellbeing scores from the whole cohort and per individual utilised a novel analytical technique (Chapter 4, Page 274) with added sensitivity compared to methodologies currently used in the literature for symptom score analysis

(Alahmari et al., 2014). Thus the scores were shown to be able to identify an early onset change in symptom trajectory prior to the onset of an acute exacerbation of COPD and also highlighted symptom trajectories during treatment. Furthermore, this approach also identified potential changes around a re-exacerbation post-treatment completion. Such information is of huge clinical relevance and demonstrates how patient-derived symptoms can be made to be effective in practical everyday monitoring tools; specifically, how they could be key determinants of COPD health status monitoring algorithms alongside other viable metrics such as salivary biomarkers and spirometry (Section 7.3, Page 436).

7.5. Conclusion and Future Directions

This thesis has sought to develop a holistic approach to the near-patient management of a chronic condition. A key factor has been the research-patient partnership in guiding the design of key instruments and understanding COPD patients' "needs and wants". Consideration has been given to the most ideal body-fluid to sample and methods involved in sample reproducibility. A content-valid reproducible PRO has been developed that was successfully incorporated into a proactive self-management model and conducted exploratory analysis to define clusters in COPD patients that could be used to monitor disease state over time.

Further work to the modified immunoassays to prepare them for commercial availability would involve several additional validation steps: (1) robustness of the method to small variations in method parameters for example, incubation time, working assay temperature; (2) sample stability based on different storage conditions, freeze-thaw cycles and testing the same sample at repeated time interval and further experiments involving parallelism for biomarker spiked saliva tested against a spiked substitute matrix (Andreasson et al. 2015)

Additional work to enable the PRO to be commercially ready would involve a further clinical trial involving a larger cohort of COPD patients and observation of the COPD Wellbeing score over 1 year. This will confirm content validity and test-retest reproducibility during stable disease phase. Qualitative research methods can then be employed to confirm overall content validity of the score.

The next aspect of this thesis that could be exploited in future are the use of the parameter datasets to generate predictive/diagnostic algorithms based on a combination of inputs from wellbeing scores, FEV_1 and salivary biomarker panel levels. Their usefulness for COPD patient

health status surveillance could then be explored longitudinally in community-based studies to determine whether they could reduce hospitalisations and improve patient outcomes when compared to best supportive care.

Characterisation of the observed stable-prodromal phase clusters for both spirometry and biomarkers requires a longer longitudinal study of perhaps over at least one year. For the baseline clusters that have been identified as possible "candidate" phenotypes an iterative validation process will need to take place (Han et al., 2010b). This would provide further information on whether these clusters can identify differences in disease progression, re-exacerbation and whether COPD patients remain in the same cluster over a protracted time-frame (1 to 5 years). This study could also incorporate the multidimensional score/clusters that have been generated in this thesis (Table 4.36, Page 351) to understand whether they can provide additional information than each component in isolation.

The second aspect for exploitation would be to expand the repertoire of the salivary panel of biomarkers for COPD, for example eosinophilic cationic protein which acts as a biomarker for eosinophilic inflammation (Saha and Brightling, 2006) in saliva. Exacerbations as previously discussed do not appear to be simply defined by pathogen or air pollution but that distinctive phenotypes/clusters do exist. Factor analysis (Chapter 4, Page 352) was utilised in an attempt to understand the key components when assessing acute exacerbations of COPD; however, this needs to be further explored to understand the relationship to the recently described COPD exacerbation phenotypes. In addition, direct markers of bacterial load, the commonest cause of an acute exacerbation (Butorac-Petanjek et al., 2010), and the role of specific pathogens during acute exacerbations warrant further attention. New technologies are emerging (such as the new biomarker assays from Aseptika Limited, Cambridge (Activ8rlives)) for the quantification of

specific bacteria (for example, Pseudomonas aeruginosa, Haemophilus influenzae) which are present in the sputum of patients with COPD (Bafadhel et al., 2015, Millares et al., 2014). Bronchial colonisation by Haemophilus is associated with an increased systemic inflammatory response in stable COPD subjects (Marin et al., 2012). Both Haemophilus and pseudomonas constitute approximately 30-40% of the bacterial aetiology in acute exacerbations of COPD. Isolation of these organisms is also more prevalent in acute exacerbations of COPD patients with severe disease (Ko et al., 2005, Miravitlles et al., 1999). I would look to calibrate these non-saliva-based assays and understand the reflection of saliva, to sputum-based biomarkers. Colonisation by bacterial pathogens has been associated with a clinically significant moderate increase in daily symptoms (Desai et al., 2014). Direct knowledge of bacterial levels may also provide an additional aspect to exacerbation prediction, by identifying a non-pathogenic rise from baseline bacterial load. This could act as a direct predictor of bacterial-driven acute exacerbations. Questions that need addressing would include: (1) does a non-pathogenic rise from baseline bacterial load immediately translate into a pathogenic rise? (2) does total baseline bacterial load equate to accelerated lung function decline? The need for an improved understanding of the direct role of respiratory pathogens in COPD is being recognised; specifically, in longitudinal monitoring to assess how changes in the COPD airway microbiome may contribute to the incidence and severity of COPD (Bourne et al., 2014).

The third aspect for further exploitation would be the further development of a bespoke prototype saliva collector (Chapter 6, Page 394), specifically in regards of an appropriate filtration system that would by-pass the need of centrifugation yet not compromise recovery and measurement of the 3 target biomarkers studied in this thesis. Ultimately if the bespoke saliva collector is to be used at POC with a near-patient saliva analyser, a docking interface will need to be developed.

Improvements to the electronic Wellbeing and Self-Assessment diary have already been suggested based on the workshops in Chapter 5, Page 361. Further updates to the diary could consider incorporating features for multiple daily entries. An enhanced electronic COPD Wellbeing and Self-Assessment diary could also be part of a virtual care suite that could also connect to a future developed near-patient saliva analyser.

In conclusion, COPD is a complex heterogeneous disease that requires improved classification, disease monitoring and tailored interventions for better self-management of acute exacerbations. This thesis has created the tools to improve the classification and monitoring of COPD and opens new avenues for future research which have already been recognised as innovative (Horizon, 2015). The ultimate goal would be to have COPD patients accurately clustered/phenotyped with personalised treatment plans and actively engaged in self-management utilising a composite suite of POC tools (saliva-based testing, spirometry and PROs). This ideal eco-system would in turn help reduce the burden of disease for COPD patients and hopefully increase both the quality and quantity of their life.

"We chose to go to the moon in this decade and do other things, not because they are easy, but because they are hard"

John F Kennedy.

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Appendix 1:

Please tick the most appropriate answer

Week Commencing: .....

1. How is your breathing today?

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Excellent							
Good							
Fair							
Bad							
Very Bad							

2. How is your breathing affecting your ability to perform?

    a. Activities of daily living. e.g. Self-wash/Dress; cooking, housework

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Not at all							
Little							
Fair Amount							
Much							
Very Much							

    b. Physical Activities. e.g. Walking, shopping, gardening

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Not at all							
Little							
Fair Amount							
Much							
Very Much							

3. Do you have a cough?

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
No							
Yes							
Much Better							
Better							
Usual							
Worse							
Much Worse							

4. How much sputum do you produce daily?

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
None							
5mls (1 teaspoon)							
15mls (1 tablespoon)							
30mls (1 egg cup)							
50mls or more (1 cup or more)							

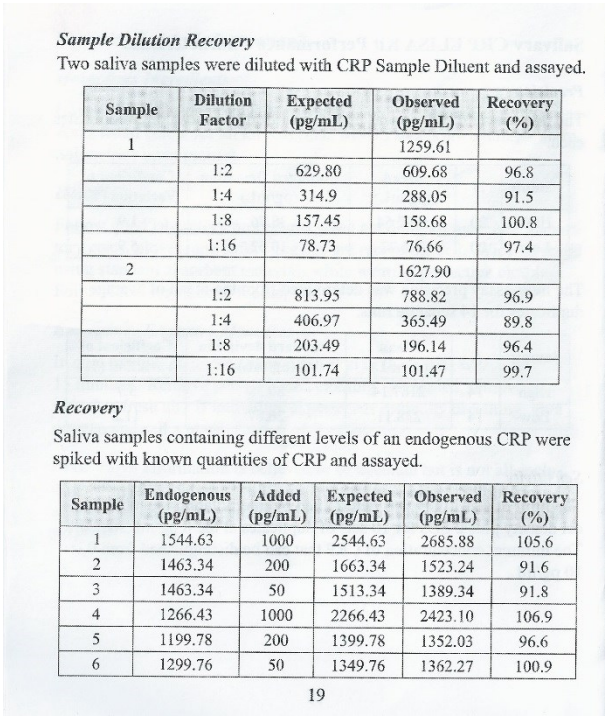
5. What is the colour?

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Watery, clear, transparent							
Watery, cloudy, colourless							

Figure S1.1: Paper-based version of the electronic COPD Wellbeing and Self-Assessment Diary.

This paper-based version of the COPD Wellbeing Score on the electronic Wellbeing and Self-Assessment diary was available to COPD patients in the community-based study (Chapter 4, Page 251) in lieu of the electronic Wellbeing and Self-Assessment diary.

Appendix 2:



Sample Dilution Recovery

Two saliva samples were diluted with CRP Sample Diluent and assayed.

Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			1259.61	
	1:2	629.80	609.68	96.8
	1:4	314.9	288.05	91.5
	1:8	157.45	158.68	100.8
	1:16	78.73	76.66	97.4
2			1627.90	
	1:2	813.95	788.82	96.9
	1:4	406.97	365.49	89.8
	1:8	203.49	196.14	96.4
	1:16	101.74	101.47	99.7

Recovery

Saliva samples containing different levels of an endogenous CRP were spiked with known quantities of CRP and assayed.

Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1544.63	1000	2544.63	2685.88	105.6
2	1463.34	200	1663.34	1523.24	91.6
3	1463.34	50	1513.34	1389.34	91.8
4	1266.43	1000	2266.43	2423.10	106.9
5	1199.78	200	1399.78	1352.03	96.6
6	1299.76	50	1349.76	1362.27	100.9

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Figure S1.2: Salimetrics: salivary C-reactive protein ELISA kit manual.

This figure represents page 19 of the manual, documenting the manufacturer's recovery experiment results.