LEUKOCYTE-MEDIATED DEGRADATION OF LUNG EXTRACELLULAR MATRIX & SERUM MOLECULES IN CHRONIC INFLAMMATORY DISEASE, AS DISCERNED THROUGH URINARY BIOMARKERS

A Thesis submitted to Cardiff University in accordance to the

requirements for the degree of Doctor Philosophy in the School of Medicine

by

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Summary

Chronic Obstructive Pulmonary disease (COPD) is an irreversible inflammatory disease of the lung, characterised by abnormal inflammation of the lungs in response to inhalation of noxious particles or toxic gases, especially cigarette smoke. COPD exacerbations, defined as acute sustained worsening of symptoms from usual stable state, accounts for significant morbidity and mortality. Improved diagnostics which give advanced warning of an exacerbation could help prevent further declines in lung function. The quest to identify a marker or a combination of markers associated with COPD exacerbations has been pursued for some time. Many groups have studied biomarkers in plasma, serum, sputum and bronchoalveolar lavage (BAL) fluid and uncovered useful markers for prediction of exacerbations, disease severity and mortality. However, there is limited research on urine biomarkers. Profiling inflammatory mediators in urine samples presents a simple, convenient, non-invasive measure of inflammation in COPD patients and can be done repeatedly within their own home or in the clinic, allowing easier monitoring of time-dependent changes in biomarker levels. The research described in this thesis is the first investigation where a large panel of biomarkers has been evaluated in urine samples from subjects in various stages of COPD. This has provided new insights into the relevance and origin of the biomarkers. Prototype point-of-care tests were developed that could be used routinely by patients in their own homes to monitor their inflammation status and predict pulmonary exacerbations. This was evaluated in a prospective observational study, results of which were used to develop a simple algorithm that showed the potential for differentiating between stable state and exacerbation events.

The research described here is part of a major research initiative carried out within the Mologic R&D group and constitutes investigations designed and directed by the author, and conclusions derived from the author's analysis of the data collected by the biomarker immunoassays. The findings constitute a key scientific foundation for a new approach to personalised medicine for COPD sufferers.

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I would like to thank my family, especially my mother, sister and brother-in-law for supporting me throughout the writing this thesis.

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Publications and presentations

PUBLICATIONS

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PRESENTATIONS

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Abbreviations

A1AT	α_1-antitrypsin / Alpha-1-antitrypsin
Ac-PGP	N-acetyl Proline-Glycine-Proline
AECOPD	Acute Exacerbation of Chronic Obstructive Pulmonary Disease
AERIS	Acute Exacerbation and Respiratory InfectionS in COPD clinical study (Clinical Trial Study Ref. NCT01360398, sponsored by GSK)
AUC	Area Under the Curve
B2M	β_2 Microglobulin / Beta-2 Microglobulin
BAL	Bronchoalveolar lavage
BL	Baseline
CAT	Com
CC16	Club (Clara) Cell secretory protein-16
CHI3L1	Chitinase 3 like protein
COPD	Chronic Obstructive Pulmonary Disease
CF	Cystic Fibrosis
BEAT-COPD	Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbations (clinical study)
CRF	Case Report Form (used in clinical studies)
CRP	C-reactive protein
DES	Desmosine
DPD	Deoxypyridinoline
ECLIPSE	Evaluation of COPD Longitudinally to identify Predictive Surrogate Endpoints. GSK sponsored clinical study (www.eclipse-copd.com)
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EDN	Eosinophil Derived Neurotoxin
EPO	Eosinophil peroxidase
ELISA/EIA	Enzyme-Linked ImmunoSorbent Assay
ELTABA	Enzyme Linked Transformation Affinity Binding Assay (Mologic patent)
EXACT-PRO	EXAcerbations of Chronic Pulmonary Disease Tool for Patient Reported Outcomes (www.exactproinitiative.com)
Fib	Fibrinogen
fMLP	N-formyl-Methionine-Leucine-Phenylalanine
HNE	Human Neutrophil Elastase

HSA	Human Serum Albumin
IL-1β	Interleukin 1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
ICS	Inhaled Corticosteroids
KLH	Keyhole limpet hemocyanin
LTB_4	Leukotriene B4
LF	Lateral Flow
MBP	Major Basic protein
MMP	Matrix Metalloproteinase (e.g. MMP-2, MMP-12 etc.)
MPO	Myeloperoxidase
NF-kB	Nuclear factor kappa B
NGAL	Neutrophil gelatinase-associated lipocalin
PEx	Pulmonary Exacerbation
PG	Prostaglandin
pNPP	p-Nitrophenyl Phosphate
PSA	Polystreptavidin
PYD	pyridinoline
QEH	Queen Elizabeth Hospital, Birmingham, UK
RBP4	Retinol Binding Protein 4
RNS	Reactive Nitrogen Species
ROC	Receiver operator curve
ROS	Reactive Oxygen Species
RT	Room temperature
SLPI	Secretory Leukocyte Protease Inhibitor
SOD	Superoxide Dismutase
SP-D	Surfactant Protein- D
sRAGE	soluble Receptor for Advanced Glycation End products
TIMP	Tissue inhibitor of metalloproteinase (e.g. TIMP-1, TIMP-2)
TNF-α	Tumour necrosis factor-α
UTI	Urinary tract infection

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Chapter 1. Introduction

1.1 Definition and epidemiology of Chronic obstructive pulmonary disease (COPD)

1.1.1 COPD

COPD is an inflammatory disease of lung, characterised by progressive airflow limitation that is not fully reversible (1). It is defined as a preventable and treatable respiratory disease and characterised by abnormal inflammation of the lungs in response to inhalation of noxious particles or toxic gases, especially cigarette smoke (2). COPD is a major global epidemic that predominantly affects the elderly population (3). Its prevalence is increasing, and it contributes to substantial morbidity and mortality. There are an estimated 80 million people that have moderate to severe COPD worldwide. COPD has an estimated annual death rate of over 4 million people globally. By 2020 it is predicted to be the third leading cause of mortality worldwide. In developed countries COPD now affects female and male subjects equally, reflecting the equal prevalence of smoking (4).

1.1.2 COPD exacerbations

COPD exacerbations, defined as an acute sustained worsening of the patient's symptoms from their usual stable state, which is beyond normal day-to-day variations, are a particularly important feature of the disease, accounting for significant morbidity, mortality and health care costs. They are responsible for about 15% of all medical admissions (5), one million bed days and an annual UK NHS expenditure of £500 million [NICE 2010]. COPD and in particular COPD exacerbations are of a high public health and financial relevance associated with a significant negative impact on the quality of life (6). Nowadays, the recognized criterion used to classify AECOPD according to symptoms is the Anthonisen standard (7). Anthonisen *et al* divided exacerbations into three types. Type 1 exacerbations involve increased dyspnea, sputum volume, and sputum purulence, Type 2 involve any two of the latter symptoms, and Type 3 just involves one of those symptoms combined with cough, wheeze, or symptoms of an upper respiratory tract infection. It has been shown that a single exacerbation (the first) may result in significant increase in the rate of decline in lung function (8).

1.2 Pathology of COPD

COPD is primarily characterised by the presence of airflow limitation resulting from chronic inflammation and remodelling of small airways or 'chronic bronchitis' and is often associated with lung parenchymal destruction, resulting in loss of the alveolar attachments or 'emphysema' (9) (Figure 1.1). Chronic inflammation causes structural changes, small airways narrowing, and destruction of lung parenchyma as demonstrated by tissue biopsies, sputum analysis, and post-mortem samples (10).

In COPD, repeated exposure to noxious particles triggers an inflammatory cascade in the small airways and lung parenchyma involving several different cell types (eg, neutrophils, macrophages, lymphocytes) and inflammatory mediators (eg, growth factors, cytokines, chemokines, proteases) (11). These changes are believed to result in mucus hypersecretion, extracellular matrix degradation leading to chronic bronchiolitis, and injury to alveolar epithelial cells, leading to emphysematous changes (10). A fraction of people with COPD has inherited PiZZ (Glu342Lys) alpha-1 antitrypsin deficiency (A1ATD), a major genetic determinant influencing the development of early-onset COPD with emphysema, especially in cigarette smokers (12). A1ATD-related COPD is associated with low circulating levels of A1AT (10-15% or normal levels) (13). Consequences of this is explained in more detail later.

1.3 COPD diagnosis and treatment

Currently, COPD patients self-report when they perceive symptoms that result from deteriorating lung function and or gross physiological changes that result from relatively advanced host responses and/or pathogen virulence. Current practice investigations are chest X-ray, SaO2, ABG, ECG, FBC, U+E/glucose, Theophylline level if appropriate, Sputum MC & S if purulent, BCs if pyrexial. At present, the best method for recognising the onset of an exacerbation is through the recording and monitoring of patient symptoms or physiological measures, which can be subjective.

Upon presentation of symptoms, it is likely that the damage to the lung has already started or occurred. Advanced warning of an exacerbation would give reassurance and additional information to help patients manage their condition more efficiently

Current guidelines advocate the use of oral corticosteroids and antibiotics for people with COPD exacerbations. The clinical response to treatment varies considerably and is associated with significant side effects; the inability to target therapy means some patients are inappropriately treated placing a vulnerable population at further risk i.e. the elderly population. The benefit of antibiotics in mild to moderate AECOPD remains controversial and their overuse can contribute to the development of bacterial resistance. Systemic corticosteroids bear the risk of adverse side effects (hyperglycaemia, increased risk of diabetes and cardiovascular disease), especially in patients with co-morbidities. Furthermore, in some patients oral corticosteroid therapy is associated with increased treatment failures (defined as retreatment, hospitalisation, or death within 30 days) (14). This has led to strategies to reduce the duration of oral corticosteroid treatment (15). A recent review concluded that current COPD guidelines are of little help in identifying which AECOPD patients might benefit from treatment with corticosteroids and antibiotics in a primary care setting (16) and stresses the importance of developing better methods for targeting corticosteroid treatment as well as gaining a better understanding of COPD phenotypes.

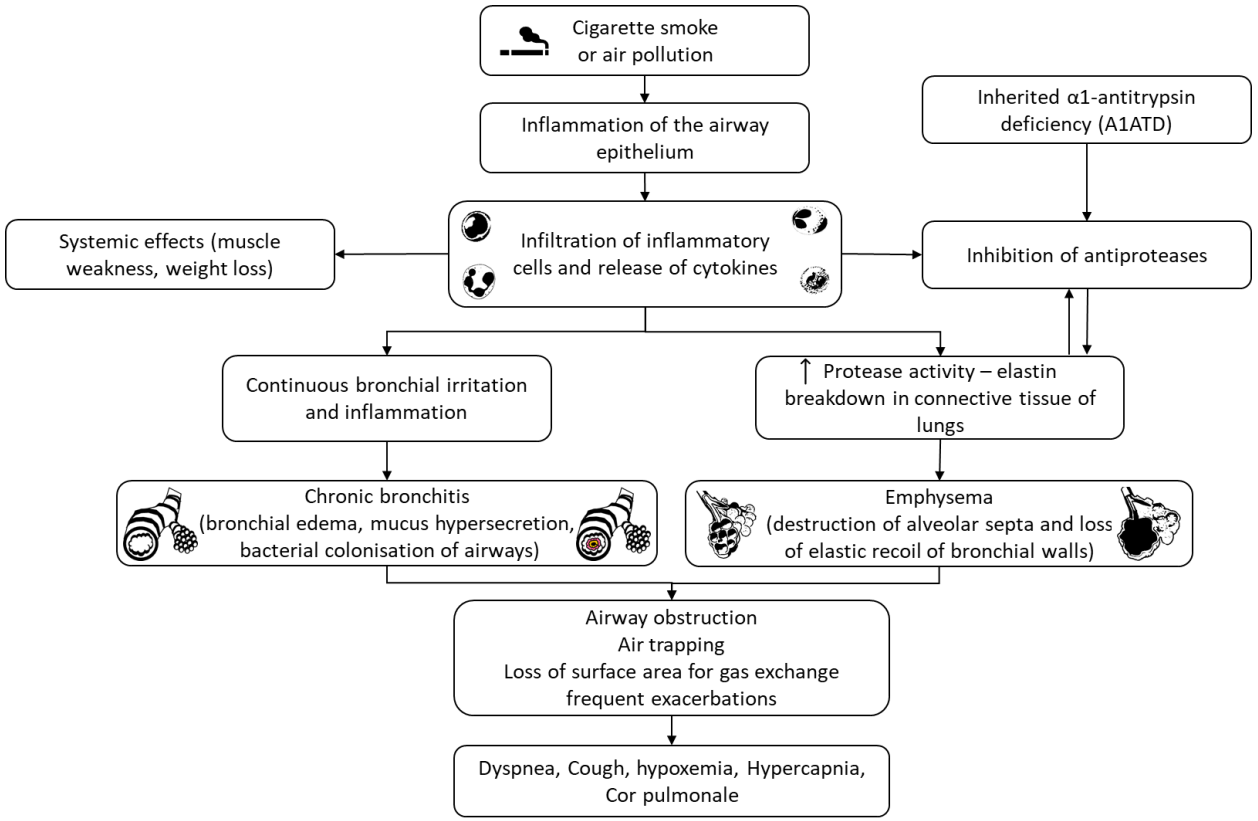


Figure 1.1. COPD, a combination of chronic bronchitis and Emphysema. The most important risk factor for chronic obstructive pulmonary disease (COPD) is cigarette smoking. Other exposures including passive smoke and biomass fuel use also play roles. The innate immune system includes mucociliary transport and coughing that, together clear the airway surface. After distress the production of mucus is increased, and the epithelial barrier is disrupted. Epithelial cells and resident monocytes/macrophages respond by generating a wide variety of cytokines and chemokines that control the movement of migrating innate inflammatory immune cells (all originating in the bone marrow) into the injured tissue. These include polymorphonuclear leukocytes (PMNs); monocytes/ macrophages; eosinophils; as well as a smaller number of natural killer and dendritic cells (Hogg, 2006). Continuous bronchial irritation and inflammation is associated with an increased number of epithelial, goblet and squamous cells, dysfunction, damage and loss of cilia and enlarged submucosal mucous secreting glands, which result in the mucous hypersecretion that is characteristic of chronic bronchitis (17). In the lung parenchyma, which includes the gas exchanging surface of the lungs (bronchioles, alveoli, pulmonary capillary system), destruction can occur in the form of emphysema, which involves dilation and destruction of the bronchioles and surrounding alveoli (17).

1.4 Systemic manifestations and Comorbidities

The impact of COPD extends beyond the lung and several systemic manifestations can further impair functional capacity and health-related quality of life. In addition, COPD is associated with several other diseases, such as cardiovascular diseases, osteoporosis, diabetes, and metabolic syndrome, more commonly than expected by chance. It is believed that the link between them is in the form of a spill over of inflammatory mediators from the lung (9, 18).

1.5 Heterogeneity and exacerbation phenotypes

There is increasing recognition that COPD is a heterogeneous condition with variability between sufferers in terms of their symptoms, age of onset, lung function, exercise capacity, microbiome shifts and patterns, comorbidities, medication, and airway inflammation. The frequency of COPD acute exacerbations (AECOPD) varies as does disease progression defined by loss of lung function. There is a series of clinically relevant subgroups in COPD i.e. patients with clearly defined clinical characteristics (phenotypes) with prognostic or therapeutic implications (19, 20). Comorbidities do not represent a specific subgroup as they are treated independently (20). The Linkages between phenotype, disease progression and intervention are summarised in figure 1.2.

A distinct patient group expressing the ''frequent exacerbation phenotype'' has greater susceptibility to exacerbations irrespective of disease severity. It is now widely recognised, having been identified by data collected as part of the Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) study. In this study, 2138 patients with a range of disease severity were observed over 3 years. It was concluded that exacerbations became more frequent and more severe as the severity of the disease increased and that history of exacerbations is the single most effective predictor of a frequent exacerbator phenotype (21). This represented 12% of the study population and has now been incorporated into the current GOLD multidimensional assessment of COPD (22). Alternatively, data from the ECLIPSE study led to the identification of a 'systemic inflammatory COPD phenotype' with persistent systemic inflammation (elevated levels of 2 or more of WBC, CRP, IL-6, Fibrinogen) for 1 year. These patients presented with significantly increased all-cause mortality and elevated exacerbation frequency at follow up visits (23). The group, representing 16% of the study population, is different to the frequent exacerbation phenotype group, as only 40% of the inflamed group were frequent exacerbators (2+), 28% had a single exacerbation and 32% had no exacerbations. This suggests that a diagnostic criterion other than exacerbation history would improve the ability to predict susceptibility to exacerbations. Other studies support the findings from the ECLIPSE study with the observation that even those patients with milder COPD (and in those without previous exacerbations) can be subject to increased risk of exacerbations in the following year (24). These

patients had simultaneously elevated levels of inflammatory biomarkers (plasma CRP, fibrinogen and white blood leukocyte counts). In the latter study it was suggested that high levels of inflammatory biomarkers reflected bacterial colonisation or latent viral infections persisting in airway epithelial cells, after a previous exacerbation. This would be a good explanation for the frequent exacerbators but, for the inflamed group, it could be that low-grade systemic inflammation had a negative effect on the immunological response, thereby increasing susceptibility to exacerbations (24).

It has also been demonstrated that patients with frequent exacerbations have a faster rise in systemic inflammation over time compared to those with infrequent exacerbations (25). In the Donaldson study, plasma fibrinogen, sputum interleukin-6 and neutrophils were significantly increased over 1-7 years across 148 patients. Longitudinal studies of populations with COPD over many years demonstrated that only about 50% of patients given a diagnosis of COPD had an accelerated decrease in lung function, whereas the remainder had a normal age-related decrease but started from a lower value, presumably because of impaired lung development (26). This implies that only half of patients with COPD have inflammation, whereas those with normal decreases in lung function presumably do not. Aaron *et al* (27) divided the exacerbations of COPD into two distinct patterns, such as sudden and gradual onsets, according to worsening respiratory symptoms from diary cards. Patients who experienced sudden onset exacerbations had greater mean daily symptom scores, greater peak symptom scores, earlier peak symptoms, and shorter median recovery times back to baseline health status. The frequent exacerbators are further divided into the following two types: those with emphysema predominant and those with chronic bronchitis predominant. The treatment for the two types is also different. For the emphysema phenotype, the basis of pharmacological treatment is long-acting bronchodilators, and in some cases with Inhaled Corticosteroids (ICS). The bronchitis-predominant exacerbator patients may be treated with bronchodilators and ICS, and in contrast to exacerbators with emphysema, they respond to treatment with Roflumilast (19).

With regard to response to treatment, one study demonstrated that frequent exacerbators had a reduced response to treatment of exacerbation, although there were limitations to its validity. Although there were significant differences between levels of biomarkers (MPO, IL-6 and CRP) in infrequent and frequent exacerbators from exacerbation to recovery at each timepoint (over 2 weeks)(28), only CRP gave no difference at admission and a significant change during the recovery timepoints. The recovery period was also variable and could be seasonal. A study has demonstrated that COPD exacerbations in colder periods of the year take longer to recover, are more likely to involve cough or coryzal symptoms and more likely to cause hospital admission (29). A different study looking at sputum (neutrophils, MPO, IL-8) and serum inflammatory markers (IL-6, CRP) revealed a time lag between the resolution of airway and systemic inflammation, which were correlated with the

improvements in different clinical indices (lung function, Dyspnea score and COPD assessment test (CAT) score. This supports the requirement for completing long-term treatment (14 days) to ensure resolution of exacerbation.

It has been suggested that eosinophil-driven inflammation is characteristic of viral exacerbations, whereas neutrophil-derived mediators are associated with both viral and bacterial exacerbations (10). However, exacerbations are heterogeneous with respect to inflammation and aetiology, typically neutrophilic driven exacerbations and some associated with eosinophilic airway inflammation. How these alter lower airway inflammation and relate to treatment response is not clear, although, in stable state, sputum eosinophilia is associated with corticosteroid responsiveness whereas high bacterial load and sputum purulence associate with favourable outcomes with antibiotics. Subpopulations were identified by the Bafadhel study (14) in which 4 biologic clusters were determined, relating to identifiable patterns of inflammation and potential causative pathogens. The biologic exacerbation clusters were bacterial-, viral-, or eosinophilic-predominant, and a fourth was associated with limited changes in the inflammatory profile and was termed “pauciinflammatory’’ (14). In this study, it was found that the best serum biomarkers associated with bacterial and viral associated exacerbations were CRP with an AUC of 0.65 and CXCL10 (IP-10) with an AUC of 0.76 respectively. The best sputum biomarkers associated with bacterial and viral associated exacerbations were IL-1β with an AUC of 0.89 and CCL5 with an Area Under the Curve (AUC) of 0.69 respectively. Blood eosinophil, sputum CCL17, sputum IL5, were the most strongly associated with sputum eosinophilia with an AUC of 0.85, 0.8 and 0.73 respectively. A further study identified subgroups for improved treatment stratification, Eosinophilic (EO), Neutrophilic (NE), mixed granulocytic (MG) and paucigranulocytic (PG). The MG and NE group had higher sputum inflammatory cells, higher levels of sputum MMP-9, IL-6 and CRP and serum SAA, lower lung function, and longer hospital stay (30). 83% with NE displayed evidence of bacterial infection and responded poorly to standard therapies. Patients with EO had a better response to corticosteroids. The stratification in the latter study was based on sputum eosinophils and neutrophils, the EO group >2.5% sputum eosinophils, the NE group >61% neutrophils, the PG group <2.5% eosinophils and <61% and neutrophils and the MG group >2.5% eosinophils and >61% neutrophils.

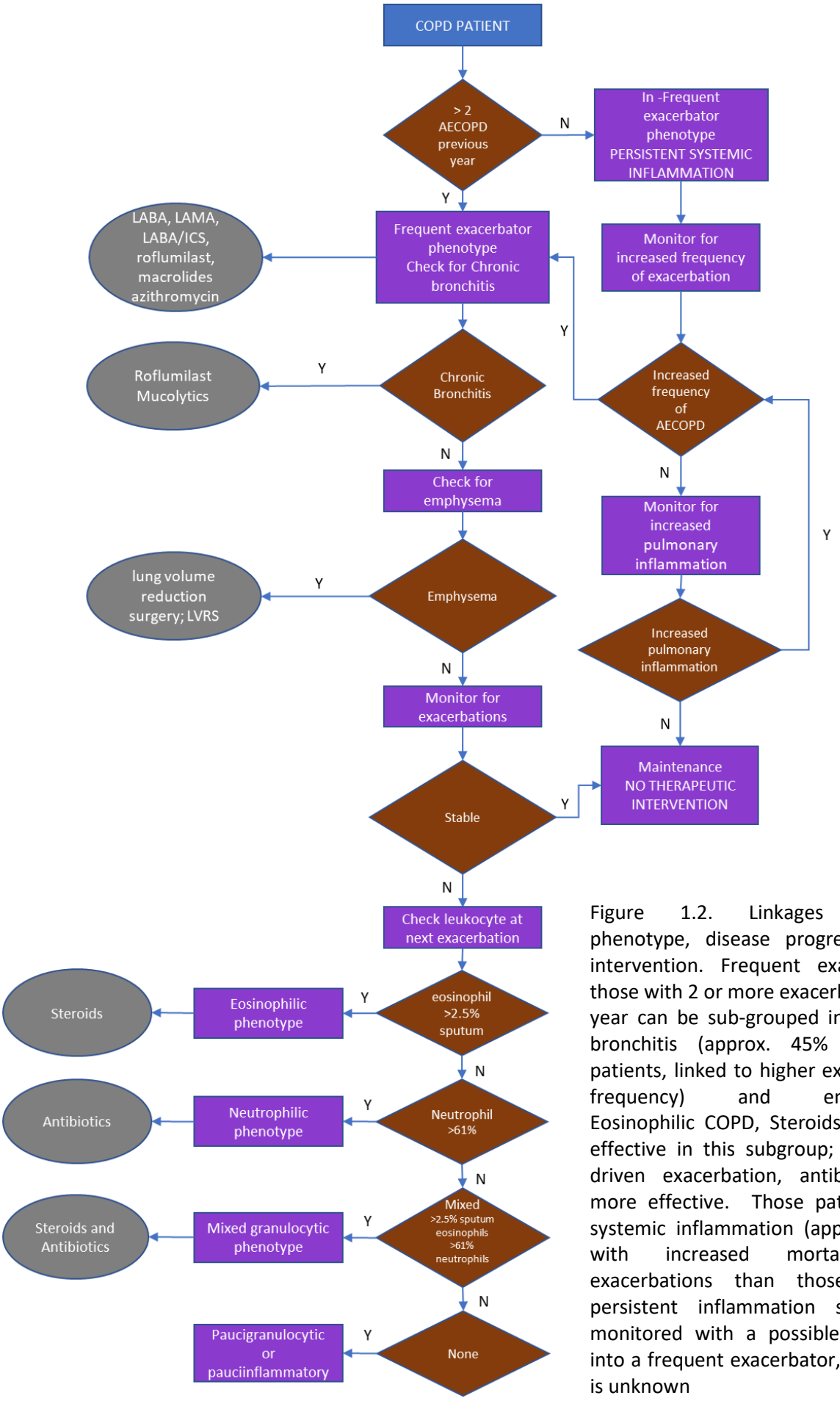


Figure 1.2. Linkages between phenotype, disease progression and intervention. Frequent exacerbators, those with 2 or more exacerbations per year can be sub-grouped into chronic bronchitis (approx. 45% of COPD patients, linked to higher exacerbation frequency) and emphysema; Eosinophilic COPD, Steroids are more effective in this subgroup; neutrophil driven exacerbation, antibiotics are more effective. Those patients with systemic inflammation (approx. 16%), with increased mortality and exacerbations than those without persistent inflammation should be monitored with a possible transition into a frequent exacerbator, treatment is unknown

1.6 Cystic Fibrosis

Unlike COPD, In Cystic Fibrosis (CF), the inflammatory response is driven mainly by bacterial infections, especially *Pseudomonas* species, which leads to tissue breakdown and severe lung damage (31). Early eradication of *P. aeruginosa* infection results in improvement of overall survival and better patient care (32). CF produces progressive lung disease and related morbidity and mortality in >90% of patients (33). Abnormalities in the CF transmembrane conductance regulator protein result in abnormal airway surface liquid that impairs mucociliary clearance. Mucus becomes a site for bacterial colonization and a resulting neutrophilic inflammatory response. These neutrophils release oxidants and proteases that degrade tissue and eventually cause permanent fibrotic change of the airways and lung parenchyma of patients with CF.

1.7 Asthma

Asthma, like COPD, is associated with airway inflammation, but the components of inflammatory response and the site of inflammation differ between both conditions. Asthma is a condition of inflammation predominantly in the large airways, although the chronic condition is also associated with inflammation in the small airways or bronchiolitis (17). In both diseases, there is chronic inflammation of the respiratory tract, which is mediated by the increased expression of inflammatory proteins including cytokines, chemokines, adhesion molecules, inflammatory enzymes and, in both diseases, there are acute episodes of exacerbations (34). Exacerbations in Asthmatic individuals are usually triggered by rhinoviruses, and less commonly by inhaled allergens and air pollution, unlike COPD which is triggered by either bacteria or viral infections and inflammatory stimuli – eg smoke (34). Patients often have overlapping clinical features of both Asthma and COPD which makes it difficult to diagnose (35). An estimated 15%-50% of patients with obstructive airway disease older than 50 years show a mixture of criteria. This group was classified as the asthma-COPD overlap syndrome (ACOS) by the Global Initiative for Asthma (GINA) committee back in 2016 but this is no longer advised.

1.8 COPD and oxidative stress

Oxidative stress in known to play an important role in the development of COPD (36) and during acute exacerbations (37). It occurs when there is an imbalance between the formation of reactive oxygen species (ROS) and antioxidant defence mechanisms (in favour of oxidants) resulting in harmful effects, including cell damage, mucous hypersecretion, antiprotease inactivation and increased pulmonary inflammation through the activation of transcription factors (2). The 'harmful effects' are a consequence of 'carbonyl stress', where oxidative damage to the surrounding tissues leads to the formulation of highly reactive organic molecules that can modify proteins nonenzymatically (37) targeting specific residues, such as lysine, arginine, cysteine, and histidine. ROS include hydroxyl radical (·OH) and superoxide anion ($O_2{\cdot}^-$) which contain unpaired electrons, the unstable nature of these ROS permit transfer of electrons to other molecules via oxidation, resulting in damage, inactivation or creation of further ROS (36).

1.8.1 Sources of ROS

Cell-derived ROS: The inflammatory-immune response results in activation of epithelial cells and resident macrophages, and the recruitment and activation of neutrophils, eosinophils, monocytes and lymphocytes, particularly during exacerbations (37). Activation of these inflammatory and structural cells in response to various stimuli including cytokines produce ROS (17). There are 4 key processes described that result in different ROS elements summarised in figure 1.3. a) The generated superoxide anion ($O_2{\cdot}^-$) is rapidly converted to (catalysed by the enzyme- superoxide dismutase (SOD)) to Hydrogen peroxide (H_20_2) and OH is formed nonenzymatically in the presence of Fe^{2+} as a secondary reaction. b) ROS and reactive nitrogen species (RNS) can be generated intracellularly from several sources, such as mitochondrial respiration, NADPH oxidase system, Xanathine Oxidase system (37). Nitrotyrosine is considered an indicator of the production of NOS (38). c) Activity of haem peroxidases (myeloperoxidase) or eosinophil peroxidase (EPO) formulate potent oxidant hypochlorous acid (HOCl) and hypobromous acid (HOBr) from H_20_2 in the presence of chloride and bromide ions, respectively.

Inhaled oxidants and cigarette smoke: Inhalation of cigarette smoke and airbourne pollutants results in direct lung damage as well as the activation of inflammatory responses in the lungs. Oxidants present in cigarette smoke can stimulate alveolar macrophages to produce ROS and to release a number of mediators, some of which attract neutrophils and other inflammatory cells into the lungs. Neutrophils and macrophages are known to be increased in the lungs in smokers compared to non-smokers and generate ROS via the NADPH oxidase system. Smoking is associated with increased content of MPO in neutrophils. Smokers and people with COPD are have increased levels particularly

during exacerbations (39). While exposure to cigarette smoke can drive the onset of COPD once the disease has been established cessation of smoking does not stop the continued presence of oxidative stress and progression of disease (37).

1.8.2 The consequences of oxidative stress in relation to COPD

Oxidative stress and neutrophil traffic in the lungs: Neutrophils from people with COPD have been shown to release increased amounts of ROS spontaneously and following stimulation (40). During migration, neutrophils release proteases and ROS as they move through lung tissues, this migration is affected (inaccurate migration) by ROS which in turn may result in moving across a larger surface area (36).

Oxidative stress and protease/antiprotease imbalance – leading to emphysema: Oxidative stress can also impair the function of the antiproteases, such as Alpha-1 antitrypsin (A1AT) and Secretory Leukocyte Protease Inhibitor secretory (SLPI), as a consequence, the imbalance accelerates the breakdown of elastin in lung parenchyma by human neutrophil elastase (HNE). ROS are able to inactivate A1AT via oxidation of the methionine 358 residue in the active site (36) promoting inflammation. In addition, it has been shown in various studies that active HNE, cathepsin G and proteinase-3 can activate matrix metalloproteinase-2 (MMP-2), and this activation is blocked by A1AT but not by a proteinase inhibitor (41). There is evidence that altered protease and antiprotease balance during a COPD exacerbation contributes to mucus obstruction (42).

Oxidative Stress and inflammation in the airways: ROS activate transcription factors such as nuclear factor-kappaB (NF-kB), which switches on multiple inflammatory genes, resulting in amplification of the inflammatory response (17). Genes for many inflammatory mediators such as interleukin-8, TNF-a, are regulated by such transcription factors. It is believed that oxidants cause the release of inflammatory mediators that are also associated with increased expression of the genes. Carbonyl stress in the form of electrophilic carbonyls can also impact on many different signalling pathways. As with oxidative stress, this is propagated through the targeting of critical cysteine residues in susceptible signalling molecules (37). Phagocytosis is impaired in COPD as a consequence of Carbonyl stress, the failure to remove apoptotic cells can lead to continued inflammation in COPD (37).

Oxidative stress and aging: Oxidative stress reduces the expression and activity of sirtuin-1, a key repair molecule that is implicated in aging, which could contribute to the accelerated aging response seen in patients with COPD. The accelerated aging lung increases the likelihood of developing emphysema (37). In addition, reduced sirtuin-1 is believed to an increased expression of MMP-9.

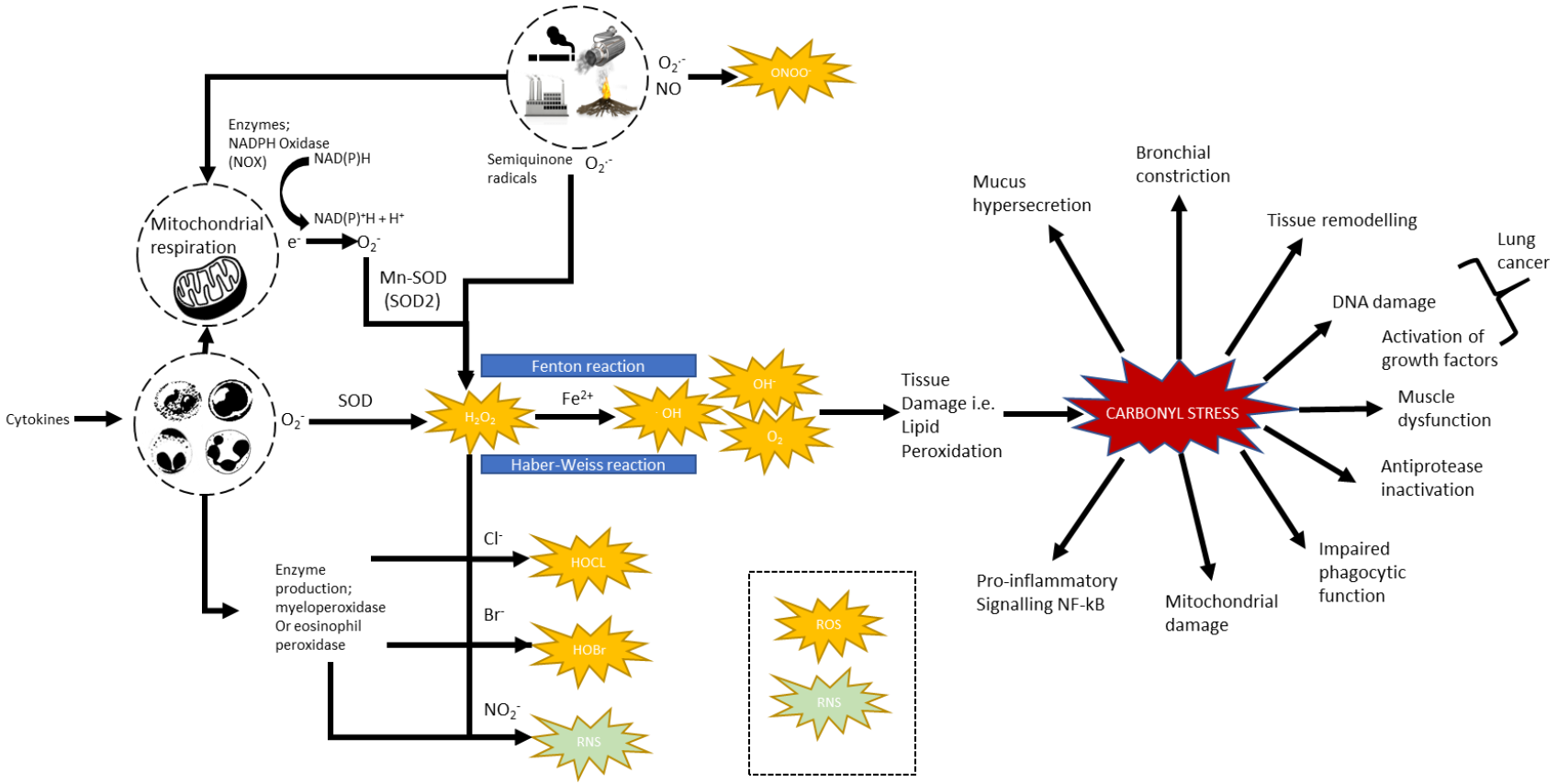


Figure 1.3. Origins of ROS and RNS and oxidative stress in COPD. Overproduction of ROS/NOS from environment and cellular sources cause tissue damage through lipid peroxidation and the oxidation of proteins and carbohydrates resulting in the formulation of carbonyl stress. Biological systems are continuously exposed to oxidants which can be either generated endogenously by metabolic reactions e.g. from mitochondrial electron transport during respiration or during activation of phagocytes or exogenously such as inhaled from air pollutants or cigarette smoke. $O_2^{\cdot-}$, a free radical with a short biological lifespan is reduced to the more stable H_20_2 mediated by a key antioxidant defence enzyme -SOD (of which there are 3 different forms and SOD 1 and SOD3 expression is reduced in the lung and blood of tobacco smokers (38). Fe2+, implicated in the formulation of hydroxyl radicals is a critical factor related to toxicity induced by ROS generation (Fenton reaction). Abbreviations: ROS, reactive oxygen species; NOS, Reactive nitrogen species; SOD, Superoxide dismutase; $O_2^{\cdot-}$, superoxide; NO, nitric oxide; H_20_2, hydrogen peroxide; HOCL, Hypochlorous acid; HOBr, hypobromous acid; ONOO-, peroxynitrite; NOX, NADPH oxidase.

1.9 Phagocytes

Phagocytes detect surface molecules that are present on pathogen cells but not on host cells. They detect evolutionarily conserved surface molecules shared by many microorganisms. Toll-like receptors (TLR) on our own phagocytes cells bind to pathogen surface molecules. The binding is a signal to the phagocyte to engulf its target and release cytokines that recruit other immune cells to the site of injury or infection. It is not possible to cover all of these in this thesis, therefore focus will be on neutrophils and eosinophils and their role in COPD.

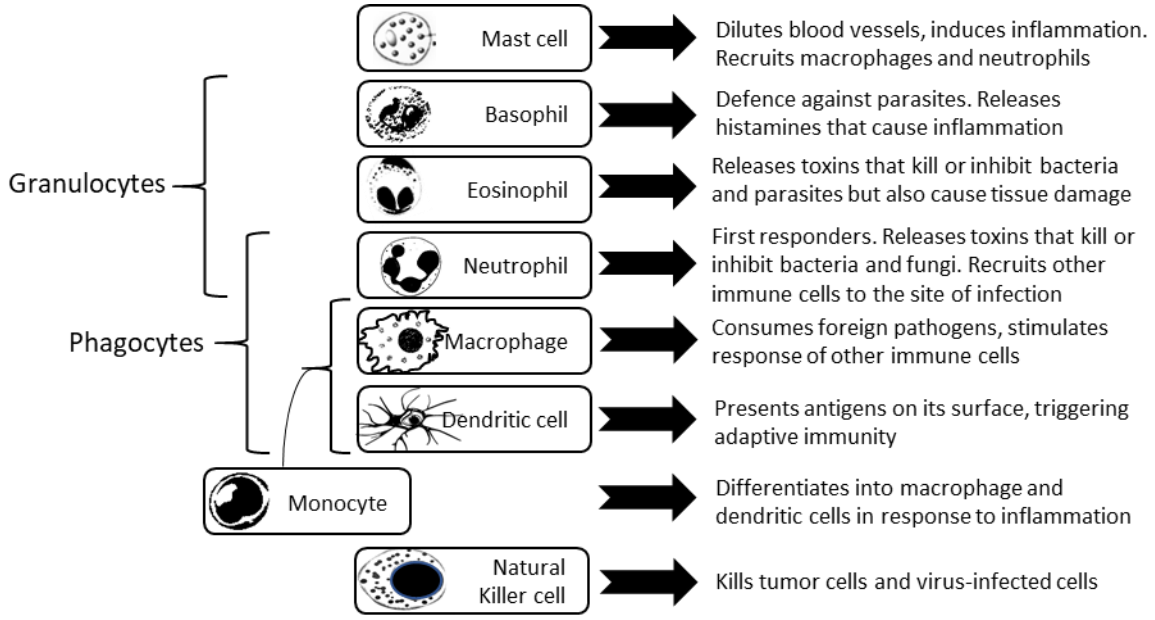


Figure 1.4. Cells involved in the Innate immune system. Phagocytes are immune cells that engulf and destroy foreign cells; i.e. macrophages, neutrophils and dendritic cells. Neutrophil and macrophages migrate from blood vessels into tissues.

1.10 The role of neutrophils in COPD

Neutrophils are the most abundant leukocytes in blood and are part of our native or innate immunity, and together with NK cells, platelets and macrophages, they act as part of our defence to protect against microbes (18). However, in COPD, there is excessive neutrophil recruitment, activation, and defective apoptosis resulting in the production of reactive oxygen species, the release of serine proteases, matrix metalloproteinases, myeloperoxidase, and lysozymes that then contribute to lung tissue damage and airway remodelling (18). Neutrophils for a long time have been recognised to be involved in smoking-induced tissue injury, as the major destroyers of the elastic matrix of the alveoli, as shown by numerous findings of increased numbers of activated neutrophils

in sputum and BAL fluid that correlate with disease severity. However, the location of the neutrophils accounting for circulating neutrophil-derived enzymes remains uncertain (40).

Several proteins are involved in the chemoattraction, adhesion and transmigration of neutrophils. Neutrophil recruitment to the airways and parenchyma from the circulation involves initial adhesion to vascular endothelial cells through E-selectin (upregulated in people with COPD (4)) and tight adhesion using integrins followed by migration across the epithelial monolayer through the paracellular space, where they are retained on the luminal side as a defence barrier and to clear invading pathogens (18). The process is propagated by increased neutrophil chemotactic factors, including but not restricted to LTB4, CXCL1, CXCL5, and CXCL8 (IL-8). These are derived from alveolar macrophages, T cells, and epithelial cells, but the neutrophil itself might be a major source of CXCL8 (4). A host- microorganism interaction-associated chemoattractant, formyl-met-leu-phe (fMLP) produced in bacteria and mitochondria is also suspected to contribute to driving neutrophils to infiltrate the damaged lung parenchyma (43) via the seven-transmembrane G-protein-coupled receptor FPR1 (44). In vitro, blockade of neutrophil FPR1 with inhibitory antibodies or the selective antagonist cyclosporin H (CsH) significantly attenuated neutrophil chemotaxis toward necrotic cells (45). Bacterial derived fMLP is a consequence of their protein processing mechanisms and/or from degraded proteins (which start out as pathogen associated molecular pattern molecules or ''PAMPs''). Mitochondrial derived fMLP results from damaged eukaryotic cells by degradations of proteins similar to bacterial proteins (in this case, molecules known as damage associated molecular patterns or ''DAMPs'').

Neutrophils store an assortment of molecules in three types of granules, primary; antimicrobial proteins and proteases (e.g. MPO, neutrophil serine proteases NSPs), secondary; lactoferrin, and tertiary; gelatinases (e.g. MMP-9). In addition, secretory vesicles contain a reservoir of membrane-associated proteins (44). During chronic neutrophilic inflammation, an increasing number of activated neutrophils secrete granule contents into the extracellular spaces, where the excess of proteases can become destructive, especially in the absence of pathogens. Myeloperoxidase (MPO), produced in the neutrophil and monocyte precursor cells in the bone marrow, is an enzyme that contributes to the destruction of bacteria during activation of the host immune system (46). Confirmation that the neutrophils are activated comes from increased concentrations of granule proteins, such as MPO and human neutrophil lipocalin (HNL, also known as NGAL). NSPs include Human neutrophil elastase (HNE), Proteinase 3 (PR3) and Cathepsin G (CG). Together they are capable of degrading most of the extracellular matrix components such as elastin and collagen (18). The imbalance of HNE and its inhibitor, A1AT (as presented in A1ATD and oxidative stress) is believed to result in emphysema. It also worsens mucus-driven airway obstruction (hypersecretion),

which is a common feature in cystic fibrosis, bronchiectasis, and COPD. Two known processes activate the sodium channel and indirect degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) leading to dehydration of the airway surface and further weakening of the ability of the airways to effectively clear not only mucus, but also any pathogens (18). It has been suggested that PR3 can modify key cytokines such as IL-8, leading to enhanced stability and potency, and release of IL-1β and TNF-a from monocytic cells. NSPs have also been reported to inactivate the IL-6 trans-signalling pathway (47), this would have a negative impact on leukocyte recruitment.

MMPs are proteolytic enzymes that degrade matrix components both in stable and exacerbation states. There are 26 different types of MMP, stratified according to structure, substrate specificity and function (48), classified into subgroups of collagenases (MMP-1, 8, 13), gelatinases (MMP-2,9), stromelysins (MMP-3,10), stromelysin-like (MMP-11, 12), matrilysins (MMP-7, 26), transmembrane (MMP-14, 15, 16, 24), glycosyl-phosphatidly-inositol-type (MMP-17, 25), MMP-19-like (MMP19, 28), and other MMPs (MMP-18, 20, 23) (48). Most MMPs are secreted as latent pro-enzymes and are activated by proteolytic conversion (49). It is the active form in which they become directly disruptive. Their activity is regulated or inhibited by Tissue Inhibitors of MMPs (TIMPs) (50). Matrix metalloproteinases MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B) regulate extracellular matrix turnover and can degrade matrix components such as elastin and, similar to the NSPs, when in excess their activity can lead to tissue destruction. The protease-antiprotease imbalance might also be altered by the degradation of the TIMPs by HNE (51), similarly, MMP-9 and MMP-12 may also inactivate A1AT and at the same time, HNE may activate MMP-9 (18). Macrophage elastase (MMP-12), is mainly produced by macrophages and has been shown to be involved in COPD (elastin degradation). It has been suggested that MMP-12 gene polymorphism may account for this disease variability and one of the causative factors in smoking related injury (52). It has also been shown in vitro that MMP-12 can also cause production and release of IL-8 (49) and TNF-α (52).

As a consequence of the collagen degradation that occurs during neutrophil degranulation and release of the molecules described, the consequent fragments can activate inflammatory cells and drive chronic inflammation further. The increase in elastase activity in patients with COPD might contribute to the development of emphysema and neutrophilic inflammation through generation of chemotactic peptides, such as N-acetyl Pro-Gly-Pro (Ac-PGP, a matrikines), which are potent neutrophil chemoattractants that activate CXCR2.This might be self- perpetuating because neutrophils release MMP-9 which, in turn, generates more PGP (4, 53), which can readily diffuse through the dense ECM. Furthermore, its unusual structure owing to the cycling back of the proline side chains onto the backbone amino group results in a matrikine that is resistant to generic

protease degradation (54). PGP is normally degraded by leukotriene A4 hydrolase, limiting neutrophil influx, but this mechanism is disrupted by cigarette smoke, thus enabling increased neutrophil inflammation to continue (49, 55). PGP can be chemically acetylated to Ac-PGP through the action of reactive aldehydes present in cigarette smoke.

Very recently, it has been reported that neutrophils can be 'de-primed' or revert back to the unprimed quiescent state. This is a novel finding because it has been assumed that neutrophil priming was an irreversible process (56).

1.11 The role of eosinophils in COPD

Eosinophils are generated in the bone marrow, and circulating eosinophils migrate to, and largely reside in, the gastrointestinal tract and thymus. Under certain condition, they are recruited to tissues, where they secrete chemokines, cytokines, and cytotoxic granular products that facilitate an inflammatory reaction. Until recently, COPD was considered to be a mainly neutrophil-mediated inflammatory disease (in contrast to asthma which is mainly eosinophilic). However, the growing body of evidence now indicates that some exacerbations can be eosinophil driven. Airway biopsies and numerous studies evaluating levels in blood have shown higher levels of eosinophils in the exacerbation state compared to stable state (57). Human eosinophils contain four basic proteins stored in secondary granules which have been shown to exert toxic effects on numerous cell types (58). Major basic protein (MBP) is found in the core, while eosinophil derived neurotoxin (EDN), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) are found in the matrix (59). EPO is believed to cause oxidative tissue injury; MBP may disrupt the epithelial barrier allowing the penetration of inhaled antigens; ECP and EDN are believed to cause apoptosis of airway epithelial cells (60). The mechanisms for increased eosinophil counts in people with COPD is not clear, but there has been interest in the role of type 2 innate lymphoid cells (ILC2s), which are regulated by epithelial mediators, such as IL-33 released as a result of epithelial cell injury (61). In the general population, sputum and blood eosinophil levels are typically <1.1% (of total white blood cells) and below an absolute count of 300 cells/µl respectively (60). Blood being a less invasive sample is preferable and a 2% cut-off (approximately 150 cells/µl absolute count) has been used as the specified threshold in the majority of studies to date (majority of which failed to meet their primary outcomes). In a prospective cohort study (SPIROMICS) of approximately 3200 participants, it was found that blood, sputum eosinophil counts related to clinical outcomes and subsequently demonstrated the usefulness of eosinophil counts to advance management decisions (62). Using eosinophil cut-offs of more than 1.25% for sputum and 200 cells/µL for blood to categorise high and low eosinophil counts, the SPIROMICS investigators found that baseline characteristics were

different according to eosinophil counts, including the proportion of current smokers (lower in the high blood eosinophil group vs low), a number of whom had taken inhaled corticosteroid medication (higher in the high vs low blood and sputum eosinophil groups), serum IgE levels (higher in the high vs low blood eosinophil group), and quality of life or lung function (most parameters worse in the high vs low blood and sputum eosinophil groups). The degree of emphysema, quantified by CT, was associated with high sputum but not blood eosinophils, particularly in the upper lobes. Furthermore, blood but not sputum eosinophils were found to be more reproducible in participants with repeated measures. Other studies suggest that blood eosinophils can be used as a biomarker in severe COPD exacerbations for predicting 12- month readmissions (63). It was found in this study that higher blood eosinophil counts at admission were associated with a more than threefold increase in 12-month readmission and a shorter time to first COPD-related admission. Evidence is now accumulating to show that in patients with COPD and a risk of exacerbations, blood eosinophil count identifies a group of patients with an increased risk, which could be modifiable with treatment. However, target eosinophil concentrations might need to be different, depending on the outcome that is to be modified— eg, whether aiming to decrease exacerbation risk, improve lung function, or relieve symptoms. Further studies that investigate mechanisms and risk modification will be required to clarify the role of eosinophils in COPD.

1.12 Bacterial infections in COPD

Acute infection results when higher loads of bacteria overcome the local defences, leading to acute inflammation involving both innate and adaptive defences. Bacterial colonisation may result from abnormal innate defences, chronic infection occurs when an inflammatory response generated by host defence mechanism fails to clear the bacteria, with continued tissue destruction (64). In bacterial exacerbations, purulent sputum (yellow or green) is the typical symptom, as is neutrophil-driven inflammation in both blood and airway (65). Bacteria overcome primary host defences by a number of pathogenic mechanisms, including release of ciliary toxins, pneumolysin, endotoxin, and IgA proteases, thereby disrupting mucociliary clearance (hence the change in sputum production). Subsequently, bacteria adhere to the epithelium, resulting in activation of dendritic cells, macrophages, and epithelial cells through toll like receptors (TLRs), initiating an inflammation response (64).

In patients with COPD, bacterial detection in lower airway derived samples is associated with increased airway inflammation, reduced lung function and more frequent exacerbations (66). Bacteria are isolated from sputum in 40-60% of acute exacerbations of COPD. The three-predominant bacterial species isolated were *Haemophilus influenzae, Moraxella catarrhalis*, and

Streptococcus pneumoniae, although Gram negative *enteric bacilli*, and *Pseudomonas spp* are also frequently isolated in patients with severe COPD (67).

The Acute Exacerbation and Respiratory InfectionS in COPD (AERIS) study is the first longitudinal study that includes molecular microbiological assessments (including viruses as potential airway pathogens) (68). After 1 year into the study, the finding was that at exacerbation, the most common bacterial species were *Haemophilus influenzae* and *Moraxella catarrhalis* (69), *Haemophilus influenzae* driven with a greater risk and frequency during high season (October-March). In addition, it was found that the lung microbiome shows significantly less variation within an individual than between individuals, that exacerbations within individuals showed higher microbiome variability during exacerbations compared to stable timepoints which was more significant in frequent exacerbators (70). It is also well known that *Haemophilus*, *catarrhalis* and *Pseudomonas* produce biofilms protecting the microbes from the immune system and antibiotics, a component of antibiotic resistance.

1.13 Viral infections in COPD

Viruses activate the innate immune system through cell surface and cytosolic PRRs, which detect viral components (especially nucleic acids) (64). Patients with detectable respiratory pathogens have been shown to exhibit a more marked impact on lung function and longer duration of hospitalisation than patients with exacerbations of non-infectious etiology (71). Viral exacerbations are associated with higher IL-6 levels, lower levels of CRP, and longer duration of hospital stay (average 9 days) (65). The frequency of dual viral and bacterial infections is low (72).

Respiratory viruses commonly associated with AECOPD are diverse and include human rhinoviruses, influenza and parainfluenza viruses, respiratory syncytial virus, coronavirus and adenovirus (68). Viruses are implicated as a major cause of exacerbations and are detected in approximately half of severe COPD exacerbations (73). Findings from the AERIS study indicate that the most common virus was rhinovirus with 23% of sputum samples positive at exacerbation (69). Rhinovirus has been shown to increase cytokine production in an epithelial cell line and thus repeated viral infection may lead to upregulation of airway cytokine expression (74). It has also been shown that viral infection alone is sufficient to induce COPD exacerbation and to lead to a secondary bacterial infection. Rhinovirus infections are frequently followed by secondary bacterial infections in COPD (36%). However, 71% of bacterial driven exacerbations had reported symptoms of a viral infection before onset. Cleavage of the antimicrobial peptides SLPI and elafin by virus-induced neutrophil elastase may precipitate these secondary bacterial infections (72).

1.14 Biomarkers in COPD exacerbations

There are biomarkers at different cellular and subcellular levels in exacerbation, which can provide information before, during and after the exacerbation (22). Sputum sampling reflects biofluid in the central airways rather than the lower and peripheral lungs, whereas, bronchoalveolar lavage (BAL) samples the more peripheral airways and alveoli. Exhaled breath condensate reflects inflammation in the respiratory tract (75) and blood carries the biomarkers from the lungs where they can be cleared from the body via the urine.

Barnes *et al* in 2006 and members of the American Thoracic Society (ATS)/European Respiratory Society (ERS) task force, reviewed biomarkers in COPD and concluded that, although there are many biomarkers of inflammation and oxidative/nitrative stress in the airways of patients with COPD, there was still a lack of information about a) how they related to disease severity; b) how reproducible they were, and c) could they be affected by concurrent therapies (76). An ideal biomarker is reproducible, derived from a standardised procedure, demonstrates disease specificity and has the ability to detect changes attributed either to therapeutic interventions or exacerbations (77). The review of > 600 published studies suggested that few of these biomarkers were validated, reproducible and related to disease development, severity, or progression (78). This meta- analysis covered 146,255 patients with COPD and revealed the poor sensitivity of current biomarkers to define clinical status and quantify the effect of treatment. Only sputum neutrophils and interleukin-8 (IL-8), as well as serum tumour necrosis factor-α (TNF-α) and C-reactive protein (CRP), showed any trend toward separating different stages of COPD (76). In another review of COPD-related biomarkers, Lock-johansson *et al*, noted that surfactant protein-D (SP-D), Club cell protein-16 (CC16), IL-8, CRP and fibrinogen did not fit the criteria individually but, in combination with each other or with additional biomarkers, they may be more useful (79). A similar study showed that different combinations of 5 plasma biomarkers: CC16, soluble Receptor for Advanced Glycation End products (sRAGE), fibrinogen, CRP and SP-D could differentiate between airflow limitation ($p<0.001$), emphysema, ($p<0.01$), decline of FEV1 ($p<0.05$), progression of emphysema ($p<0.01$) and all 5 for mortality ($p<0.05$) (80). A different review that included 59 studies, indicated that CRP, IL-6 and TNF-α were the most studied and only CRP showed consistent elevations in exacerbation compared to control subjects (81). It has been stated that biomarkers may come to the forefront for diagnosis of disease in the complex patient (82).

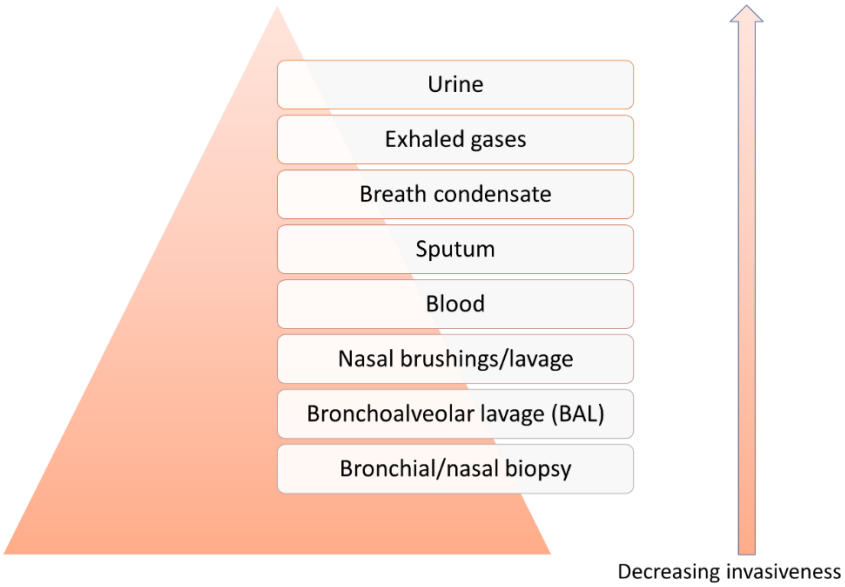


Figure 1.5. sample collection methods and invasiveness. The most invasive method being biopsy and the most non-invasive method being urine.

It is not possible within the scope of this thesis to review the thousands of studies undertaken to date that evaluate the different findings of biomarkers in different sample matrices. However, table 1.1. lists examples of studies selected to provide evidence of the shortlisted biomarkers selected for evaluation in urine and what is known about these biomarkers associated with exacerbations in different sample matrices; EBC, blood, sputum, BAL fluid and urine. It is to be noted that there are a) few studies assessing biomarkers in urine, b) few longitudinal studies due to the inability to collect other sample types frequently over a long period of time (see figure 1.5) and c) most of the exacerbation studies have less than adequate numbers of samples/patients due to the same reasons stated previously. The short list of biomarkers selected are shown in figure 1.6. these include:

- Signalling molecules that recruit neutrophils to the inflammation site
- Acute phase proteins regulated by signalling molecules
- Degranulation molecules including proteases released by the neutrophils to fight off the infection that can become self-destructive
- Protease inhibitors that usually regulate the proteases that are impaired thus causing a further imbalance
- Degradation molecules that are a consequence of the damage caused by the proteases to the lung structure such as elastin and collagen
- Other additional biomarkers which are contributed by the kidneys themselves as a result of the downstream damage

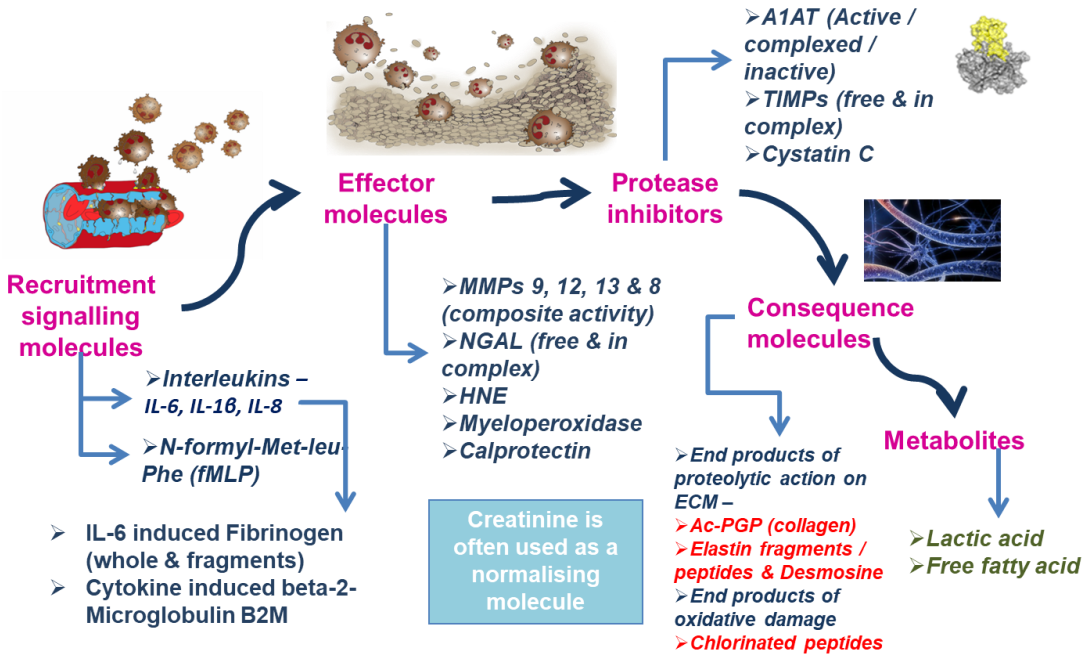


Figure 1.6. Biomarker selection rationale (diagram courtesy of Prof. Paul Davis, Mologic). Inflammatory leukocytes active in the lung cause a wide range of biomarkers to be released into lung fluid and blood, some originating from the leukocytes, some from the damage they cause to the surrounding tissue and some as a consequence of the signalling pathways that call them into the lung or control their activity

Table 1.1. A review of studies evaluating biomarkers in AECOPD in various sample matrices

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
TNF-a	Canada	2001	(83)	Stable- AECOPD		1 month and 9-15 months	50		↑ AECOPD vs. BL ($p<0.01$)			
TNF-a	Germany	2005	(84)	AECOPD - recovery	ICU n= 11 General ward n = 34	ICU after 10 days GW after 1 week	45	↑ AECOPD vs. recovery (both groups)				
IL-6	UK	2000	(74)	Stable - AECOPD.	37 AECOPD		57		↑ AECOPD vs. stable ($p<0.05$)			
IL-6	Germany	2005	(84)	AECOPD - recovery	ICU n= 11 General ward n = 34	ICU after 10 days GW after 1 week	45	↑ AECOPD vs. recovery (both groups)				
IL-6	China	2014	(28)	AECOPD-stable.	non-frequent (n=78) frequent (n=57)	Day 0, 4,7,14 week 8 after discharge	135			↑ AECOPD vs. stable (serum)		
IL-6	China	2014	(85)	AECOPD - stable	Resolved day 4 in sputum and day 14 serum	Day 0, 4, 7, 14, week 8 (after discharge)	93			↑ AECOPD vs. stable ($p=<0.001$)		
Il-1β	Germany	2005	(84)	AECOPD - recovery	ICU n= 11 General ward n = 34	ICU after 10 days GW after 1 week	45	↑ AECOPD vs. recovery (both groups)				
IL-8	Canada	2001	(83)	Stable- AECOPD		1 month and 9-15 months	50		↑ AECOPD vs. stable ($p<0.05$)			
IL-8	Netherlands	2004	(86)	AECOPD - recovery		Day 1, 3, 7	14			↑ AECOPD vs. recovered ($p=<0.002$) (serum)		
IL-8	Germany	2005	(84)	AECOPD - recovery	ICU n= 11 General ward n = 34	ICU after 10 days GW after 1 week	45	↑ AECOPD vs. recovery (both groups)				
IL-8	China	2014	(85)	AECOPD - stable.	Resolved day 4 in sputum and day 14 serum	Day 0, 4, 7, 14, week 8 (after discharge)	93		↑ AECOPD vs. stable ($p<0.001$)			
IL-8	**USA**	2009	(33)	AECOPD-recovery	71 expectorated sputum samples during 26 hospitalisations (19 patients).	Day 0-3, day 3-8 days and days 8-12 days.	19		↑ AECOPD vs. 1wk stable ($p = 0.01$) but not significantly decreased from measurement 2- 3.	↔AECOPD vs. 1wk & not significantly decreased from measurement 2- 3. (plasma)		
Chitinase 3 like protein	China	2016	(87)	AECOPD vs stable COPD vs Healthy controls	AECOPD (n=37) Stable (n=44) Controls (n=47)	N/A	128			↑ AECOPD vs. stable ($p= 0.0005$) (serum)		

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
MMP-8	Finland	2008	(88)	AECOPD - recovery. healthy non smokers healthy smokers stable COPD	AECOPD (n=10) recovery (n=8) Healthy NS (n=32) Healthy S (n=28) stable (n=15)	Day 1, 4 weeks	85		↑ AECOPD - Recovery (p=0.04) ↑ AECOPD - stable (p < 0.001) ↑ AECOPD - controls (p<0.0001)			
MMP-9	Poland	2012	(89)	Stable- AECOPD + Control group	COPD (n=17) Asymptomatic smokers (n=22)	N/A	17	↑ AECOPD vs. stable p<0.005		↑ AECOPD vs. stable p<0.005		
MMP-9	UK	2005	(90)	Stable- AECOPD	Stable (n=12) AECOPD (n=19)	Pre-Stable sample taken 2-8 months	19	↑ AECOPD vs. stable p <0.01		↑ AECOPD vs. stable p <0.01 (serum)		
MMP-9	Switzerland	2015	Papakonstantinou	AECOPD vs stable COPD	AECOPD (n=44) stable (n=53)		97					↑ AECOPD vs. stable P=0.012)
MMP-9	UK	2016	(91)	AECOPD - recovery		Day 1, day 5-7 and 4 weeks				↑ AECOPD – recovery (not MMP-8 or urinary MMP-8/9) (serum)		
HNE	Finland	2008	(88)	AECOPD - recovery. healthy non-smokers healthy smokers stable COPD	AECOPD (n=10) recovery (n=8) Healthy NS (n=32) Healthy S (n=28) stable (n=15)	Day 1, 4 weeks	85		↑ AECOPD - Recovery (p=0.03) ↑ AECOPD - stable (p < 0.001) ↑ AECOPD - controls (p<0.0001)			
HNE	**USA**	2009	(33)	AECOPD-recovery	71 expectorated sputum samples during 26 hospitalisations (19 patients).	Day 0-3, day 3-8 days and days 8-12 days.	19		↑ AECOPD vs. 1wk stable (p = 0.05) & decreased from measurement 2- 3 (p=0.05)			
NGAL	Turkey	2014	Gumus,	AECOPD - recovery + healthy controls	AECOPD (n=30) Controls (n=20)	day 1 (within 24hrs) and day 70	50			↑ AECOPD – recovery p<0.001		
MPO	Sweden	2015	(92)	Stable- AECOPD + controls	Smokers, 38/60 had at least 1 AECOPD Asymptomatic smokers (n=10) Never smokers (n=10)	over 60 weeks (every 15 weeks)	60			↑ AECOPD (p<0.01). (+neutrophils and CRP, not HNE)		
MPO	China	2014	(28)	AECOPD- stable	non-frequent (n=78) frequent (n=57)	Day 0, 4, 7, 14, week 8 (after discharge)	135		↑ AECOPD vs. stable			

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
MPO	China	2014	(85)	AECOPD- stable	Resolved day 4 in sputum and day 14 serum	Day 0, 4, 7, 14, week 8 (after discharge)	93		↑ AECOPD - vs. stable (p=<0.001)			
A1AT	Germany	2012	(93)	AECOPD vs stable + healthy controls	AECOPD (n=18) Stable (n=17) Healthy (n=10)	N/A	45	↑ AECOPD vs. stable (p=0.00003)		↑ AECOPD vs. stable (p=0.04) (serum)		
TIMP-1	UK	2005	(90)	Stable- AECOPD	Stable (n=12) AECOPD (n=19)	Pre-Stable sample taken 2-8 months	19	↑ AECOPD vs. stable p <0.01		↔ AECOPD vs. stable (serum)		
TIMP-1	Poland	2012	(89)	Stable- AECOPD + Control group	COPD (n=17) Asymptomatic smokers (n=22)	N/A	17	↑ AECOPD vs. stable p<0.005		↔ AECOPD vs. stable		
TIMP-1	Switzerland	2015	(94)	AECOPD vs stable COPD	AECOPD (n=44) stable (n=53)		97					↑ AECOPD vs. stable P=0.028)
TIMP-2	Switzerland	2015	(94)	AECOPD vs stable COPD	AECOPD (n=44) stable (n=53)		97					↑ AECOPD vs. stable P=0.030)
Cystatin C	China	2016	(95)	AECOPD - stable. + healthy controls	AECOPD (n=90) Controls (n=90)	day 0, day 10 (8-13 days) and convalescent	180			↑ AECOPD vs. stable (p<0.01) ↑ AECOPD vs. controls (p<0.01) ↑ stable vs. controls (p<0.001)		
Desmosine	Egypt	2014	(96)	AECOPD - stable		Day 0 and month 1	20			↑ AECOPD vs. stable (p<0.001) (Blood - not clear if serum, plasma or whole blood).		
Desmosine	UK	2011	(97)	GP1: Stable COPD vs AECOPD vs Healthy controls vs stable asthma GP2: Healthy volunteers, COPD	Stable (n=53) AECOPD (n=105) Control non-smokers (n=26) control smokers (n=20) Stable Asthma (n=53) Healthy (n=81), COPD (n=105)	N/A	204			↔ AECOPD vs. stable COPD	↑ AECOPD vs. all other groups (p<0.001)	
Desmosine	Italy	2002	(98)	AECOPD (Mod/severe)- stable	N/A	Within 24hrs, day 3-5 and 2 months	9				↑ AECOPD vs. stable (p=0.049) (no difference from day 0 to day 5).	

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
Desmosine	USA	2012	(99)	AECOPD-recovery	155 spot urine samples from 53 patients during 63 exacerbations.	Day 0-3, day 3-8 days and days 8-12 days.	53				↑ AECOPD vs. stable (SG adjusted and non-adjusted p =0.01). No correl with sputum DES. SG used instead of creatine as Ucr concentration significantly decreases during hospilisations.	
CC-16	Canada	2016	(100)	AECOPD-recovery		Day 0, 15, 30, 90, 180	38			↓ AECOPD vs day 15 (same as RelB, whereas higher levels of SPD during AECOPD)		
CC-16	Italy	2007	(101)	AECOPD	GOLD2 (n=10) GOLD3 (n=10)	N/A	20		↓GOLD3 vs GOLD2 (p=0.027)			
CRP	UK	2011	(14)	Stable-AECOPD.	Predominantly GOLD2,3. 182 Exacerbations	1yr and at AECOPD	145			Association with bacterial AECOPD AUC-0.65 (serum)		
CRP	Sweden	2015	(92)	Stable- AECOPD + controls	Smokers, 38/60 had at least 1 AECOPD Asymptomatic smokers (n=10) Never smokers (n=10)	over 60 weeks (every 15 weeks)	60			↑ AECOPD (p<0.001). (+neutrophils and MPO, not HNE) (serum)		
CRP	Turkey	2015	(102)	AECOPD - stable	N/A	Day 0, 7	43			↑ AECOPD vs. stable (p=0.001) (serum)		
CRP	China	2014	(28)	AECOPD- stable.	non-frequent (n=78) frequent (n=57)	Day 0, 4,7,14 week 8 after discharge	135			↑ AECOPD vs. stable (serum)		
CRP	China	2014	(85)	AECOPD - stable	Resolved day 4 in sputum and day 14 serum	Day 0, 4, 7, 14, week 8 (after discharge)	93			↑ AECOPD vs. stable (p=<0.001) (serum)		
CRP	Egypt	2014	(103)	AECOPD - stable	N/A	day 0 and 12 months	98			↑ AECOPD vs. stable (p<0.001) (serum)		
CRP	China	2014	(104)	AECOPD vs stable	AECOPD (n=27) Stable (n=26)	N/A	54			↑ AECOPD vs. stable (Plasma)		
CRP	Greece	2014	(105)	AECOPD - stable	90 patients	Day 0, 7	90			↑ AECOPD vs. stable (p<0.0001) (Serum)		
CRP	Austria	2014	(106)	AECOPD - stable		Day 0, week 8	29			↑ AECOPD vs. stable (p<0.025) (Plasma)		

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
CRP	China	2014	(107)	AECOPD - stable		Day 0 and 12-17 days (discharge)	44			↑ AECOPD vs. stable (p<0.01) (Serum)		
CRP	Australia	2008	(108)	AECOPD - stable	Cross-sectional		62			↑ AECOPD vs. stable (p<0.01) (Serum)		
CRP	Australia	2008	(108)	Stable-AECOPD-recovery	Longitudinal 78 episodes in 37 individuals		37			Predicting severe AECOPD using the ratio of AECOPD onset to stable baseline values for each inflammatory marker AUC of 0.71 (95% CI, 0.56–0.87)		
CRP	UK	2013	(109)	Stable-AECOPD-recovery	98 patients, 55 with recovery samples	Days 3, 7, 14, and 35	98			↑ AECOPD vs. stable (p<0.001) (Serum)		
CRP	China	2016	(110)	AECOPD vs stable + healthy controls	AECOPD (n=40) Stable (n=71) Controls (n=60)		171			↑ AECOPD vs. stable (p<0.001) (Serum)		
CRP	Chile	2012	(111)	Stable-AECOPD.	70 patients, 120 exacerbations	day 1, 15, 30	70			↑ AECOPD vs. stable (p<0.0001) (Serum)		
CRP	China	2016	(95)	AECOPD - stable. + healthy controls	AECOPD (n=90) Controls (n=90)	day 0, day 10 (8-13 days) and convalescent	180			↑ AECOPD vs. stable (p<0.001) ↑ AECOPD vs. controls (p<0.001) ↑ stable vs. controls (p<0.001)		
CRP	Germany	2012	(93)	AECOPD vs. stable COPD vs healthy controls	AECOPD (n=18) Stable (n=17) Controls (n=10)		45			↑ AECOPD vs. stable (p<0.003) (Serum)		
Periostin	Greece	2017	(112)	AECOPD - recovery		day 1 and discharge	155			↑ AECOPD vs. stable (p<0.0003) (Serum)		
RBP-4	China	2013	(113)	AECOPD vs. stable vs. healthy controls	AECOPD (n=100) Stable (n=46) Controls (n=50)		196			↓ AECOPD vs stable and controls (p<0.001) Gender differences, ↓predictor of mortality		
Fibrinogen	Turkey	2015	(102)	AECOPD - stable		Day 0, 7	43			↑ AECOPD vs. stable (p<0.001) (Serum)		
Fibrinogen	Egypt	2014	(103)	AECOPD - stable	N/A	day 0 and 12 months	98			↑ AECOPD vs. stable (p<0.001) (Serum)		
Fibrinogen	China	2014	(107)	AECOPD - stable		Day 0 and 12-17 days (discharge)	44			↑ AECOPD vs. stable (p<0.01) (plasma)		

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
Fibrinogen	UK	2013	(109)	Stable-AECOPD-recovery	98 patients, 55 with recovery samples	Days 3, 7, 14, and 35	98			↑ AECOPD vs. stable (p<0.015) (Serum)		
Fibrinogen	Chile	2012	(111)	Stable-AECOPD.	70 patients, 120 exacerbations	day 1, 15, 30	70			↑ AECOPD vs. stable (p<0.0002) (Serum)		
sRAGE	Austria	2014	(106)	AECOPD - stable		Day 0, week 8	29			↓AECOPD vs. recovery (p<0.001)		
sRAGE	China	2014	(107)	AECOPD - stable		Day 0 and 12-17 days (discharge)	44			↑ AECOPD vs. recovery (p<0.03)		
pneumococcal urinary antigen	Spain	2010	(67)	AECOPD vs. stable	AECOPD (n=17) Stable (n=29) (Pneumococcal exacerbations)		46				↑13/17 AECOPD (77% sensitivity) ↑12/ 29 stable (59% specificity) (conc. urine)	
Cts-LTs	Poland	2012	(39)	AECOPD-recovery		Day 1, 2-4 (during therapy), 4-6 (end of therapy, 21-28 (follow up)	16	↑AECOPD vs. recovery (p<0.02)				
Leukotriene B_4 (LTB_4)	Poland	2012	(39)	AECOPD-recovery		Day 1, 2-4 (during therapy), 4-6 (end of therapy, 21-28 (follow up)	16	↑AECOPD vs. recovery (p<0.003)				
Leukotriene B_4 (LTB_4)	UK	2003	(114)	COPD vs healthy controls	COPD- no steroid treatment (n=20) COPD – steroid treated (n=25) Controls (n=15)	N/A	60	↑COPD (both groups vs. controls (p<0.001)				
Leukotriene B_4 (LTB_4)	UK	2003	(115)	AECOPD-recovery		Day 0, 2 weeks and 2 months	21	↑AECOPD vs. recovery (p<0.0001)				
Leukotriene B_4 (LTB_4)	Hungary	2014	(116)	AECOPD-recovery + stable ex-smokers	AECOPD (n=62) Control (n=25)	day 1 at admission and after treatment.	62		↑AECOPD vs. stable (p<0.05)			
Prostaglandin E_2 (PGE_2)	Poland	2012	(39)	AECOPD-recovery		Day 1, 2-4 (during therapy), 4-6 (end of therapy, 21-28 (follow up)	16	↑AECOPD vs. recovery (p<0.004)				
Prostaglandin E_2 (PGE_2)	Hungary	2014	(116)	AECOPD-recovery + stable ex-smokers	AECOPD (n=62) Control (n=25)	day 1 at admission and after treatment.	62		↑AECOPD vs. stable (p<0.001) ↑AECOPD vs. recovery (p<0.01)			

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
Prostaglandin E_2 (PGE_2)	UK	2003	(114)	COPD vs healthy controls	COPD- no steroid treatment (n=20) COPD – steroid treated (n=25) Controls (n=15)	N/A	60	↑COPD (both groups vs. controls (p<0.001)				
8 Isoprostane	Poland	2012	(39)	AECOPD-recovery		Day 1, 2-4 (during therapy), 4-6 (end of therapy, 21-28 (follow up)	16	↑AECOPD vs. recovery (p<0.001)				
8 Isoprostane	UK	2003	(115)	AECOPD-recovery		Day 0, 2 weeks and 2 months	21	↑AECOPD vs. recovery (2wks) (p<0.0001)				
8 Isoprostane	Hungary	2014	(116)	AECOPD-recovery + stable ex-smokers	AECOPD (n=62) Control (n=25)	day 1 at admission and after treatment.	62		↑AECOPD vs. stable (p<0.01)			
H_2O_2	Poland	2012	(39)	AECOPD-recovery		Day 1, 2-4 (during therapy), 4-6 (end of therapy, 21-28 (follow up)	16	↑AECOPD vs. recovery (p<0.001)				
H_2O_2	Netherlands	2004	(86)	AECOPD-recovery		Day1, 3, 7	14	↑AECOPD vs. recovery (p<0.001)				
CXCL10	UK	2011	(14)	Stable-AECOPD.	Predominantly GOLD2,3. 182 Exacerbations	1yr and at AECOPD	145			Association with bacterial AECOPD AUC-0.76 (serum)		
sICAM-1	Netherlands	2004	(86)	AECOPD-recovery		Day1, 3, 7	14			↑AECOPD vs. recovery (p<0.001) (serum)		
IL-10	Germany	2005	(84)	AECOPD - recovery	ICU n= 11 General ward n = 34	ICU after 10 days GW after 1 week	45	↑AECOPD vs. all groups				
IL-12p70	Germany	2005	(84)	AECOPD - recovery	ICU n= 11 General ward n = 34	ICU after 10 days GW after 1 week	45	↑AECOPD vs. all groups				
Neutrophils	Sweden	2015	(92)	Stable- AECOPD + controls	Smokers, 38/60 had at least 1 AECOPD Asymptomatic smokers (n=10) Never smokers (n=10)	over 60 weeks (every 15 weeks)	60			↑ AECOPD (p<0.01). (+MPO and CRP, not HNE)		
Neutrophils	China	2014	(28)	AECOPD- stable	non-frequent (n=78) frequent (n=57)	Day 0, 4, 7, 14, week 8 (after discharge)	135		↑AECOPD vs. stable			

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
Neutrophils	China	2014	(85)	AECOPD - stable	Resolved day 4 in sputum and day 14 serum	Day 0, 4, 7, 14, week 8 (after discharge)	93		↑AECOPD vs. stable (p=<0.001)			
Neutrophils	Austria	2014	(106)	AECOPD - stable		Day 0, week 8	29			↑AECOPD vs. stable (p<0.004)		
Neutrophils	China	2014	(107)	AECOPD - stable		Day 0 and 12-17 days (discharge)	44			↑AECOPD vs. stable (p<0.01)		
Neutrophils	Egypt	2014	(103)	AECOPD - stable	N/A	day 0 and 12 months	98			↑AECOPD vs. stable (p<0.001) (serum)		
Serum soluble urokinase-type plasminogen activator receptor (suPAR)	Turkey	2015	(102)	AECOPD - stable		Day 0, 7	43			↑AECOPD vs. stable (p<0.001) (serum)		
Microfibrillar-associated protein-4	Denmark	2014	(117)	stable - AECOPD	AECOPD (n=14)	day 0 and day 1, 3-5, 6-8, 9-11, 4 weeks, 3 months and 6 months	69			↓AECOPD vs. stable week 4 (p<0.00001) (plasma)		
Surfactant Protein-D (SP-D)	Denmark	2014	(117)	stable - AECOPD	AECOPD (n=14)	day 0 and day 1, 3-5, 6-8, 9-11, 4 weeks, 3 months and 6 months	69			↑AECOPD vs. stable week 4 (p<0.008) (serum)		
Surfactant Protein-D (SP-D)	China	2016	(110)	AECOPD vs stable + healthy controls	AECOPD (n=40) Stable (n=71) Controls (n=60)		171			↑AECOPD vs. stable (p<0.001) (serum)		
Osteopontin	Korea	2013	(118)	AECOPD vs stable	AECOPD (n=64) Stable (n=68)		132			↑AECOPD vs. stable (p<0.001) (plasma		
Adrenomedullin (ADM)	China	2014	(119)	AECOPD-recovery vs stable vs healthy controls	AECOPD (n=79) Stable (n=29) Controls (n=20)	Day 0 and follow up 6 months	207			↑AECOPD vs. stable/controls (p<0.001, AUC 0.97) ↑AECOPD vs. recovery (p<0.01) (plasma)		
Angiopoietin-2 (Ang-2)	Greece	2014	(105)	AECOPD - stable		Day 0, 7	90			↑AECOPD vs. stable (p<0.0001) (serum)		
B-type natriuretic peptide (BNP)	Japan	2014	(120)	Stable-AECOPD-recovery	Exacerbations (n=87) Included patients (excluded those with readmissions) n=43 All 3 timepoints (n=15)	Day 1, day 10-14, 30 days post discharge.	61			N= 43: ↑AECOPD vs. recovery (p<0.001) N=15: ↑AECOPD vs. stable (p=0.0033) ↑AECOPD vs. recovery (p=0.0013)		

Biomarker	Country	Year	Ref	Study detail	Notes	Follow up visits	N	EBC	Sputum	Blood	Urine	BAL
Adiponectin	Egypt	2014	(121)	AECOPD vs. Stable	Male only AECOPD (n=40) stable (n=15)		55			↑AECOPD vs. stable (p<0.05) (serum)		
Adiponectin	Egypt	2014	(122)	AECOPD vs. Stable vs. controls	Male only. AECOPD (n=34) Stable (n=34) (non-obese (n=17), obese (n=17)) Controls-non-obese (n=10) Controls-obese (n=10)		88			↑AECOPD vs. stable (p<0.001) (serum)		
High mobility group protein B1 (HMGB1)	hhhji	2014	(107)	AECOPD - stable	Day 0 and 12-17 days (discharge)		44			↑AECOPD vs. stable (p<0.01) (plasma)		
Aalpha-Val360	UK	2013	(123)	stable - exacerbation. Seen at each timepoint. N=40 studied over 4 years			81			↑AECOPD vs. stable (p=0.036) (plasma)		
Serum Amyloid A	Australia	2008	(108)	AECOPD - stable	Cross-sectional		62			↑AECOPD vs. stable (p<0.01) (serum)		
Serum Amyloid A	Australia	2008	(108)	Stable-AECOPD-recovery	Longitudinal 78 episodes in 37 individuals		37			Predicting severe AECOPD using the ratio of AECOPD onset to stable baseline values AUC of 0.84 (95% CI, 0.74–0.94)		
Procalcitonin	USA	2012	(124)	AECOPD - stable		Day 1, day 2 and 1 month	224			↑AECOPD vs. stable (serum) (more severe illness and maybe indicative of pneumonia)		
Procalcitonin	Spain	2011	(125)	AECOPD vs. stable vs pneumonia	AECOPD (n=217) Stable (n=46) Pneumonia (n=55)	Day 1 and 1 month for n=23 AECOPD events	318			↑AECOPD vs. stable (p<0.0001) (serum)		
Procalcitonin	Egypt	2012	(126)	AECOPD vs. controls	Bacterial AECOPD (n=20). Non-bacterial AECOPD (n=30) Healthy controls (n=10)	Day 1 and recovery	60			↑bacterial AECOPD vs. stable (p<0.001) (serum)not significantly different with non-bacterial infection group		

1.14.1 Exhaled Breath Condensate

Exhaled breath consists of two components. The first 150 ml is “dead-space” air from the upper airway in which no gaseous exchange between blood and air occurs. The remaining 350 ml, known as “alveolar” breath, comes from the lungs, where gaseous exchange between blood and air occurs. EBC is saturated with water vapour which can be condensed by cooling and used to sample a wide range of biomarkers (127), the collection of which is a non-invasive method that reflects biochemical changes in the airway lining fluid (77). Commonly used EBC methods are shown to have considerable variability, due to technical issues concerning both sample collection (pattern of breathing, collection device, effect of oral contamination), and analysis (76). There is also limited data regarding the repeatability and stability of the samples (77).

Markers of oxidative stress: H_2O_2 is generated via non-enzymatic and enzymatic dismutation of superoxides in the upper and lower airways. In healthy individuals H_2O_2 production is ongoing oxygen reduction process that occurs due to electron transport in mitochondrial respiration (128). As described in section 1.8 an imbalance occurs in COPD and COPD exacerbations. Most of the published studies have reported elevated levels of H_2O_2 with a clear discrimination between healthy subjects and COPD patients H_2O_2 is not a specific biomarker for the disease but is further elevated during exacerbations (39, 86).

Leukotrienes and prostaglandins: Leukotriene B_4 (LTB_4) is produced by constitutive cells (eg, mast cells and macrophages) and infiltrating cells (eg, neutrophils and eosinophils). LTB_4 (a leukotriene) has no direct action on airway smooth muscle, but it may contribute to bronchoconstriction by increasing vascular permeability and mucus secretion. It is considered as one of the main mediators responsible for neutrophil recruitment. Increased levels of LTB_4 have been reported in stable COPD (114) with further increase during exacerbations (77). Reviewed are three studies that show increased levels of LTB_4 in exacerbation state compared to the recovery samples (39, 115, 116). PGE_2 (a prostaglandin) on the other hand is an airway smooth muscle relaxant and is likely to have bronchoprotective and anti-inflammatory actions (116). Increased levels of PGE_2 have been reported in stable COPD (114) with further increase during exacerbations (39).

- 8-Isoprostane: Isoprostanes are prostaglandin-like compounds formed by the free-radical lipid peroxidation of arachidonic acid and represent in vivo markers of oxidative stress (77). Concentrations of 8- isoprostane are greater in patients with COPD than in normal smokers, are related to disease severity and are further increased during exacerbations (39, 115)

- Cytokines (and chemokines): increased levels of TNF-α, IL-6, IL1β, IL-10, IL-12p70 and IL-8 are all reported biomarkers found in exacerbation (84).

- Increased levels of Matrix Metalloproteinase (MMP) have been found in exacerbation compared to stable COPD (89, 90).

- Increased levels of the protease inhibitors, alpha-1 Antitrypsin (A1AT), tissue inhibitor of metalloproteinase-1 (TIMP-1) have been found in exacerbation compared to stable COPD (89, 90, 93) .

1.14.2 Biomarkers in Sputum

Collection and analysis commonly used are semi-invasive. In spontaneous sputum, there are large quantities of dead cells, thereby induced sputum is a more reliable method, however, this induces a local inflammatory response (76) therefore repeated inductions within a 48 hour period is recommended (75). Processing the sputum sample which is variable between different laboratories is expensive, time consuming and require specialised equipment and trained personnel. Mucolytic agents such as dithiothreitol (DTT) or dithioerythritol (DTE) are commonly used to assist in homogenising the sample by breaking the disulphide bonds in the mucin molecules (75), this has been shown to affect the measurement of mediators, require addition of BSA for example and further processing. The lack of a validated dilution factor may lead to incomparable measurements among authors (129). Finally, it is not a pleasant experience for the patient, compliance is poor and sputum induction should not be performed in patients with severe persistent bronchoconstriction and/or co-morbidities such as cardiovascular disorders (129).

Inflammatory cytokines, including IL-8, IL-6 and TNF-α have been extensively studied and reported to be increased in sputum of COPD patients compared to normal smokers and their concentrations are related to the severity of the disease and further increased with exacerbations (83, 84). TNF-α is a potent activator of the nuclear factor kappa B (NF-kB), and this might amplify the inflammatory response (4). IL-8, is a major important chemokine and chemoattractant of neutrophils, produced and released by neutrophils, alveolar macrophages, and other activated cells. IL-8 induces the release of myeloperoxidase (MPO) from neutrophils and contributes to further recruitment of inflammatory cells, helping to sustain inflammation. IL-1β markedly activates macrophages from patients with COPD to secrete inflammatory cytokines, chemokines, and MMP-9. There is an increase in the concentration of IL-1β in sputum of COPD, which is correlated with disease severity (84). IL-6 is increased in sputum of patients with COPD, particularly during exacerbations. Plasma

concentrations of IL-6 are correlated with increased levels of CRP, and IL-6 is the major mechanism that stimulates CRP release from the liver (9).

Other biomarkers found elevated in exacerbations are neutrophils and neutrophil granulocyte proteins such as MPO, MMP-8, human neutrophil elastase (HNE) (28, 88). In the Ilumets study, MMP-9 was also studied and shown to be significantly higher in exacerbation compared to stable COPD and control samples but did not reach significance for the 1-month recovery sample. This is most likely due to the small sample size (10 AECOPD and 8 recovery), MMP-8 and HNE only just met the criteria with p values of 0.04 and 0.03 respectively. The biological role of the proteins is described in more detail in section 1.10.

In sputum, LTB_4 and PGE_2 and 8-isoprostane were found in elevated concentrations in COPD exacerbations compared to recovery samples (129). These were also found elevated in EBC and the roles of which have been described in more detail above.

1.14.3 Biomarkers in BAL

In BAL fluid, metabolites deep in the tissue at the level of bronchioles and alveolar ducts are sourced. BAL can generally be safely performed and is an advantage over bronchial biopsies, however, it may cause discomfort to the patients (78) and bronchospasm, mild fever and transient asymptomatic pulmonary infiltrates are occasional complications (75). This makes this sample matrix not suitable for large studies and is certainly not for frequent collection. Induction of BAL fluid samples, this is reflected by the limited published studies. In a study conducted by Papakonstantinou *et al* (94) the results indicate that during exacerbations there are increased expression of TIMP-1, TIMP-2 and MMP-9

1.14.4 Plasma/serum/blood biomarkers

The most studied and consistent blood biomarkers are CRP, IL-6, Fibrinogen and neutrophils, and there are a large number of studies providing evidence that the biomarker levels are elevated in stable COPD and in exacerbation. Even so, they are still not accepted biomarkers for routine monitoring of the disease state and in the reviewed studies, although they are longitudinal, the frequency of testing in stable state is low. Most patients were recruited at exacerbation and then tested frequently over 2 weeks and then one month after discharge. However, data from samples taken during the lead up to an exacerbation are rare. The biomarkers have been used to differentiate between different phenotypes (see section 1.5), disease severity and mortality but have not been evaluated for prediction of exacerbations. Frequent exacerbators have higher sputum

IL-6 and serum CRP during their exacerbation recovery periods, making persistent post-exacerbation inflammation a possible explanation for their higher baseline inflammation. A higher CRP concentration during the recovery period is also associated with a shorter time until the next exacerbation (130).

The next group of biomarkers that have been widely studied with consistently reported elevated levels in exacerbations compared to recovered or stable state are TNF-α, IL-8, MMP-9, Neutrophil gelatinase-associated lipocalin (NGAL), MPO, and A1AT. The relevance of these biomarkers has been reported previously in line with findings in EBC, sputum and BAL fluid.

sRAGE, High mobility group protein B1 (HMGB1), CC16 and SP-D have been found to be associated with exacerbations with supporting evidence.

sRAGE and HMGB1 were found to be increased in exacerbation compared to recovery state (Zhang et al 2014). HMGB1 is a protein that acts as a cytokine when released by interacting with at least three receptors, one of them being RAGE, this signalling results in generation of ROS and activation of the transcription factor NF-kB. Fibrinogen and CRP were also significantly lower in recovery compared to exacerbation. The finding that sRAGE was higher in exacerbation is not consistent with other studies that show decreased levels in exacerbation (106). It is clear that a better understanding of these two biomarkers is required before they can be deemed useful in prediction of exacerbations. In a review of all the eclipse studies, more than 50 publications were found to report that circulating sRAGE levels were associated with the severity of emphysema (lower levels) (131). Other proteins measured, from the above list were CC16 (found to be weakly associated with lung function decline, emphysema and depression) and SP-D (which had a weak association with COPD exacerbations) (131). Both CC16 and SP-D are specific lung-derived mediators, CC16 is secreted by non-ciliated bronchiolar club cells with reduced expression with lung injury and smoking (132), whereas SP-D is produced primarily by type II pneumocytes, and is thought to play a role in innate immunity and regulation of surfactant homeostasis in the lung. As the airways become more permeable due to injury, these lung-specific mediators can escape and be detected in the peripheral circulation (100). It has been demonstrated in a study that low levels of CC16 and high levels of SP-D are found at exacerbation that increase and decrease at day 15 respectively (100). It was observed that by day 30, SP-D increased back up the same levels at exacerbation which is consistent with a study by Johansson where the same observations were repeated, a decrease up to days 9-11 and then at day 30 was increased and remained stable until 6-month follow-up (117).

COPD biomarkers associated with exacerbations with limited supporting evidence are Chitinase -3 like 1 protein (CHI3L1), Cystatin C, Periostin, RBP4, sICAM, Serum soluble urokinase-type plasminogen activator receptor (suPAR), Osteopontin, Adrenomedullin (ADM), Angiopoietin-2 (Ang-2), B-type natriuretic peptide (BNP), Adiponectin, Alpha-Val360 and Serum Amyloid A.

CHI3L1 is known to play a role in tissue remodelling but there is not a lot known of the role it plays in COPD. It is believed that it may induce the production of MMP-9 in other diseases (87). It is reported that there are elevated levels of CHI3L1 in COPD exacerbations compared to stable COPD, but limitations of this study are that the exacerbation and stable samples were not matched (87). Circulating levels of Cystatin C were found to be increased at exacerbation compared to recovery state and healthy controls (95). Cystatin C is a cysteine protease inhibitor and protects against elastolysis and tissue destruction. To further explain, as it has not been mentioned previously, proteases (secreted by alveolar macrophages and neutrophils) are classified into 4 groups, serine, metallo (MMPs that have been discussed), cysteine and aspartic. The Cathepsin family belongs in the cysteine group which degrades lung extracellular matrix, especially lung elastin which sets off a chain of events. Cystatin C is upregulated indirectly in response to the need to inhibit cathepsin activity. When there is an imbalance between proteases and their inhibitors the resulting state is considered to be pathogenic, leading to an increase in the in severity of the disease (95).

There were conflicting results regarding the trends and significance for MMP-8, desmosine and procalcitonin indicating that the relevance of these markers in blood and exacerbations is not strong or consistent or that the studies are poorly designed or executed.

1.14.5 Biomarkers in urine

Desmosine is the only COPD urinary biomarker to have received substantial attention. It has been found to be elevated in people with COPD above the concentrations observed in healthy controls (133).

Proteases such as HNE and MMP-12 are two key inflammatory elastases contributing to the pathogenesis underlying COPD (134-136). They are released by activated neutrophils and alveolar macrophages respectively. Excess leukocyte activity is a critical driver of exacerbation (135) and is thought to contribute particularly to parenchymal lung damage by way of breakdown of elastin. Peptides are released from elastin during the degradation process, the rate of release from a particular anatomical site being, in theory, directly proportional to the elastase activity at that site.

Mature elastin fibres are held together by desmosine and isodesmosine (together abbreviated as DES) working as covalent crosslinkers between the elastin polypeptide chains (137). When the

elastin is degraded, DES-containing elastin fragments are excreted from the kidney into the urine. Free DES is also passed into the urine, as well as variously sized peptide fragments with DES still attached (137). The amount of elastin-derived peptides accumulating in the urine may give an indication of how much leucocyte-driven tissue damage is occurring within the body at the time of testing.

Aside from desmosine, pneumococcal urinary antigen was reported in the reviewed literature, with a sensitivity of 77%. However, the results did not suggest that this marker would be useful in predicting pneumococcal-driven exacerbations as the specificity was very poor at just 59% (67).

1.15 Urine, a non-invasive method of monitoring COPD status

Most focus to date has been on blood biomarkers, which makes sense as it is connected to every part of the body but, although promising blood biomarkers have been identified, no blood-borne biomarkers have been adopted for diagnostic testing. This lack of uptake begs the question of whether, in fact, blood biomarkers are the right focus for biomarker discovery. It has been suggested that, as all cells rely on a homeostatic microenvironment to survive and function, the blood in which they are bathed is the key provider of an internal environment for preserving the health and performance of all tissues and organs. It is naturally responsible for maintaining the stability and balance required to protect organs (138) and that, as a result, potentially harmful substances introduced into the blood tend to be cleared by the liver, kidney and /or other organs via a variety of mechanisms to maintain homeostasis. The presence of particular products in urine, is likely to reflect physiological or pathophysiological changes more accurately than their presence in blood. In fact, urinary marker profiles can be expected to magnify any such changes that might have occurred fleetingly in blood prior to the molecules being cleared through the kidney. It has been demonstrated that more proteins can be identified in urine than in plasma (139). Our hypothesis is that the kidneys also produce elevated levels of protease inhibitors to protect the organ from destructive inflammatory factors, such as active proteases in the blood. This would also cause amplification of certain biomarkers in the urine. Despite the fact that urine might be a better biomarker source, it is also speculated that changes in urine are complex and that other nonspecific factors would influence the levels of the biomarkers, so it would be difficult to determine the root cause (140). The early and sensitive markers are believed to be present in urine in particular in early stage disease when the homeostatic mechanisms are strong and effective (141). It has been suggested that this could explain why prediction of early disease diagnosis has not been achieved when looking at blood biomarkers. The homeostatic mechanisms have already been impaired when the biomarkers appear in the blood and they are not easily cleared in the blood (141). Proteolytic

degradation may be complete prior to collection of urine and, because proteases are activated during blood collection, the urinary proteome shows much greater stability than that of plasma (139).

Profiling inflammatory mediators in urine samples presents a simple and robust measure of inflammation in COPD patients and can be done repeatedly within their own home or in the clinic. Urine samples can be collected over a long period of time, allowing for an easier monitoring of time-dependent changes in biomarker levels (139). Urinary biomarker screening could be adopted into current COPD and primary care reviews. In COPD patients at high risk of frequent exacerbations the urinary biomarkers can be tested more frequently and changes in the inflammatory profile can be used as an early warning of an exacerbation onset and also help to determine the underlying biology. This early signal of an exacerbation and, potentially. a predictor of likelihood of response to antibiotics and or oral corticosteroids will enable the patient, together with the health care provider, to improve decision making for when to treat versus not and which therapy to use. This stratification will reduce risk of adverse events associated with antibiotics and corticosteroids on an individual basis but also reduce the population risks of inappropriate antibiotic prescribing. It has been reported that approximately 30% of patients seen at hospital with an exacerbation will be seen again and possibly admitted with another exacerbation within 8 weeks (142). In addition to reducing unnecessary therapy this biomarker approach would also ensure early treatment where needed.

1.16 Selected biomarkers for evaluation and molecular weights

The core biomarker panel is shown in table 1.2. TNF-α and MBP are not included, as it has been found in previous studies (not disclosed) that urine does not contain measurable amounts of said biomarkers. The molecular weight cut-off for glomerular filtration is thought to be 30–50 kDa (143) which would rule out the presence of A1AT, Siglec 8, MMP-12 pro form, MMP-13 pro form, MMP-8 active form, Human Serum Albumin(HSA), MMP-2 active form, MMP-2 Pro form, MMP-8 pro form, MMP-9 active form, Periostin, MMP-9 pro form, CRP, MPO and Fibrinogen should this be the case. It is also likely that in urine (and other sample matrices) that fibrinogen and elastin will be broken down into smaller fragments.

Table 1.2. Biomarker panel and molecular weights (kDA) sorted from small to large.

OM. Other molecule; CM, consequence molecules; SM, signalling molecule; NIRM, non-immune response molecule; PI, protease inhibitor; EM, effector molecule.

Group	Analyte	MW kDa
OM	Creatinine	0.113
CM	N-acetyl Proline-Glycine-Proline	0.311
SM	N-Formylmethionine-leucyl-phenylalanine	0.4376
CM	Desmosine	0.5266
SM	Interleukin- 8	8.4
NIRM	Club cell- 16	10
PI	Secretory leukocyte protease inhibitor	11.7
OM	beta 2 Microglobulin	11.7
PI	Cystatin C	13.3
OM	Major Basic protein	13.8
SM	Interleukin 1- beta (IL1β)	17.5
OM	Retinol binding protein-4	20.6
SM	Interleukin-6	21
PI	Tissue inhibitor of metalloproteinase-1	23
EM	Calprotectin	24
PI	Tissue inhibitor of metalloproteinase-2	24.3
EM	Neutrophil gelatinase-associated lipocalin	25
EM	Human Neutrophil Elastase	29
EM	MMP12 active form	29.2
OM	Eosinophil cationic protein	32
SM	Chitinase 3 like protein	40
OM	Soluble receptor for advanced glycation end products	41
EM	MMP13 active form	45
PI	Alpha-1 antitrypsin	52
SM	Siglec 8	54
EM	MMP12 pro form	54
EM	MMP13 pro form	57
EM	MMP-8 active form	65
OM	Human Serum Albumin	66.5
EM	MMP2 active form	67
EM	MMP2 Pro form	72
EM	MMP-8 pro form	75
EM	MMP-9 active form	82
NIRM	Periostin	92
EM	MMP-9 pro form	92
NIRM	C reactive protein	114
EM	Myeloperoxidase	150
OM	Fibrinogen	340

1.17 Aims and hypothesis

The purpose of the research is to explore if and how key biomarkers of lung tissue degradation (caused by neutrophil-driven inflammation) partition into urine, where they can be quantified and used as a new diagnostic tool. The questions to be answered: a) Which biomarkers are released by inflamed lungs and find their way into urine? b) Does molecular size influence the extent to which individual biomarkers in the urine reflect the state of lung inflammation? c) Is it possible to utilise the kidney as a "sentinel" of inflammatory activity elsewhere in the body? d) Are there any patterns in the urinary biomarker profile to indicate which organ/tissue is the source?

The hypothesis is that the biomarkers arise in the urine is as follows:

- As neutrophil leukocytes, in particular, infiltrate the lungs and become activated, large amounts of proteases and other molecular biomarkers are produced and these spill-over into the blood.
- Any active protease in the blood will quickly encounter the kidneys.
- As the kidneys have a copious vascular supply and high blood perfusion rates, they cannot risk any protease-mediated tissue damage, so, they produce their own inhibitor supplies
- During episodes of acute inflammation, the kidneys are affected by the presence of active inflammatory mediators in the blood, causing changes in the molecular permeability of the glomeruli and, consequently, measurable changes in urinary concentration certain biomarker molecules.
- Consequently, kidneys become sentinel organs, releasing molecular messages that warn of impending exacerbation, although the messages need to be de-convoluted.
- Small but measurable amounts of inflammation biomarkers traverse the kidney to become detectable in the urine. The relative amounts depend on molecular size of the biomarkers, so the process of traversing the kidney effectively scrambles the overall biomarker message.

Specifically, this project aims to:

- explore the presence of urinary biomarkers in COPD regarding the presence, influence of gender and age, severity of disease.
- Determine biomarkers in urine that can measure the heterogeneity of the inflammatory profile, predict future risk of exacerbations
- Develop a point of care diagnostic test that can be used repeatedly in the home to monitor the inflammation status and predict pulmonary exacerbations.
  Evaluate the test and biomarker levels in a real-life study and develop a simple algorithm to assess the performance and ability to predict and diagnose exacerbations

Chapter 2. Methods

2.1 Introduction

A shortlist of 35 biomarkers were selected for analysis in urine and blood samples. For most biomarkers, commercially available assays were available, for novel biomarkers, good quality assays were not always available and needed to be developed and subsequently validated for use. Methods for each assay are described in part 1. Two examples of the in-house developed assays (desmosine and active MMP Lateral flow assay) are described in the appendix, both assays required specialised reagents, therefore immunisations were undertaken to generate sheep or rabbit polyclonal antibodies. In part 2, the various statistical methods used for data analysis are described.

Table 2.1. Biomarker panel and assay format (24 commercial (C) and 11 developed in-house (IH))

No	Biomarker	Assay	Analyte (full description)
Signalling molecules			
1	IL-6	ELISA (C)	Interleukin-6
2	fMLP	ELISA (IH) & Lateral flow (IH)	N-Formylmethionine-leucyl-phenylalanine
3	Il-1β	ELISA (C)	Interleukin-1β
4	IL-8	ELISA (C)	Interleukin- 8
5	Siglec 8	ELISA (IH)	Siglec 8
6	CHI3L1	ELISA (C)	Chitinase 3 like protein
Effector molecules			
7	Active MMP	Lateral flow (ultimate ELTABA) (IH)	Active protease (Composite MMP 2,8,9,12,13)
8	Active MMP	Plate assay (substrate assay) (C)	Active protease (Composite MMP 2,8,9,12,13,7)
9	Active HNE	Plate assay (substrate assay) (C)	Active Human Neutrophil Elastase
10	MMP-8	ELISA (C)	Total Matrix Metalloproteinase -8
11	MMP-9	ELISA (C)	Total Matrix Metalloproteinase -9
12	HNE	ELISA (IH)	Human Neutrophil Elastase
13	NGAL	ELISA (C)	Neutrophil gelatinase-associated lipocalin
14	Calprotectin	ELISA (C)	Calprotectin
15	MPO	ELISA (C)	Myeloperoxidase
Protease Inhibitors			
16	A1AT	ELISA (IH) & Lateral flow (IH)	Alpha-1 antitrypsin
17	TIMP-1	ELISA (C) & Lateral flow (IH)	Tissue inhibitor of metalloproteinase-1
18	TIMP-2	ELISA (C) & Lateral flow (IH)	Tissue inhibitor of metalloproteinase-2
19	SLPI	ELISA (IH)	Secretory leukocyte protease inhibitor
20	Cystatin C	ELISA (C)	Cystatin C
Consequence molecules			
21	Ac-PGP	ELISA (IH)	N-acetyl Proline-Glycine-Proline
22	Desmosine	ELISA (IH) & Lateral flow (IH)	Desmosine
23	LEF	ELISA (IH)	Large Elastin Fragments
24	Desmosine Fragments	ELISA (IH)	Desmosine fragments
Non- immune response molecules and other molecules			
25	CC16	ELISA (C)	Club cell- 16
26	CRP	ELISA (C) & Lateral flow (IH)	C reactive protein
27	Periostin	ELISA (C)	Periostin
28	Creatinine	Plate assay (C)	Creatinine
29	B2M	ELISA (C)	beta 2 Microglobulin
30	RBP-4	ELISA (C)	Retinol binding protein-4
31	HSA	ELISA (C)	Human Serum Albumin
32	Fibrinogen	ELISA (C)	Fibrinogen
33	sRAGE	ELISA (IH)	Soluble receptor for advanced glycation end products
34	RNASE-3	ELISA (C)	Eosinophil cationic protein
35	MBP	ELISA (C)	Major Basic protein

2.2 Part 1 Methods for measuring biomarkers in clinical samples

Biotek 405LS Plate washer

Flurostar plate reader (BMG Labtech)

Plate shaker

2.2.1 Commercial DuoSet kits (R&D systems)

2.2.1.1 Materials and reagents

- SigmaFast OPD Substrate composing of:
    - Stable peroxidase buffer [Fisher 11889270]
    - OPD tablet: Sigma 10 mg P8287
    - 1 tablet of each added to 20ml de-ionised water
- Costar high bind clear polystyrene plate: cat # 9018
- R&D Reagent Diluent: R&D Systems DY955 concentrate diluted 1 in 10 with deionised water before use
- Wash buffer (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20)
- Streptavidin-HRP part number 893975

2.2.1.2 Generic method for DuoSet assays (table 2.2 details dilutions used)

The plate was sensitised with capture antibody at working concentration diluted in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (R&D reagent diluent) with 120µl/well for 1hour at room temperature.

Assay running procedure: The specific standard was diluted in the reagent buffer to give the top standard stated in table 2.2. and then serially diluted ½ in reagent buffer to prepare the 7-point standard curve (buffer only for the negative standard). The standard and urine sample (diluted as specified in reagent diluent) was added to the plate 100µl/well after a wash step and incubated for 2 hours at room temperature with gentle agitation. After a further wash step, the detection antibody diluted in reagent buffer to working concentration was added 100µl/well and incubated for 2 hours at room temperature with gentle agitation. After a further wash step, the streptavidin HRP conjugate diluted 1/200 in reagent diluent was added 100µl/well and incubated for 20 minutes at room temperature with gentle agitation After the final plate wash, the colour reaction was initiated with the addition of 100µl of OPD substrate to each well and incubated in the dark. Once colour had been allowed to develop (approximately 30 minutes), the absorbance was measured at 450nm using an Omega plate reader and the standard curve was approximated in a sigmoid 4 parameter logistic model.

Table 2.2. DuoSet details, catalogue number, reagent dilutions (capture, detection and standard) and sample dilutions for both blood and urine. Mouse Anti-Human (MAH), Goat anti-Human (GAH)

Assay	Cat. No	Capture antibody		Detection antibody		Standard		Sample dilution	
		Part. No	Working conc µg/ml (dilution) GAH or RAH	Part. No Biotinylated Goat Anti-Human	Working conc µg/mL (dilution)	Part. No	Working conc µg/ml (dilution)	urine	blood
IL -6	DY206	MAH 840113	2µg/ml	840114	50ng/ml	840115	9.38 - 600pg/ml	1:2	1:2
IL-1β	DY201	MAH 840168	4µg/ml	840169	200ng/ml	840170	3.91 - 250pg/ml	1:2	1:2
IL-8	DY208	MAH 890804	4µg/ml	890805	20ng/ml	890806	31.3 - 2000pg/ml	1:2	1:2
CHI3L1	DY2599	RAH 842869	2µg/ml	842870	200ng/ml	842871	31.25 - 2000 pg/ml	1:10	1:100
MMP-8	DY908	MAH 841031	2µg/ml	841032	122.2ng/ml	841033	62.5 – 4000pg/ml	1:10	1:1000
MMP-9	DY911	MAH 841028	1µg/ml	841029	100ng/ml	841030	31.25 - 2000 pg/ml	1:10	1:1000
MPO	DY3174	RAH 842842	4µg/ml	842843	50ng/ml	842844	62.5 - 4000 pg/ml	1:10	1:750
NGAL	DY1757	843371	2µg/ml	842272	25ng/ml	842273	0.078 - 5ng/ml	1:100	1:100
TIMP-1	DY970	MAH 840294	2µg/ml	840295	50ng/ml	840296	31.25 - 2000 pg/ml	1:10	1:4000
TIMP-2	DY971	MAH 840528	2µg/ml	840529	12.ng/ml	840530	31.25 - 2000 pg/ml	1:10	1:4000
CC16	DY4218	RAH 843195	2µg/ml	843196	2µg/ml	843197	31.25 - 2000 pg/ml	1:10	1:1000
CRP	DY1707	MAH 842676	2µg/ml	842677	90ng/ml	842678	15.6 – 1000pg/ml	1:10	1:100K
Periostin	DY3548b	MAH 844441	2µg/ml	844442	500ng/ml	843260	62.5 – 4000pg/ml	1:2	1:1000
HSA	DY1455	843250	2µg/ml	843251	125ng/ml	843252	2.5 - 160ng/ml	1:100	1:100K
RBP4	DY3378	842954	2µg/ml	842955	500ng/ml	842956	23.4 - 1500pg/ml	1:2000	1:100K
Cystatin C	DY1196	842942	4µg/ml	842943	250ng/ml	842944	62.5- 2000ng/ml	1:500	1:1000

2.2.2 Commercial full kits

2.2.2.1 Calprotectin measurements

The Calprotectin Kit (Biolegend cat. 439707) was provided with pre-coated plates (Anti-Human MRP8/14) and specific reagents for each kit, this included the standard, detection reagent, substrate (solution D), sample buffer (Assay Buffer A) required to run the assay. The standard used was lyophilized and required storage at -20°C upon arrival, remaining assay components was stored at 2-8°C. The wash buffer used was 50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20.

Assay running procedure: Calprotectin was diluted in buffer A to give concentrations between 3.13 and 200ng/ml to generate the standard curve. Urine samples or serum samples were diluted 1 in 10 or 1 in 200 respectively in buffer A. Equal volumes of standard or diluted sample and buffer A were added per well such that the final volume was 100µl. These mixtures were incubated for 1hour at room temperature with gentle agitation (600rpm). The plate was washed 3 times (100µL per well) with wash buffer followed by addition of 100µl Human MRP8/14 Detection Ab to each well and incubated at RT for 30mins with shaking. After a second wash step, 100µl Avidin-HRP B solution was added to each well and incubated at RT for 30mins with shaking. After a third wash step, the colour reaction was initiated with the addition 100µl Substrate Solution D was added to each well and incubated in the dark for 25 minutes and then stopped by adding 100µl of Stop solution to each well. The absorbance was measured at 450nm using the Omega plate reader and the standard curve was approximated in a sigmoid 4 parameter logistic model.

2.2.2.2 Creatinine measurement

The Parameter creatinine assay (R&D solutions cat. KGE005) was supplied as a complete kit including plates (part 892880), Creatinine standard (stock at 100mg/dl, stored 2-8°C, part 892890), picric acid reagent (0.13% stored at RT, part 892891) and NaOH (1N, part No. 891236).

Assay running procedure: Standards were prepared at 0.3-20mg/dl in distilled water (1 in 2 dilution). Urine samples were centrifuged at 3,000 x g for 10 minutes and diluted 1 in 20 in distilled water. The Alkaline Picrate solution was prepared according to the manufacturer's instructions. To the plate, 50ul of standards and samples were added in duplicate with 100ul Alkaline Picrate Solution, the plate was incubated for 30 minutes at room temperature and subsequently read at 490nm with the Omega plate reader using a prepared programme.

2.2.2.3 Beta 2 Microglobulin measurement

the Human beta 2 Microglobulin ELISA kit (Abcam, 108885) was supplied as a complete kit (stored at 2-8°C unless specified) that included pre-coated plates, B2M standard (lyophilized), 10x diluent concentrate, Biotinylated B2M antibody (stored at -20°C), 100x streptavidin-peroxidase conjugate (stored at -20°C), chromogen substrate and stop solution. The wash buffer used was 50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20.

Assay running procedure: prior to running the assay, the 10x diluent was diluted to 1x in distilled water, the biotinylated B2M antibody was diluted to the specified concentration with the 1x diluent and the streptavidin-peroxidase conjugate was diluted 1:100 with the 1x diluent. The standards were prepared at 0.049-50ng/ml in 1x diluent (1 in 4 dilution). Urine samples were centrifuged at 3,000 x g for 10 minutes and diluted 1:100 in 1x diluent and serum samples were diluted 1:1000 in 1x diluent. To the microtitre plate, 50µl of standard or diluted sample was added per well in duplicate and incubated for 2 hours at room temperature with gentle agitation (600rpm). After a wash step, 50µl of 1x biotinylated B2M antibody was added per well and incubated for 1 hour with shaking. After a second wash step, 50µl of 1x SP conjugate was added to each well and incubated for 30 minutes at room temperature with shaking. After a final wash step, 50µl of chromogen substrate was added, incubated for 10 minutes in the dark before addition of 50µl of stop solution. The colour changed from blue to yellow. The absorbance was read at 450nm with the Omega plate reader.

2.2.2.4 Fibrinogen measurement

the Human Fibrinogen ELISA kit (Abcam, 108841) was supplied as a complete kit (stored at 2-8°C unless specified) that included pre-coated plates, Fibrinogen standard, 10x diluent concentrate, Biotinylated fibrinogen antibody (stored at -20°C), 100x streptavidin-peroxidase conjugate (stored at -20°C), chromogen substrate and stop solution. The wash buffer used was 50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20.

Assay running procedure: prior to running the assay, the 10x diluent was diluted to 1x in distilled water, the biotinylated fibrinogen antibody was diluted to the specified concentration with the 1x diluent and the streptavidin-peroxidase conjugate was diluted 1:100 with the 1x diluent. The standards were prepared at 1.25-80ng/ml in 1x diluent (1 in 2 dilution). Urine samples were centrifuged at 3,000 x g for 10 minutes and diluted 1:2 in 1x diluent and serum samples were diluted 1:200 in 1x diluent. Remaining methodology same as B2M assay described above but using 1x biotinylated fibrinogen antibody.

2.2.2.5 Major Basic Protein measurement

The Human MBP ELISA kit (Cloud-clone Corp, SEB650Hu) was supplied as a complete kit that included pre-coated plates, MBP standard (lyophilised), standard diluent, Detection reagent A, Assay diluent A, Detection reagent B, Assay diluent B, TMP substrate, Stop solution. The wash buffer used was 50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20.

Assay running procedure: prior to running the assay, the standard was reconstituted with 1ml of standard diluent and standard curve was prepared at 3.12-100ng/ml in standard diluent (1 in 2 dilution). Detection reagent A and B were diluted to working concentration 100-fold with assay diluent A and B respectively. Samples were diluted 1 in 5 in standard diluent. To the microtitre plate, 100µl of standard or diluted sample was added per well in duplicate and incubated for 1 hour at 37°C. After the incubation period, 100µl of detection reagent A was added and incubated for 1 hour at 37°C. After a wash step, 100µl of detection reagent B was added and incubated for 30 minutes at 37°C. A final wash step was undertaken before adding 90µl of substrate solution to each well, incubated for 10-20 minutes at 37°C (in the dark) followed by 50µl of stop solution. The colour changed from blue to yellow. The absorbance was read at 450nm with the Omega plate reader.

2.2.2.6 Eosinophil cationic protein (RNASE3) measurement

The Human RNASE3 ELISA kit (Cloud-clone Corp, SEB758Hu) was supplied as a complete kit that included pre-coated plates, RNASE3 standard (lyophilised), standard diluent, Detection reagent A, Assay diluent A, Detection reagent B, Assay diluent B, TMP substrate, Stop solution. The wash buffer used was 50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20.

Assay running procedure: prior to running the assay, the standard was reconstituted with 1ml of standard diluent and standard curve was prepared at 0.078-5ng/ml in standard diluent (1 in 2 dilution). Detection reagent A and B were diluted to working concentration 100-fold with assay diluent A and B respectively. Samples were diluted 1 in 4 in standard diluent.

Assay was run as described above for the MBP assay.

2.2.3 Substrate assays

2.2.3.1 MMP substrate assay (Active protease (Composite MMP 2,8,9,12,13,7))

MMP-9 standard (Alere pro MMP standard activated in house with APMA) was prepared at concentrations ranging from 2.7 – 2000ng/ml (1 in 3 dilution) diluted in MMP buffer (50mM Tris, 10mM Calcium chloride dihydrate, 100mM Sodium chloride, 0.05mM Zinc chloride, 0.025% 2/vol Brij-35 and 0.05% w/vol Sodium azide). The MMP fluorogenic substrate (Enzo BML-P276-001, sequence: Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) was diluted in MMP buffer to give a final

concentration of 10µM. To a plate (Costar, black, Cat. 3925), 5µl of standard or neat sample was added (2 replicates) followed by 195µl of diluted substrate solution. The Plate was immediate read (1-minute cycles for 30 minutes) with the Omega plate reader using a prepared programme for EX330 EM400. Mca fluorescence is quenched by the Dpa group until cleavage separates them (MMPs cleave between Gly-Leu).

2.2.3.2 HNE activity

HNE standard (Lee BioSolutions 342-40) was prepared at concentrations ranging from 0.04 – 30ng/ml (1 in 3 dilution) diluted in MMP buffer (50mM Tris, 10mM Calcium chloride dihydrate, 100mM Sodium chloride, 0.05mM Zinc chloride, 0.025% 2/vol Brij-35 and 0.05% w/vol Sodium azide). The HNE fluorogenic substrate (Bachem 1-1270, sequence: MeOSuc-AAPV-AMC) was diluted in MMP buffer to give a final concentration of 20µM. To a plate (Costar, black, Cat. 3925), 5µl of standard or neat sample was added (2 replicates) followed by 195µl of diluted substrate solution. The Plate was immediate read (1-minute cycles for 30 minutes) with the Omega plate reader using a prepared programme for EX380 EM450.

2.2.4 Mologic in-house developed assays

For all assays after addition of substrate and subsequent colour development or stop solution, the absorbance was measured at 405 (for pNPP) or 450nm (for TMB) using an Omega plate reader and the standard curve was approximated in a sigmoid 4 parameter logistic model.

2.2.4.1 Materials and reagents

Nunc plates (Maxisorp™ flat bottomed 442404)
Fisher Scientific plates (Corning ™ 9018 flat bottomed 9018)
PBS buffer (10mM phosphate buffered saline pH7.5)
Wash buffer (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20)
Buffer 1 (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20 and 1% (w/v) BSA)
Buffer 2 (10mM phosphate buffered saline pH7.5, supplemented with 0.1% (v/v) Tween20 and 1% (w/v) BSA)
Buffer 3 (10mM phosphate buffered saline pH7.5, supplemented with 1% (w/v) BSA)
Buffer 4 (50mM tris buffered saline pH8, supplemented with 1% (v/v) Tween20)
Buffer 5 (MMP buffer; 50mM Tris, 10mM Calcium chloride dihydrate, 100mM Sodium chloride, 0.05mM Zinc chloride, 0.025% 2/vol Brij-35 and 0.05% w/vol Sodium azide)
Buffer 6 (50mM tris buffered saline pH8, supplemented with 1% (w/v) BSA)
pNPP (Biopanda, cat no pNPP-001)

streptavidin-HRP (Invitrogen, Cat. SA100-01)

TMB solution (Biopanda, Cat. TMB-S-004)

stop solution (Biopanda, Cat. STP-001)

2.2.4.2 fMLP measurement - Novel assay developed in-house

Disposable 96-well polystyrene plates were obtained from Nunc. fMLP (Sigma-Aldrich, Prod. No. 47729). fMLP-ovalbumin conjugate was prepared using an "imm-link" (carboxyl) immunogen conjugation kit purchased from Innova Bioscience (Cat No 471-0500). The plate was sensitised with ovalbumin-FMLP conjugate at 2µg/ml in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 1) with 120µl/well for 1hour at room temperature.
Assay running procedure: fMLP was diluted in the buffer 1 to give concentrations between 1.56 and 100ng/ml to generate the standard curve. Equal volumes of standard or urine sample (diluted 1 in 2 in buffer 1) and sheep anti-fMLP alkaline phosphatase conjugate (at predetermined dilutions) were added to duplicate microtitre wells sequentially, such that the final volume was 100µl. These mixtures were incubated for 1hour at room temperature with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µl of pNPP solution to each well.

2.2.4.3 N-acetyl Proline-Glycine-Proline (Ac-PGP) measurement- Novel assay developed in-house

Disposable 96-well polystyrene plates were obtained from Fisher Scientific. The plate was sensitised with Sheep Anti Ac-PGP (Mologic CF1763 affinity purified) at 0.5µg/ml in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 2) with 120µl/well for 1 hour at room temperature.
Assay running procedure: Ac-PGP was diluted in buffer 2 to give concentrations between 62.5 and 4000ng/ml (1 in 2 serial dilution) to generate the standard curve. Equal volumes of standard or urine sample (diluted 1 in 5 in buffer 2) and competitor – biotinylated Ac-PGP (Peptide synthetics, custom designed) at 4µg/ml were added to duplicate microtitre wells sequentially, such that the final volume was 100µl. The final top standard was 2000ng/ml and the final sample dilution was 1 in 10). These mixtures were incubated for 1hour at room temperature with gentle agitation. After a wash step, 100µl/well of streptavidin-HRP at a 1 in 5000 was added and left to incubate for 30 minutes with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µl of TMB solution to each well followed by 100µl of stop solution after approximately 15 minutes.

2.2.4.4 Desmosine measurement – Novel assay developed in-house (appendix)

Disposable 96-well polystyrene plates were obtained from Nunc. Desmosine was supplied by Elastin Products Company, Inc. (Cat No, D866). Desmosine-ovalbumin conjugate was prepared using an "imm-link" (carboxyl) immunogen conjugation kit purchased from Innova Bioscience (Cat No 471-0500). The plate was sensitised with ovalbumin-desmosine conjugate at 1µg/ml in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 1) with 120µl/well for 1hour at room temperature.

Assay running procedure: Desmosine was diluted in buffer 1 to give concentrations between 0.82 and 200ng/ml (1 in 2.5 serial dilution) to generate the standard curve. Equal volumes of standard or urine sample (diluted 1 in 5 in buffer 1) and sheep anti-desmosine alkaline phosphatase conjugate (at predetermined dilutions) were added to duplicate microtitre wells sequentially, such that the final volume was 100µL. These mixtures were incubated for 1 hour at room temperature with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µL of pNPP solution to each well.

2.2.4.5 Large Elastin Fragments measurement – Novel assay developed in-house

As Desmosine 2.2.4.4, but the antibody used was sheep anti-large elastin fragment (CF1670) alkaline phosphatase conjugate diluted 1/4000 in buffer 3.

2.2.4.6 Desmosine fragments measurement – Novel assay developed in-house

As Desmosine 2.2.4.4, but the antibody used was sheep anti-small elastin fragment (CF1674) alkaline phosphatase conjugate diluted 1/3000 in buffer 3.

2.2.4.7 Siglec 8 measurement- Novel assay developed in-house

Disposable 96-well polystyrene plates were obtained from Fisher Scientific. The plate was sensitised with Sheep anti Siglec 8 (Mologic, SA122 purified against peptide MOL624) at 2µg/ml in PBS overnight at ambient, 120µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 3) with 120µl/well for 1 hour at room temperature.

Assay running procedure: Recombinant SIGLEC8 binding domain (Mologic, York) was diluted in buffer 3 to give concentrations between 7.81 and 500ng/ml to generate the standard curve. The standard and urine sample (diluted 1 in 10 in buffer 3) were added to the plate 100µl/well after a wash step and incubated for 1 hour at room temperature with gentle agitation. After a further wash step, sheep anti-siglec 8 (Mologic, SA122 purified against Siglec 8) alkaline phosphatase conjugate at 1 in 2000 were added 100µl/well and incubated for 1 hour at room temperature with gentle

agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µL of pNPP solution to each well.

2.2.4.8 Ultimate ELTABA (Active protease (Composite MMP's)) -Novel assay developed in-house

The peptide (MOL378 stock concentration 1mg/ml) was diluted to 2µg/ml in buffer 4. MMP-9 standard (Alere pro MMP standard activated in house with APMA) was prepared at top concentration of 500ng/ml diluted in buffer 5 and then further diluted down to 3.9ng/ml in buffer 4.
Assay running procedure: Eppendorfs were pre-loaded with 12.5µl of peptide solution. Standards or samples (neat) were added to the peptide solutions, 75µl per vial and mixed thoroughly. After a 10 minute incubation period at room temperature, the entire solution (approximately 87µl) was added to the Ultimate ELTABA cassette and then read after a further 10 minutes. The reader used for measuring the line intensity was the cube reader (Optricon) and the raw values were converted to concentration values using the 4-parameter logistic model with the 'myassay' software.

2.2.4.9 Human Neutrophil Elastase (HNE) measurement

Disposable 96-well polystyrene plates were obtained from Nunc. The plate was sensitised with mouse anti HNE (Alere, 1871) at 1.5µg/ml in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 3) with 120µl/well for 1hour at room temperature.
Assay running procedure: HNE (Lee biosolutions, 342-40) was diluted in the buffer 2 to give concentrations between 0.39 and 25ng/ml to generate the standard curve. The standard and sample (urine sample diluted 1 in 10 and serum sample diluted 1 in 100 in buffer 2) was added to the plate 100µl/well after a wash step and incubated for 1.5 hours at room temperature with gentle agitation. After a further wash step, mouse anti-HNE (Alere, 1241) alkaline phosphatase conjugate at 1 in 25000 diluted in buffer 2 were added 100µl/well and incubated for 1hour at room temperature with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µL of pNPP solution to each well.

2.2.4.10 Alpha-1 antitrypsin (A1AT) measurement

Disposable 96-well polystyrene plates were obtained from Nunc. The plate was sensitised with mouse anti A1AT (Alere, 1521) at 2µg/ml in PBS overnight at ambient, 100µl per well.
After a wash step, the sensitised-well surfaces were blocked (buffer 6) with 120µl/well for 1hour at room temperature.
Assay running procedure: A1AT (Calbiochem, Prod. No. 178251) was diluted in buffer 2 to give concentrations between 0.13 and 80ng/ml (1 in 2.5 serial dilution) to generate the standard curve.

The standard and sample (urine sample diluted 1 in 20 and serum sample diluted 1 in 200000 in buffer 2) was added to the plate 100µl/well after a wash step and incubated for 1.5 hours at room temperature with gentle agitation. After a further wash step, mouse anti-A1AT (Alere, 1951) alkaline phosphatase conjugate at 1 in 30000 diluted in buffer 2 were added 100µl/well and incubated for 1hour at room temperature with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µl of pNPP solution to each well.

2.2.4.11 Secretory leukocyte protease inhibitor (SLPI) measurement

Disposable 96-well polystyrene plates were obtained from Fisher Scientific. The plate was sensitised with sheep anti SLPI (Mologic, CF1099 IgG cut) at 20µg/ml in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 3) with 120µl/well for 1hour at room temperature.

Assay running procedure: recombinant SLPI (R&D systems cat. 1274-P1) was diluted in buffer 2 to give concentrations between 0.781 and 50ng/ml (1 in 2 serial dilution) to generate the standard curve. The standard and sample (urine sample diluted 1 in 2 and serum sample diluted 1 in 2 in buffer 2) was added to the plate 100µl/well after a wash step and incubated for 1.5 hours at room temperature with gentle agitation. After a further wash step, mouse anti-SLPI (Alere, 431) alkaline phosphatase conjugate at 1 in 2500 diluted in sample diluent were added 100µl/well and incubated for 1 hour at room temperature with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µl of pNPP solution to each well.

2.2.4.12 Soluble receptor for advanced glycation end products (sRAGE) measurement

Disposable 96-well polystyrene plates were obtained from Fisher Scientific. The plate was sensitised with sheep anti sRAGE (Mologic, SA056 affinity purified) at 1µg/ml in PBS overnight at ambient, 100µl/well. After a wash step, the sensitised-well surfaces were blocked (buffer 2) with 120µl/well for 1hour at room temperature.

Assay running procedure: recombinant sRAGE (Novoprotein cat. C423) was diluted in buffer 2 to give concentrations between 0.02 and 5ng/ml (1 in 2 serial dilution) to generate the standard curve. After a wash step, the standard and sample (neat urine) was added to the plate 50µl/well with 50µl/well of sample diluent and incubated for 1.5 hours at room temperature with gentle agitation. After a further wash step, rabbit anti-sRAGE (Mologic, RA040) alkaline phosphatase conjugate at 1 in 5000 diluted in sample diluent were added 100µl/well and incubated for 1 hour at room temperature with gentle agitation. After the final plate wash, the colour reaction was initiated with the addition of 100µl of pNPP solution to each well.

2.3 Part 2 Statistical analysis

2.3.1 T-tests

For all parametric tests, the distribution of quantitative variables in the population was assumed to be normally distributed. Data was normalized by log transforming, however, normality tests were done to ensure that the data was normally distributed. Otherwise, nonparametric tests were used for data that was not normalized such as Mann Whitney tests for unpaired data or Wilcoxon matched-pairs signed rank test for paired data. A threshold of p= 0.05 was used which has been most commonly used. When performing a large number of statistical tests (multiple comparison tests), some will have P values less than 0.05 purely by chance, even if all the null hypotheses are really true. The Bonferroni correction is one simple way to take this into account; adjusting the false discovery rate using the Benjamini-Hochberg procedure is a more powerful method. These correction methods were not taken into account for any of the analysis in particular results displayed in chapter 3 where multiple t tests were performed to compare different groups.

2.3.2 Receiver-operator characteristic (ROC) curves

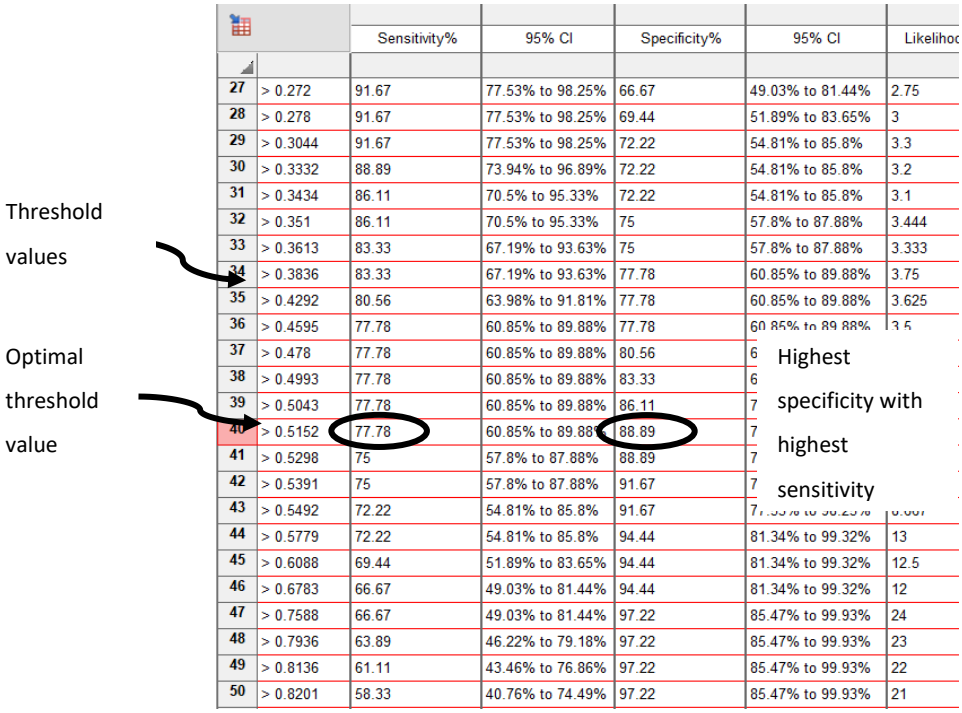
Area: The AUC was used to quantify the overall ability of the test to discriminate between those individuals with the disease and those without the disease or between different states. The general grading system used was: test not useful (<0.5), bad (0.5-0.6), sufficient (0.6-0.7), good (0.7-0.8), very good (0.8-0.9), excellent (0.9-1).

Confidence Interval of Area: These results were computed by a nonparametric method that did not make any assumptions about the distributions of test results in the different groups. Interpretation of the confidence interval was straightforward. If the patient and control groups for example represented a random sampling of a larger population, then there was a 95% sure that the confidence interval would contain the true area

P Value: The reported P value tested the null hypothesis that the area under the curve was equal to 0.50. If the p value was small then it concluded that the test was able to discriminate between the 2 groups tested.

Sensitivity and specificity: In most cases the distribution of the values within each group would overlap, therefore sensitivity and specificity derived from the ROC curve would be used to select the threshold value. Sensitivity was deemed to be the fraction of people with the disease that the test correctly identified as positive and specificity was the fraction of people without the disease that the test correctly identified as negative. In GraphPad prism, the sensitivity and specificity using each value in the data table as the cut-off value was calculated, this allowed the cut-off value to be

selected see figure 2.1 for an example. The selected threshold value would be based upon one which would give the better overall performance based on the specifications i.e. if a bias towards either sensitivity or specificity was required.



		Sensitivity%	95% CI	Specificity%	95% CI	Likelihoo
27	> 0.272	91.67	77.53% to 98.25%	66.67	49.03% to 81.44%	2.75
28	> 0.278	91.67	77.53% to 98.25%	69.44	51.89% to 83.65%	3
29	> 0.3044	91.67	77.53% to 98.25%	72.22	54.81% to 85.8%	3.3
30	> 0.3332	88.89	73.94% to 96.89%	72.22	54.81% to 85.8%	3.2
31	> 0.3434	86.11	70.5% to 95.33%	72.22	54.81% to 85.8%	3.1
32	> 0.351	86.11	70.5% to 95.33%	75	57.8% to 87.88%	3.444
33	> 0.3613	83.33	67.19% to 93.63%	75	57.8% to 87.88%	3.333
34	> 0.3836	83.33	67.19% to 93.63%	77.78	60.85% to 89.88%	3.75
35	> 0.4292	80.56	63.98% to 91.81%	77.78	60.85% to 89.88%	3.625
36	> 0.4595	77.78	60.85% to 89.88%	77.78	60.85% to 89.88%	3.5
37	> 0.478	77.78	60.85% to 89.88%	80.56	6	
38	> 0.4993	77.78	60.85% to 89.88%	83.33	6	
39	> 0.5043	77.78	60.85% to 89.88%	86.11	7	
40	> 0.5152	77.78	60.85% to 89.88%	88.89	7	
41	> 0.5298	75	57.8% to 87.88%	88.89	7	
42	> 0.5391	75	57.8% to 87.88%	91.67	7	
43	> 0.5492	72.22	54.81% to 85.8%	91.67	7	
44	> 0.5779	72.22	54.81% to 85.8%	94.44	81.34% to 99.32%	13
45	> 0.6088	69.44	51.89% to 83.65%	94.44	81.34% to 99.32%	12.5
46	> 0.6783	66.67	49.03% to 81.44%	94.44	81.34% to 99.32%	12
47	> 0.7588	66.67	49.03% to 81.44%	97.22	85.47% to 99.93%	24
48	> 0.7936	63.89	46.22% to 79.18%	97.22	85.47% to 99.93%	23
49	> 0.8136	61.11	43.46% to 76.86%	97.22	85.47% to 99.93%	22
50	> 0.8201	58.33	40.76% to 74.49%	97.22	85.47% to 99.93%	21

Figure 2.1. Example of mechanism for selecting threshold values. Displayed are many pairs of sensitivity and specificity. If a high threshold is selected, the specificity of the test is increased with a loss in sensitivity. If a low threshold is selected, the test's sensitivity is increased but with a loss in specificity.

2.3.3 Sensitivity/specificity/PPV and NPV

Table 2.3. Calculation of sensitivity/specificity/PPV and NPV. Classification of true positives (TP), false positives (FP), false negatives (FN) and true negatives (TN).

		Diagnostic test result		
		Positive	**Negative**	**Total**
Reference assay/disease status	**Positive/present**	**a (TP)**	**c (FN)**	**a+c**
	Negative/Absent	**b (FP)**	**d (TN)**	**b+d**

- Sensitivity (true positives) = TP/(TP+FN) = a/(a+c)

- Specificity (True negatives) = TN/(TN+FP) =d/(b+d)
- Relative agreement = (TP+TN)/(TP+FN+FP+TN) = (a+d)/((a+c) +(b+d))
- Positive predicted value =TP/(TP+FP) = a/(a+b)
- Negative predicted value = TN/(TN+FN) = d/(c+d)

2.3.4 Logistic regression

All data were analysed using SPSS (version 21). Logistic regression analysis was used to develop predictive models, combining biomarkers that predicted exacerbation outcome. The rules were that there were not more variables (i.e. biomarkers) than there were observations (i.e. exacerbations). This would lead to overfitting. In addition, variables that correlated would contribute to inaccuracy in the analysis. Internal validation was addressed by dividing the cases into 80% training set and 20% test set. This process was repeated 5 times using assignment to training and validation sets by random number generation in SPSS.

2.3.5 Correlation methods: Bland-Altman plots and spearman's / pearsons r

Bland-Altman: The difference scores of two measurements were plotted against the mean for each subject upon which, the mean difference was studied with constructed limits of agreements either by fold differences or % differences. The graph was plotted on the XY axis where X represented the difference of the two measurements, and the Y-axis showed the mean of the two measurements (see figure 2.2 for examples). When interpreting this kind of analysis, the limits should not be used to define acceptable ranges but should be defined before, based on varying factors, for example, inter-patient variability is known to be higher than intra-patient variability. Perhaps wrongly, bland-Altman plots are generally interpreted informally, without further analyses or limits but based on questions such as (taken from GraphPad prism guide):

- How big is the average discrepancy between methods (the bias)? You must interpret this clinically. Is the discrepancy large enough to be important? This is a clinical question, not a statistical one.
- How wide are the limits of agreement? If it is wide (as defined clinically), the results are ambiguous. If the limits are narrow (and the bias is tiny), then the two methods are essentially equivalent.
- Is there a trend? Does the difference between methods tend to get larger (or smaller) as the average increases?

- Is the variability consistent across the graph? Does the scatter around the bias line get larger as the average gets higher?

It has been recommended that 95% of the data points should lie within ±1.96 SD of the mean difference – limits of agreement (144). The ideal bias is '0'. More recently, Critchley and Critchley accepted limits of agreement within ±30% of the reference standard.

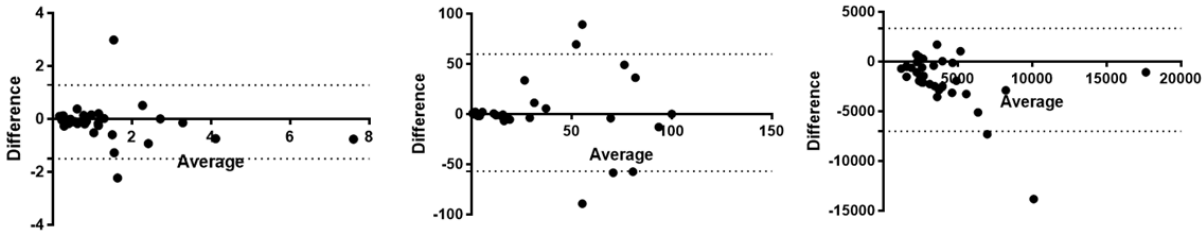


Figure 2.2. Example bland-Altman plots. A) If measurements with the two methods are similar, then the differences between them will be small, with an average near zero they will be consistent over the range of measurement values, and the limits of agreement will be narrow, B) methods do not appear to be similar post midrange values where there is no trend and points are scattered and C) standard deviation decreasing with concentration and /or with a proportional difference i.e. as the concentrations increase with one method, the concentrations decrease with the other.

Spearman's r and Pearsons r values: Spearman's rank correlation coefficient (spearman's r) was used as a non-parametric measure of correlation. Pearson's correlation was used on normalised data. In terms of the strength of relationship, the value of the correlation coefficient varies between +1 and -1. (± 1 indicates a perfect degree of association between the two variables). As the correlation coefficient value goes towards 0, the relationship between the two variables will be weaker. The results were displayed either graphically or by heatmaps, in some cases other diagrams were manually drawn to summarise the relationships.

Chapter 3. Biomarkers in urine

3.1 Introduction

This chapter explores biomarkers found in urine. Most of the biomarkers selected have been investigated in other matrices such as blood, sputum, BAL, saliva, but little is known of their presence in urine and their relative concentrations in various disease states. The questions to address are a) what biomarkers from the shortlist can be found in the urine and b) are there any influences such as age, gender and severity of disease.

Urinary biomarker profiles were explored in 5 separate studies to provide further insight into their role in inflammation. The selected urine samples were obtained from healthy controls, from donors with stable lung disease, COPD and CF and from people with suspected urinary tract infection (UTI). The justification for each population studied was to understand the biomarkers in health and stable disease before looking at changes that occur during an inflammatory insult. The UTI cohort was studied as it is hypothesised that the kidney has an influence on the biomarker levels and a local infection in the urinary tract would alter the levels in the urine non-specifically which would be unrelated to the infection in the lung.

Healthy controls. Samples from 40 'healthy' subjects were collected from Mologic Ltd. These samples were not matched to any particular disease in terms of gender or age as the number of volunteers was limited.

Stable COPD subjects. The stable samples came from the GlaxoSmithKline (GSK) ECLIPSE study (Evaluation of COPD Longitudinally to identify Predictive Surrogate Endpoints). ECLIPSE, was an observational, longitudinal study in which, after the baseline visit, participants were evaluated at 3 months, 6 months and subsequently every 6 months over 3 years. In the larger ECLIPSE study, 2164 patients with COPD were recruited and blood, sputum and urine samples were donated at each timepoint, for this analysis, urine samples from 98 patients were evaluated. Full details of the study have been reported (21), and one of the main findings was that the single best predictor of exacerbation of COPD was the patient's own history of exacerbations. Another conclusion was that exacerbations became more frequent and more severe as COPD progresses. Over the three-year study period, of 296 patients who had frequent exacerbations in years 1 and 2, 210 continued to have frequent exacerbations in year 3. Of 521 patients with no exacerbations in years 1 and 2, 388 had no exacerbations in year 3. Blood samples from the study have been extensively studied by different groups (145, 146) but the urine samples collected have never been tested or if so never reported. The feedback from GSK was that limited analysis had not provided any significant results that were worth pursuing for the specific aims towards which they were working.

Stable CF subjects (1). The wider purpose of this run-in study for the investigators was to help design a therapeutic trial and to select the patients most likely to be able to demonstrate benefit. This study was led by the UK Cystic Fibrosis Gene Therapy Consortium (Imperial College, University of Edinburgh and University of Oxford) and sponsored by Imperial College managed by Professor Eric Alton and funded by the Cystic Fibrosis Trust. Approximately 200 CF patients were recruited from two centres, measurements were taken over several years which included tests of lung function, bacterial infection and inflammation in the lungs. Measurements and samples (blood and urine) were collected at 4 timepoints over the first 12-18 months (4 monthly intervals). Patients were recruited from the adult and paediatric clinics at the Royal Brompton Hospital (approximately 150) and Scotland (approximately 50). This study helped the consortium to decide which tests would be best at showing any benefits that might result from treatment with gene therapy.

Stable CF subjects (2). These stable CF samples were obtained from Queens university Belfast (QUB). In the Belfast study, 129 clinically stable CF patients attending the adult and paediatric CF centres in Belfast were recruited to the study. The study was supported by the UK NHS NOCRI Translational Research Partnership & US Ireland Partnership Grant. Some additional samples were collected at the start and/or end of antibiotic treatment of an infective exacerbation.

Suspected UTI and recovered UTI subjects. Samples were received from adult women who consulted their GP with symptoms of UTI. Correct diagnosis of UTI is difficult. GPs will usually send a urine sample to the hospital laboratory for culture and in about 50% cases, samples are contaminated and do not provide useful information. GPs are therefore faced with the dilemma that the diagnostic results are often not very helpful, but that truly infected patients need to be treated as early as possible to avoid complications. As a consequence, GPs often inappropriately give antibiotics to patients with suspected infections even though some of those individuals may have unrelated illnesses or prescribe antibiotics to which the bacteria are resistant.

It is important to note that as the project progressed the shortlist of biomarkers expanded with time so not all 34 biomarkers were evaluated in this early work. Reference assays were used to measure the levels of biomarkers in urine as described in chapter 2.

3.2 Methods – patient selection and assessment

In all cases with the exception of the healthy controls, the samples were received from other sites. Samples were received frozen, transferred on dry ice in 1,5 or 10ml tubes and immediately stored at -80°C. At the point of testing, the samples were thawed, equilibrated to room temperature and tested with the reference assays. Remaining sample was aliquoted in to 1ml vials and stored at -80°C.

3.2.1 Healthy controls

Samples from 40 'healthy' subjects were collected from Mologic Ltd. Consent was given and samples were anonymised. The main inclusion criterion was that the volunteer felt well at the time of donating the sample. Urine samples were aliquoted, labelled and stored at -80°C upon within 3 hours of collection. The samples were thawed and equilibrated to room temperature before testing with the reference assays as described in chapter 2.

3.2.2 Stable COPD

A total of 98 patients from the ECLIPSE study with collections from each patient at 8 different timepoints as shown in table 3.1 (total of 800 samples) were received from GSK. Of the 98 patients, 50% exhibited 'infrequent' exacerbation and 50% exhibited 'frequent' exacerbations. After testing with reference assays detailed in chapter 2, biomarker measurements from samples collected at visit 1 (baseline) were taken forward for analysis, biomarkers were explored in stable state and then stratified into the two subgroups for further analysis. Analysis performed explored single biomarkers and multiplex biomarkers that could differentiate frequent and infrequent exacerbators and prediction of severity of disease over subsequent years.

Table 3.1. Urine sample collection time points and groupings for GSK ECLIPSE study

Month	*Time Point Number*	*Year*
0	1	**baseline**
3	2	**Year 1 samples**
6	3	
12	4	
18	5	**Year 2 samples**
24	6	
30	7	**Year 3 samples**
36	8	

3.3.3 Stable CF (1)

A total of 157 patients with collections from each patient at 4-6 different timepoints taken 4 months apart (total of 822 samples) were received from Imperial College London. After testing with reference assays detailed in chapter 2, biomarker measurements from samples at visit 1 (baseline) were taken forward for analysis exploring biomarkers in stable state. The total number of baseline samples taken forward for analysis was 157.

3.3.4 Stable CF (2)

A total of 146 samples from CF patients were received from QUB, stratified according to figure 3.1. For analysis, only the 1st stable visit data was included n=129.

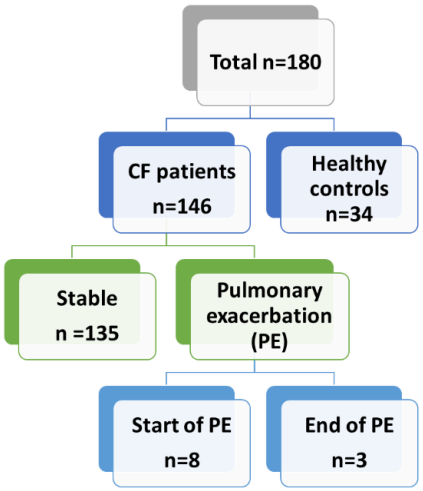


Figure 3.1. Urine samples received from Belfast City Hospital and Royal Belfast Hospital for Sick Children. All CF patient urine samples were received and analysed. After testing with reference assays detailed in chapter 2, biomarker measurements from stable samples only were taken forward for analysis exploring

3.2.5 Suspected UTI and recovered UTI subjects

A total of 202 urine samples from adult women presenting with suspected uncomplicated UTI were received from Cardiff. A second sample from some of the volunteers was subsequently collected 2 weeks once treatment had finished. Fresh urine samples from primary care sites were transported to the hospital laboratories where they were aliquoted into 1ml vials and stored at -80°C. The samples were thawed and equilibrated to room temperature before testing with the reference assays as described in chapter 2.

3.2.6 Statistical analysis

All data were analysed using SPSS (version 21) or GraphPad PRISM. Data normality was explored, and appropriate parametric or non-parametric tests chosen accordingly. Receiver-operator characteristic (ROC) analysis and Wilcoxon's signed rank test, Mann-Whitney or students t-test with significance levels $p<0.05$ was used to compare biomarker levels in different disease states,

subgroups, gender and age. Correlation matrix were performed with each study using GraphPad Prism, Spearman's rank correlation coefficient for nonparametric measure of rank correlation. Logistic regression was used to develop predictive models, combining biomarkers that determined the outcome of exacerbation.

3.3 Results

3.3.1 Patient characteristics

There was limited clinical information available for the healthy, CF and UTI samples, that which was available is summarised in table 3.2. The stable COPD ECLIPSE were much older with a median age of 62yrs whereas the median age for the CF stable donors were younger 22.8 and 24.8yrs for the 2 cohorts ranging from 10 - 68yrs collectively. The healthy cohort had median age of the 42yrs and the range overlapped both COPD and CF cohorts. There was no information available on the age of the UTI cohort. With regards to gender, the COPD, healthy and CF cohorts were similar with a predominately male population whereas the UTI cohort consisted of all females. Both the CF cohorts were similar with regards to the FEV1% prediction values.

The Stable COPD cohort was broken down into 2 sub-groups - frequent and infrequent exacerbators (table 3.3). Both subgroups were very similar in terms of age, gender, smoking status, BMI, comorbidities, the key difference was frequency of exacerbations in the year prior to recruitment >2 exacerbations, hospitalisation's, FV950 (Emphysema score) and Gold status (severity of disease).

Table 3.2. Mean (SD) values of the main characteristics for all 5 cohorts with common information available

	STABLE COPD	**HEALTHY**	**STABLE CF (1)**	**STABLE CF (2)**	**UTI**
	N = 98	**N=40**	**N=158**	**N=146**	**N=49**
Age (yrs.)	61.6 (5.2)	42	22.8 (11.7)	24.8 (14.2)	-
Age min-max (yrs.)	46-74	18-69	10-59	6-68	-
Male (%)	58 (59%)	21 (52.5%)	87 (55.4%)	75 (58.1%)	0 (0%)
FEV1, post-BD mean (SD)	1.4 (0.6)	-	2.26 (0.79)	2.3 (0.9)	-
FEV1% PRED mean (SD)	-	-	70.4 (19.5)	75.1 (21.3)	-

Table 3.3. Main characteristics of the stable COPD cohort and two subgroups- Infrequent Exacerbator (IF) & Frequent Exacerbator (F) at baseline. Mean (SD) values and number (%)

		Stable COPD	Infrequent Exacerbators	Frequent Exacerbators
		N = 98	**N=49**	**N=49**
Demographics				
Age (yrs.)	Mean (SD)	61.6 (5.2)	61.5 (5.1)	61.8 (5.4)
Age min-max (yrs.)	Min-max	46-74	46-74	50-74
Male	No (%)	58 (59%)	29 (60.4%)	29 (60.4%)
Smoking, pack-years	No (%)	47.1 (27.6)	48.4 (28.5)	45.8 (26.9)
BMI, kg/m^2	Mean (SD)	25.3 (4.4)	25.8 (4)	24.8 (4.8)
Frequent exacerbators (≥2 pa)	No (%)	49 (50)	0	100%
mMRC Score	Mean (SD)	1.5 (1)	1.2 (0.9)	1.8 (1)
SGRQ-C Total Score	Mean (SD)	46.6 (18.7)	36.9 (18.1)	56.5 (13.5)
Exacerbations in year prior to recruitment	Mean (SD)	1.2 (1.5)	0.2 (0.6)	2.2 (1.5)
ICS use	No (%)	13 (13.3%)	5 (10.2%)	8 (16.3%)
Oral CS use		1 (1%)	0	1 (2%)
Cardiovascular disease	No (%)	31 (31.6%)	15 (30.6%)	16 (32.7%)
Hx Osteoporosis	No (%)	12 (12.2%)	4 (8.2%)	8 (16.3%)
Diabetes	No (%)	5 (5.1%)	2 (4.1%)	3 (6.1%)
Statin Use	No (%)	22 (22.4%)	9 (18.4%)	13 (26.5%)
Physiology and Imaging				
FEV1 (L), post-BD	Mean (SD)	1.4 (0.6)	1.65 (0.6)	1.2 (0.4)
FVC (L), post-BD	Mean (SD)	3.3 (0.9)	3.5 (0.9)	3.1 (0.8)
FEV1/FVC, post-BD	Mean (SD)	42.6 (12.1)	46.3 (10.9)	38.8 (12.3)
6MWD, m	Mean (SD)	425.6 (124.2)	462 (115.6)	388.9 (122.6)
BODE index	Mean (SD)	2.7 (2)	1.9 (1.8)	3.5 (1.8)
Oxygen sat	Mean (SD)	95.2 (2.3)	95.8 (2.3)	94.7 (2.2)
FV950	Mean (SD)	15.5 (10.6)	11.4 (8.2)	19.6 (11.2)
GOLD Risk index				
A	No (%)	18 (18.4%)	18 (36.7%)	0
B	No (%)	3 (3%)	3 (6.1%)	0
C	No (%)	28 (28.6%)	28 (57.1%)	0
D	No (%)	49 (50%)	0	49 100%)

3.3.2 Biomarker levels in normal, stable COPD, stable CF and UTI

The biomarker levels in each of the 5 groups are shown in table 3.4-3.8. The COPD and CF cohorts were all compared to the biomarker levels measured in the healthy samples whereas the suspected UTI samples were compared to the 2-week recovery samples (post antibiotics) which should have been similar to the 'healthy' ranges. A graphical representation of all the data is shown in figure 3.2 for 22 biomarkers.

A summary of the significance levels is shown in table 3.9. There were 8 biomarkers that unexpectedly behaved differently with both the CF cohorts irrespective of the age and gender similarities. These were fMLP, IL-1β, active MMP (ultimate ELTABA and substrate assay), MMP-8, NGAL, MPO, B2M and desmosine. There may have been other parameters that made these 2 cohorts different such as bacterial species or even genotype.

It was expected that the normal and UTI recovery samples would contain similar levels, however, the UTI recovery patients may still have an active infection (UTI or other dependent on actual diagnosis that have been overlooked). Those biomarkers that were significantly different ($p<0.05$) between healthy and UTI recovered were active MMP (ultimate ELTABA and substrate assay), HNE, NGAL, Cystatin C, Desmosine and HSA. UTI markers that were significantly different from both the healthy and recovered samples were IL-1β, active MMP (as measured by Ultimate ELTABA), IL-8, MMP-9, MMP-8, HNE, NGAL, HSA and fibrinogen. Biomarkers that were only significantly different in relation to recovered samples were IL-6 and RBP4. The 1 biomarkers that was significantly different in relation to healthy samples was cystatin C.

There were 3 biomarkers that were significantly higher in stable COPD compared to healthy (and specific to COPD) with a p value <0.05. These consisted of 2 signalling molecules – IL-1β and IL-6 and fibrinogen which is regulated by IL-6. There were 4 biomarkers that were significantly higher in stable CF (and specific to CF) in relation to healthy state which were 2 effector molecules -MMP-8 and NGAL, creatinine and RBP4 (RBP4 was significantly higher in only one of the CF cohorts). There were three biomarkers that were significantly different in COPD and CF (cohort 1 and 2), these were IL-8, active MMP (as measured by ultimate ELTABA) and TIMP-1. To add to the list of biomarkers associated with both COPD and CF which had conflicting results between the 2 CF cohorts were fMLP, MMP-9, A1AT, desmosine, HSA, and Cystatin C. lastly biomarkers that remained the same regardless of either respiratory condition was HNE, calprotectin, B2M, TIMP-2 and MPO.

Table 3.4. Biomarker results in healthy urines- Median (IQR)

Biomarker assay	Unit	Number of values	Minimum	Maximum	Median (IQR)
IL-6	pg/ml	40	0	23.61	0.4625 (0.18- 1.614)
fMLP	ng/ml	40	0.648	19.98	6.9 (4.675-11)
IL-1β	pg/ml	40	1.119	38.71	5.489 (4.587-7.2410
Siglec 8	ng/ml	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-
Ultimate ELTABA	ng/ml	40	181	4496	442 (270.5-1010)
MMP substrate assay	ng/ml	40	0	177.4	19.58 (8.319-37.82)
HNE substrate assay	ng/ml	40	0	30	0 (0-5.5)
IL-8	pg/ml	40	0	61.66	0 (0-0)
MMP-8 Total	ng/ml	40	0	7.639	0 (0-0.6733)
MMP-9 Total	ng/ml	40	0	9.363	0.21 (0-1.529)
HNE	ng/ml	40	0	250	2.059 (0-23.61)
NGAL	ng/ml	40	0	102.4	0 (0-18.27)
Calprotectin	ng/ml	40	0	80.8	20.29 (3.9-44.28)
MPO	ng/ml	40	0	42.27	0.9747 (0.1716-11.35)
RNASE-3	ng/ml	-	-	-	-
A1AT	ng/ml	40	1.5	252.3	26.4 (11.2 -86.25)
TIMP-1	ng/ml	40	0	4.592	0 (0-0.207)
SLPI	ng/ml	-	-	-	-
Cystatin C	ng/ml	40	63.76	216.1	107.1 (84.79-128.2)
Creatinine	mg/dl	40	17.5	219.9	83.6 (39.38-120.8)
Beta 2 Microglobulin	ng/ml	40	4.252	86.76	24.68 (15.52-37.97)
RBP4	ng/ml	40	66.61	272.9	142.9 (112.2-198.5)
TIMP-2	ng/ml	40	0.264	13.14	2.853 (1.009-6.34)
Ac-PGP	ng/ml	-	-	-	-
Desmosine V1 ELISA	ng/ml	40	0	33.21	3.55 (0.625 -8.625)
LEF	ng/ml	-	-	-	-
Desmosine fragments	ng/ml	-	-	-	-
CC16	ng/ml	-	-	-	-
CRP	ng/ml	-	-	-	-
Periostin	ng/ml	-	-	-	-
H.S.A	ng/ml	40	253.9	11780	1021 (528-1557)
Fibrinogen Abcam	ng/ml	40	1.753	177.3	8.626 (5.583-20.13)
sRAGE	ng/ml	-	-	-	-

Table 3.5. Biomarker results in stable COPD urines, median (IQR) and differentiation from healthy urines. Significant p values <0.05 highlighted in bold.

Biomarker assay	unit	Number of values	Minimum	Maximum	Median (IQR)	P value
IL-6	pg/ml	100	0	43.94	0.4145 (0-5.321)	***0.0391***
fMLP ELISA	ng/ml	100	0	38.71	4.741 (2.222-7.9790	***0.0150***
IL1b	pg/ml	100	0	20.47	0.3165 (0-5.141)	***0.0011***
Siglec 8	ng/ml	-	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-	-
Ultimate ELTABA	ng/ml	100	0	282.9	37.37 (17.93-66.78)	***<0.0001***
MMP substrate assay	ng/ml	100	0	65.14	2.585 (0-7.627)	***<0.0001***
HNE substrate assay	ng/ml	100	0	482	0 (0-0)	0.7567
IL-8	pg/ml	100	0	226.7	0 (0-7.57)	***0.0369***
MMP-8 Total	ng/ml	70	0	40	0 (0-0.7588)	0.2515
MMP-9 Total	ng/ml	100	0	21.44	0.6645 (0.05075-2.717)	***0.0191***
HNE	ng/ml	100	0	284.3	0 (0-10.09)	0.1660
NGAL	ng/ml	100	0	500	15.25 (6.997-26.78)	0.0691
Calprotectin	ng/ml	100	0	90.27	17.69 (7.575-44.21)	0.8611
MPO	ng/ml	100	0	56.3	4.867 (0.999-20.44)	0.2699
RNASE-3	ng/ml	-	-	-	-	-
A1AT	ng/ml	100	0	1735	65.5 (15.33-160.6)	***0.0313***
TIMP-1	ng/ml	100	0	16.16	1.327 (0.7153-3.437)	***<0.0001***
SLPI	ng/ml	100	0	15.83	1.226 (0.02525-5.332)	-
Cystatin C	ng/ml	100	0	484.3	68.7 (35.65-109.8)	***0.0207***
Creatinine	mg/dl	100	10.4	307.4	96.02 (47-141.5)	0.1157
Beta 2 Microglobulin	ng/ml	100	3	12500	72 (29.72-183.8)	0.1420
RBP4	ng/ml	100	14.06	1706	122 (85.46-177.9)	0.8050
TIMP-2	ng/ml	100	0	16.71	4.033 (2.049-6.388)	0.4079
Ac-PGP	ng/ml	100	0	21114	270 (0-775.6)	
Desmosine V1 ELISA	ng/ml	100	0	68.1	6.485 (2.825-16.1)	***0.0105***
LEF	ng/ml	40	0	2986	449.4 (111.1-1029)	-
Desmosine fragments	ng/ml	100	15.2	6850	349.4 (122-995.6)	-
CC16	ng/ml	100	0	205.9	11.76 (1.791-35.09)	-
CRP	ng/ml	100	0	10000	399.8 (0-916.2)	-
Periostin	ng/ml	-	-	-	-	-
H.S.A	ng/ml	100	178.1	16000	1796 (1005-4256)	***0.0038***
Fibrinogen Abcam	ng/ml	100	2	222	23.55 (11-44)	***0.0364***
sRAGE	ng/ml	-	-	-	-	-

Table 3.6. Biomarker results in stable CF (1) urines (median (IQR) and differentiation from healthy urines. Significant p values <0.05 highlighted in bold.

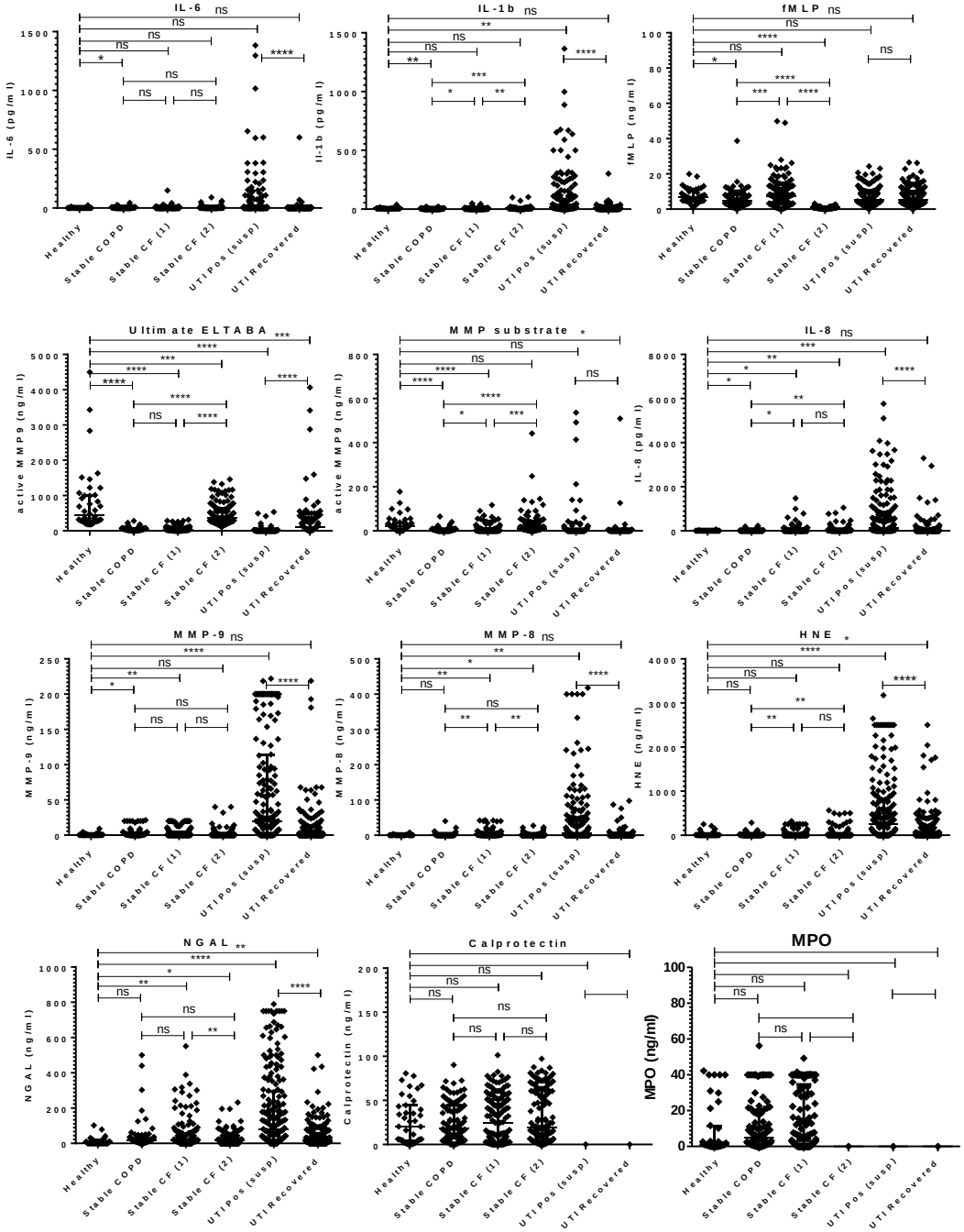
Biomarker assay	unit	Number of values	Minimum	Maximum	Median	P value
IL-6	pg/ml	157	0	150	0 (0-3.422)	0.2566
fMLP ELISA	ng/ml	155	0	49.91	7.091 (3.113-11.91)	0.5076
IL1b	pg/ml	157	0	48.9	3.597 (0.3705-5.972)	0.2327
Siglec 8	ng/ml	-	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-	-
Ultimate ELTABA	ng/ml	157	0	314.7	35.24 (13.68-78.38)	***<0.0001***
MMP substrate assay	ng/ml	157	0	117	1.892 (0-10.92)	***<0.0001***
HNE substrate assay	ng/ml	157	0	322	0 (0-0)	0.7858
IL-8	pg/ml	157	0	1480	0 (0-47.54)	***0.0344***
MMP-8 Total	ng/ml	157	0	42.65	1.669 (0-5.162)	***0.0021***
MMP-9 Total	ng/ml	157	0	20	0.421 (0-4.678)	***0.0078***
HNE	ng/ml	157	0	322.5	0 (0-44.49)	0.4011
NGAL	ng/ml	157	0	550.9	17.68 (0-56.73)	***0.004***
Calprotectin	ng/ml	157	0	101.2	24.68 (2.519-50.7)	0.6436
MPO	ng/ml	157	0	49.29	3.02 (0-34.57)	0.0805
RNASE-3	ng/ml	-	-	-	-	-
A1AT	ng/ml	157	0	2005	41.05 (11.1-116.8)	0.1201
TIMP-1	ng/ml	157	0	15.45	0.5036 (0.2214-1.316)	***0.0037***
SLPI	ng/ml	-	-	-	-	-
Cystatin C	ng/ml	157	0	224.7	56.05 (32.51-111.4)	***<0.0001***
Creatinine	mg/dl	157	8.343	345.7	107.2 (55.88-155.3)	***0.0156***
Beta 2 Microglobulin	ng/ml	157	0	5262	82.93 (29.14-162.5)	0.0592
RBP4	ng/ml	157	0	873.3	75.94 (47.15-112.5)	***<0.0001***
TIMP-2	ng/ml	157	0	18.15	4.561 (2.524-6.958)	0.0859
Ac-PGP	ng/ml	157	0	28204	4984 (1542-11866)	-
Desmosine V1 ELISA	ng/ml	157	0	167.9	17 (5.515-35.35)	***<0.0001***
LEF	ng/ml	156	0	1177	371.3 (172.2-631.2)	-
Desmosine fragments	ng/ml	156	2.831	1376	281.1 (82.33-797.9)	-
CC16	ng/ml	157	0	200	0 (0-12.81)	-
CRP	ng/ml	157	0	14705	365.5 (20.29-1369)	-
Periostin	ng/ml	-	-	-	-	-
H.S.A	ng/ml	157	14.35	16000	1454 (686.7-3439)	***0.0135***
Fibrinogen Abcam	ng/ml	157	0	195.8	10.27 (4.738-24.36)	0.8269
sRAGE	ng/ml	-	-	-	-	-

Table 3.7. Biomarker results in stable CF (2) urines (median (IQR) and differentiation from healthy urines. Significant p values <0.05 highlighted in bold.

Biomarker assay	unit	Number of values	Minimum	Maximum	Median	p value
IL-6	pg/ml	129	0	90.35	2.19 (0.59-4.4.39)	0.0582
fMLP ELISA	ng/ml	129	0.034	3.173	0.86 (0.49-1.34)	***<0.0001***
IL1b	pg/ml	129	0	103.4	6.6 (3.38-10.18)	0.3227
Siglec 8	ng/ml	-	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-	-
Ultimate ELTABA	ng/ml	129	107	1462	377 (262-643)	***0.0005***
MMP substrate assay	ng/ml	129	0	441.4	10 (4.28-28.99	0.4831
HNE substrate assay	ng/ml	129	0	288	0 (0-6)	0.1814
IL-8	pg/ml	129	0	1048	14.22 (0.62-50.87)	***0.0085***
MMP-8 Total	ng/ml	129	0	27.36	0 (0-2.75)	***0.0323***
MMP-9 Total	ng/ml	129	0	40	0 (0-1.47)	0.1351
HNE	ng/ml	129	0	565	0 (0-30.95)	0.2947
NGAL	ng/ml	129	0	231.8	15.09 (0-36.29)	***0.0333***
Calprotectin	ng/ml	129	0	97.2	19.4 (4.25-62.45)	0.2926
MPO	ng/ml	-	-	-	-	-
RNASE-3	ng/ml	-	-	-	-	-
A1AT	ng/ml	129	0	2108	126.5 (48.25-264.7)	***0.0015***
TIMP-1	ng/ml	129	0	15.31	0.69 (0.14-2.19)	***0.0015***
SLPI	ng/ml	-	-	-	-	-
Cystatin C	ng/ml	129	0	592.9	76.05 (41.54-110.9)	0.0743
Creatinine	mg/dl	129	16.3	533.5	108 (62.15-172.4)	***0.0048***
Beta 2 Microglobulin	ng/ml	129	8.864	20000	212 (77.19-432.3)	0.1111
RBP4	ng/ml	129	4.869	3864	96.78 (64.41-139.8)	0.8521
TIMP-2	ng/ml	129	0.317	20.09	4.43 (2.33-6.85)	0.1005
Ac-PGP	ng/ml	-	-	-	-	-
Desmosine V1 ELISA	ng/ml	129	0	50.4	2.81 (0.96-5.85)	0.3936
LEF	ng/ml	-	-	-	-	-
Desmosine fragments	ng/ml	-	-	-	-	-
CC16	ng/ml	-	-	-	-	-
CRP	ng/ml	-	-	-	-	-
Periostin	ng/ml	-	-	-	-	-
H.S.A	ng/ml	129	399.6	32000	1366 (849.8-2198)	0.1776
Fibrinogen Abcam	ng/ml	129	0	174	8.38 (4.4-14.91)	0.4272
sRAGE	ng/ml	-	-	-	-	-

Table 3.8. Biomarker results in suspected UTI urines, median (IQR) and differentiation from UTI recovery from urines after treatment administered. Significant p values <0.05 highlighted in bold.

Biomarker assay	unit	UTI SUSPECTED POSITIVES				UTI RECOVERY				
		Number of values	Min	Median	Max	Number of values	Min	Median	Max	p value
IL-6	pg/ml	202	0	0 (0-21.69)	1384	222	0	0 (0-1.28)	600	***<0.0001***
fMLP ELISA	ng/ml	202	0	5.57 (2.81-9.78	24.26	222	0	5.139 (2.78-8.80)	26.45	0.3566
IL1b	pg/ml	202	0	20.75 (6.83-83.48	1364	219	0	6.783 (0.003-13.39)	301.8	***<0.0001***
Siglec 8	ng/ml	-	-	-	-	-	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-	-	-	-	-	-
Ultimate ELTABA	ng/ml	69	6	151 (49-33)	1261	89	0	117 (43-384.5)	4063	0.2542
MMP substrate assay	ng/ml	96	0	0 (0-8.05)	536	92	0	0 (0-0.995)	509.1	0.1051
HNE substrate assay	ng/ml	-	-	-	-	-	-	-	-	-
IL-8	pg/ml	202	0	133.2 (3.44-702.5)	5764	222	0	3.473 (0-54.31)	3298	***<0.0001***
MMP-8 Total	ng/ml	202	0	5.744 (0-39.6)	418.2	222	0	0 (0-0)	97.63	***<0.0001***
MMP-9 Total	ng/ml	202	0	20 (1.06-113.4)	222.1	222	0	0 (0-5.92)	218.8	***<0.0001***
HNE	ng/ml	202	0	250 (32.62-769.3)	3176	222	0	27.04 (0-158.6)	2500	***<0.0001***
NGAL	ng/ml	202	0	79.16 (22.26-295.1)	789.6	222	0	19.67 (0-46.52)	500	***<0.0001***
Calprotectin	ng/ml	-	-	-	-	-	-	-	-	-
MPO	ng/ml	-	-	-	-	-	-	-	-	-
RNASE-3	ng/ml	-	-	-	-	-	-	-	-	-
A1AT	ng/ml	-	-	-	-	-	-	-	-	-
TIMP-1	ng/ml	-	-	-	-	-	-	-	-	-
SLPI	ng/ml	-	-	-	-	-	-	-	-	-
Cystatin C	ng/ml	202	0	32.03 (14.55-62.94)	255.4	222	0	28.69 (14.7-54.26)	782.2	0.6582
Creatinine	mg/dl	202	8.5	68.1 (33.06-110)	400	222	0.6	76 (41.28-125.4)	316.8	0.1536
Beta 2 Microglobulin	ng/ml	-	-	-	-	-	-	-	-	-
RBP4	ng/ml	202	0	102.7 (60.1-164.4)	1937	222	0	79.73 (44.38-128)	3000	***0.0129***
TIMP-2	ng/ml	27	0.437	3.806 (1.225-7.154)	25.94	-	-	-	-	-
Ac-PGP	ng/ml	121	0	565.6 (0-1953)	26963	157	0	807.5 (0-2542)	20000	0.5235
Desmosine V1 ELISA	ng/ml	202	0	9.5 (3.3-28.51)	1673	222	0	8.4 (3.2-20.91)	204.5	0.0785
LEF	ng/ml	121	3.061	403.9 (233.6-796.3)	2747	157	0	419.6 (219.2-688.2)	2091	0.5486
Desmosine fragments	ng/ml	121	14.95	1000 (295.8-1000)	2319	157	19.44	1000 (319.6-1000)	2404	0.8962
CC16	ng/ml	-	-	-	-	-	-	-	-	-
CRP	ng/ml	-	-	-	-	-	-	-	-	-
Periostin	ng/ml	-	-	-	-	-	-	-	-	-
H.S.A	ng/ml	202	238.5	8635 (2357-24000)	30268	222	41.14	2328 (1098-4987)	24000	***<0.0001***
Fibrinogen Abcam	ng/ml	194	0	58.75 (7-160)	407	221	0	12 (3-32)	320	***<0.0001***
sRAGE	ng/ml	-	-	-	-	-	-	-	-	-


IL-6
IL-1b
fMLP
Ultimate ELTABA
MMP substrate
IL-8
MMP-9
MMP-8
HNE
NGAL
Calprotectin
MPO
Healthy
Stable COPD
Stable CF (1)
Stable CF (2)
UTI Pos (susp)
UTI Recovered

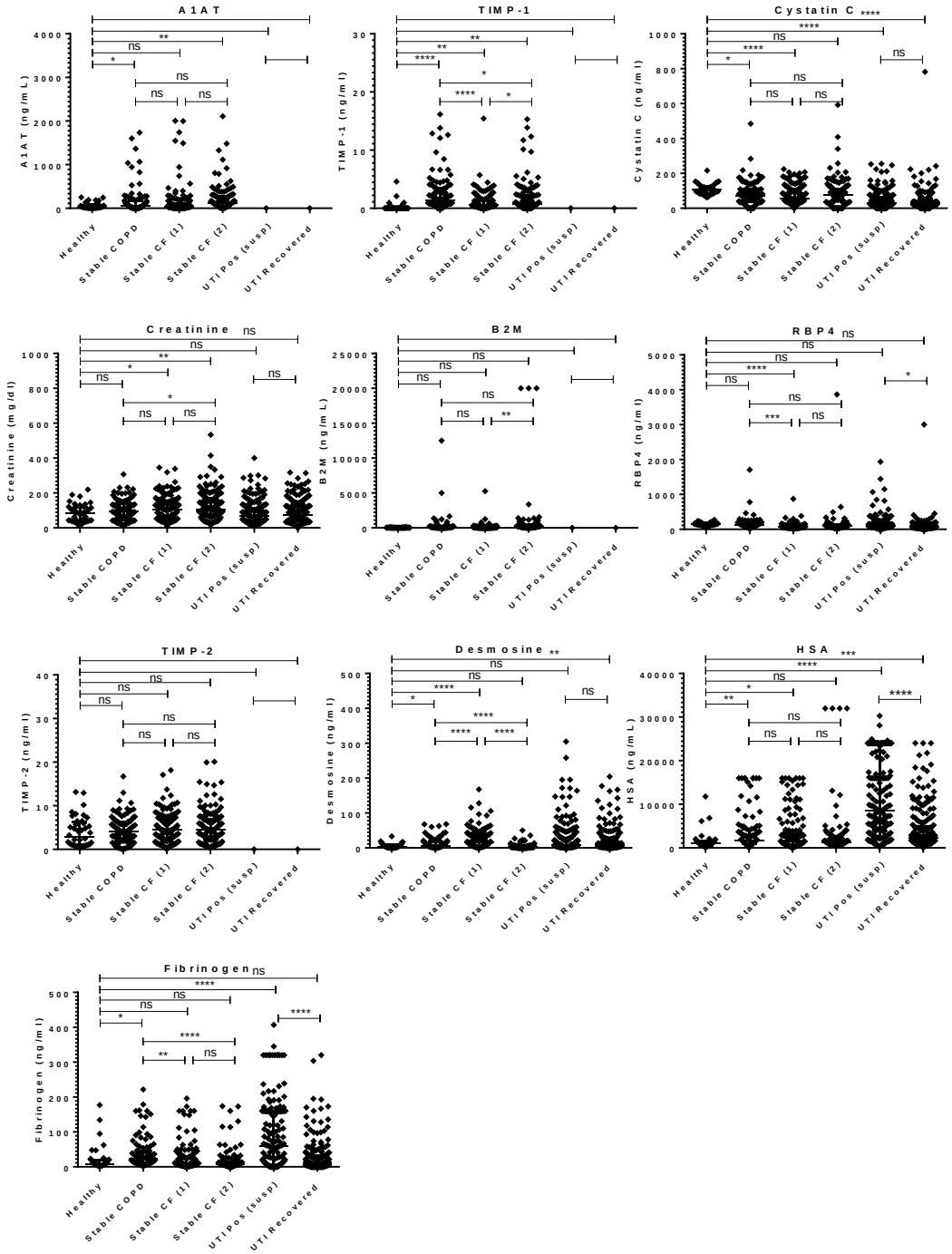


Figure 3.2. Scatter plots showing the difference between all 5 cohorts. Scatter plots for healthy controls, stable COPD, stable CF (2 cohorts) and UTI (subdivided into those with suspected UTI and recovered) for each biomarker are shown. Median with interquartile range shown for each plot. Unpaired t tests are displayed for each combination, >0.05 = not significant (ns), degree of significance indicated by *, 1 star being just significant to 4 stars being very significant.

Table 3.9. Summary table from all studies and significance levels. Unpaired t tests are displayed for each combination, >0.05 = not significant (ns), degree of significance indicated by *, 1 star being just significant to 4 stars being very significant.

	Healthy vs. stable COPD	Healthy vs. stable CF (1)	Healthy vs. stable CF (2)	Healthy vs. suspected UTI	Healthy vs. recovered UTI	Stable COPD vs. stable CF (1)	Stable COPD vs stable CF (2)	Stable CF (1) vs Stable CF (2)	Suspected UTI vs recovery
IL6	*	ns	ns	ns	ns	ns	ns	ns	****
fMLP	*	ns	****	ns	ns	***	****	****	ns
IL-1β	**	ns	ns	**	ns	*	***	**	****
Ultimate ELTABA	****	****	***	****	***	ns	****	****	****
MMP substrate	****	****	ns	ns	*	*	****	***	ns
IL-8	*	*	**	***	ns	*	**	ns	****
HNE	ns	ns	ns	****	*	**	**	ns	****
MMP-8	ns	**	*	**	ns	**	ns	**	****
MMP-9	*	**	ns	****	ns	ns	ns	ns	****
NGAL	ns	**	*	****	**	ns	ns	**	****
Calprotectin	ns	ns	ns	-	-	ns	ns	ns	-
MPO	ns	ns	-	-	-	ns	-	-	-
A1AT	*	ns	**	-	-	ns	ns	ns	-
TIMP-1	****	**	**	-	-	****	*	*	-
Cystatin C	*	****	ns	****	****	ns	ns	ns	ns
Creatinine	ns	*	**	ns	ns	ns	*	ns	ns
B2M	ns	ns	ns	-	-	ns	ns	**	-
RBP4	ns	****	ns	ns	ns	***	ns	ns	*
TIMP-2	ns	ns	ns	-	-	ns	ns	ns	-
Desmosine	*	****	ns	ns	**	****	****	****	ns
HSA	**	*	ns	****	***	ns	ns	ns	****
Fibrinogen	*	ns	ns	****	ns	**	****	ns	****

3.3.3 Biomarker correlations

Correlation matrices were performed with each study using GraphPad Prism, each study consisted of different number panels of biomarkers and number of participants.

There were clear biomarkers that correlated in all cohorts and some that may have been influenced by gender as shown by figure 3.3 – 3.7.

In the healthy samples there were 2 groups or clusters of biomarkers that correlated with a spearman's r> 0.7.

Cluster 1: MMP-8, MMP-9, HNE, NGAL, Calprotectin, MPO and IL-8 of which the greatest correlation was with MMP-9, HNE and MPO.

Cluster 2: Cystatin C, Creatinine, B2M, RBP4, TIMP-2, desmosine and fMLP of which creatine and TIMP-2 gave the greatest correlation.

The correlations observed in the 'healthy' cohort extended to all the other cohorts, however, with further stratification with gender, it was established that the strength of the correlations was influenced by gender for some biomarkers. for example, as shown in figure 3.4, IL-8 remained in group 1 only in females as did TIMP-1. Active HNE also seemed to negatively correlate in the male group compared to the female.

In general cluster 1 correlation was stronger in females and cluster 2 correlation was stronger in males and this was qualified through all the COPD and CF cohorts.

Healthy samples n = 40

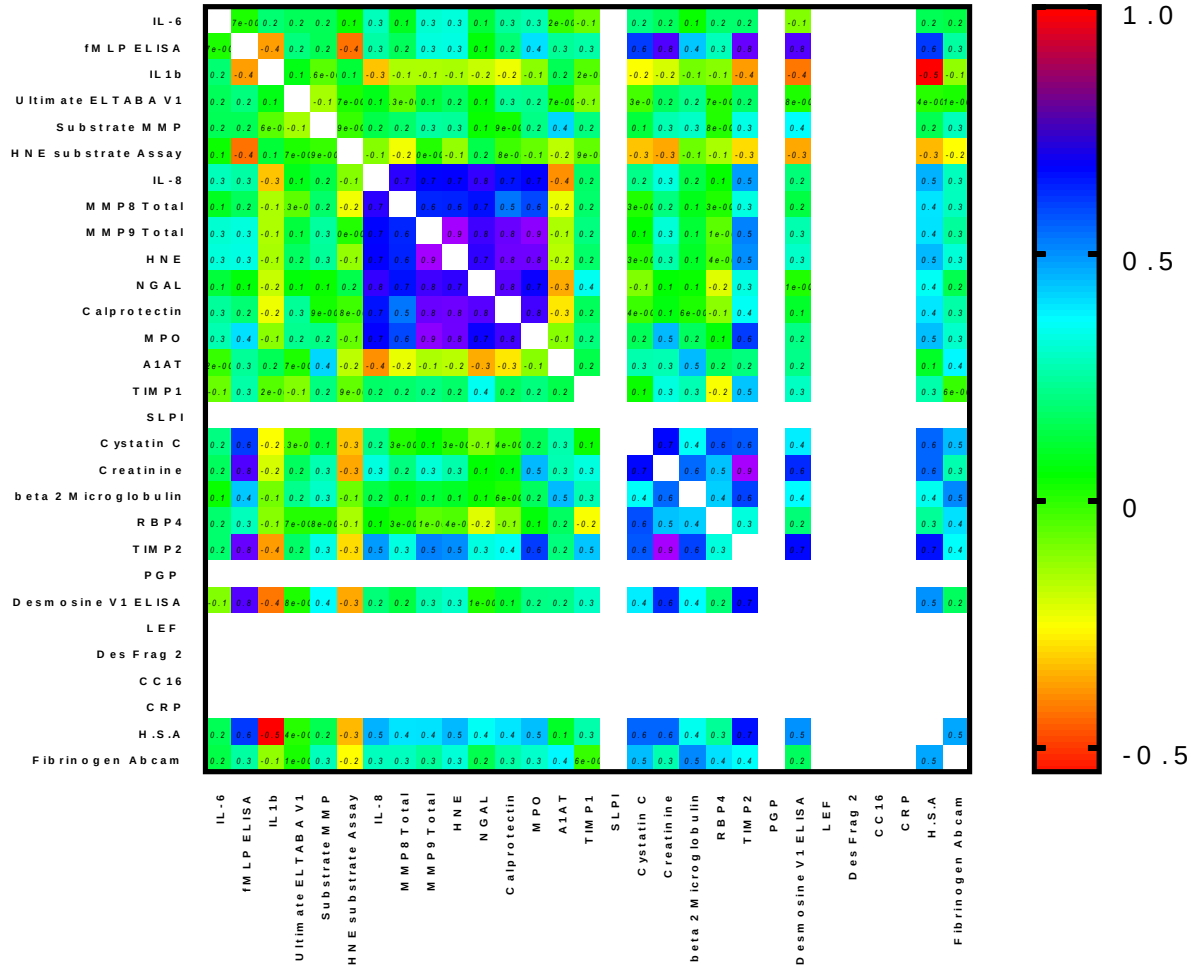


Figure 3.3. Heat map for biomarker correlation in healthy volunteers. Scale of colour is shown on the right, purple with Spearman's r closer to '1' green for no correlation and towards red for a negative correlation. Spearman's r values shown on heatmap. This scale was used for all the remaining heat maps.

COPD ECLIPSE n = 100

ECLIPSE FEMALE n = 41

ECLIPSE MALES n=59

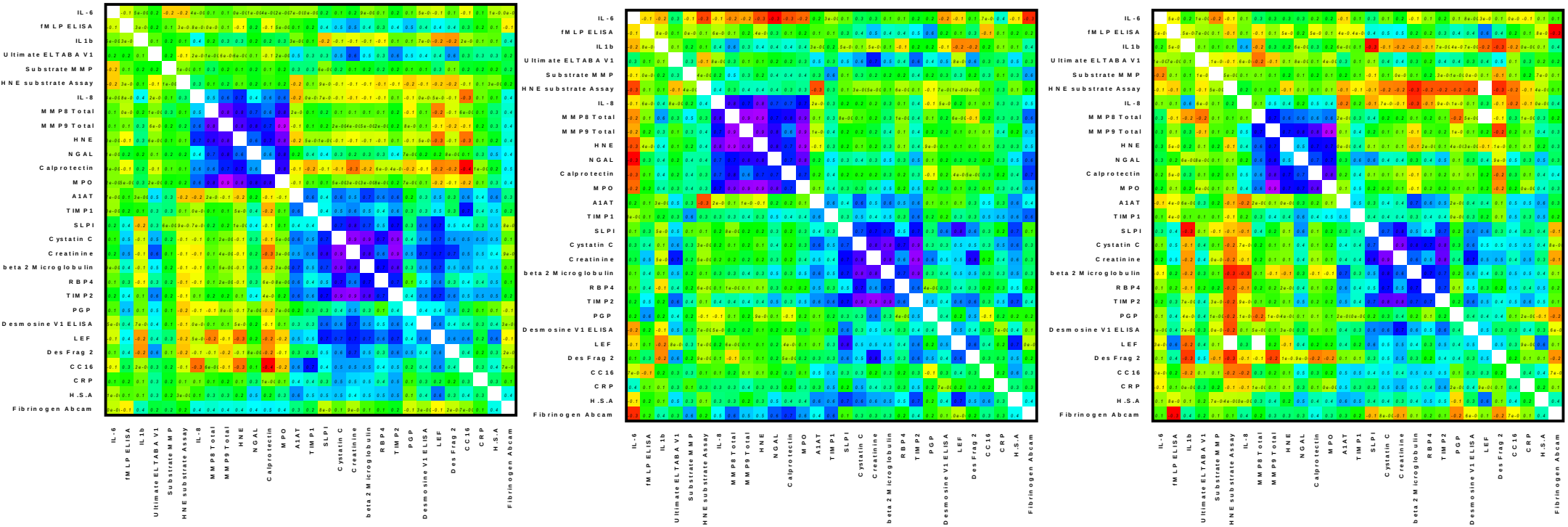


Figure 3.4. Heat map for biomarker correlation in COPD stable samples, shown with all samples then stratified by gender. Scale of colour is shown on the right, purple with Spearman's r closer to '1' green for no correlation and towards red for a negative correlation. Spearman's r values shown on heatmap.

CF Imperial n= 157

CF-imperial Females n= 71

CF-imperial Males n= 86

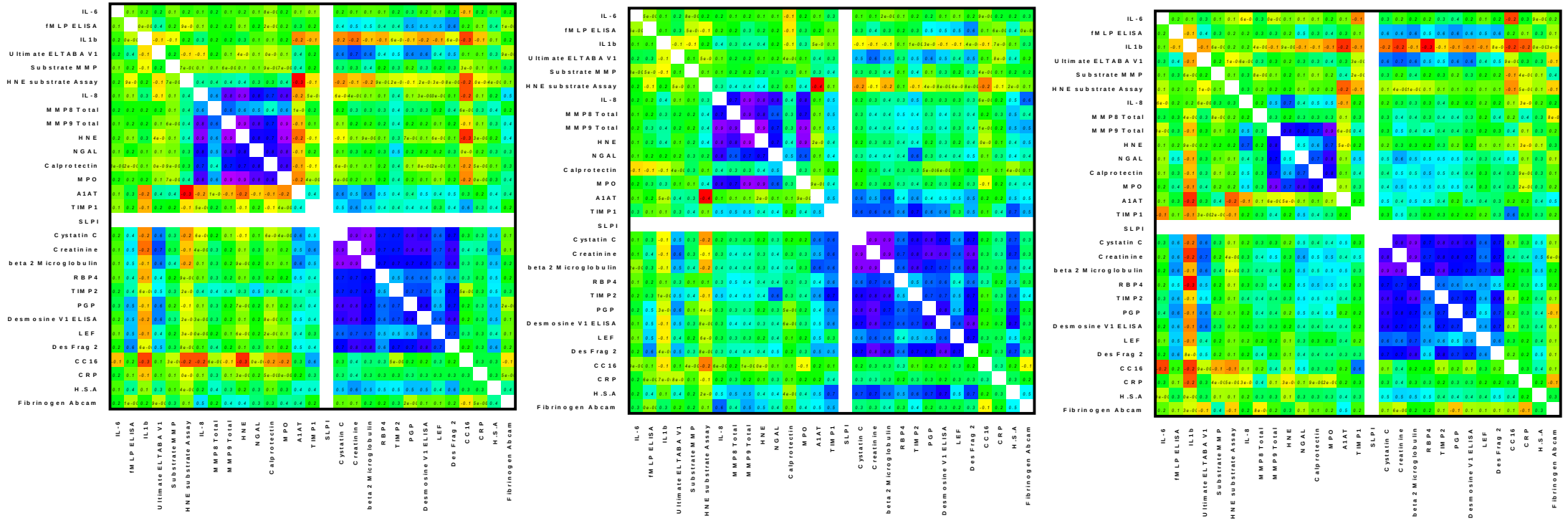


Figure 3.5. Heat map for biomarker correlation in CF stable samples (1) shown with all samples then stratified by gender. Scale of colour is shown on the right, purple with Spearman's r closer to '1' green for no correlation and towards red for a negative correlation. Spearman's r values shown on heatmap.

CF Belfast n = 129

CF Belfast Females n = 54

CF Belfast Males n = 75

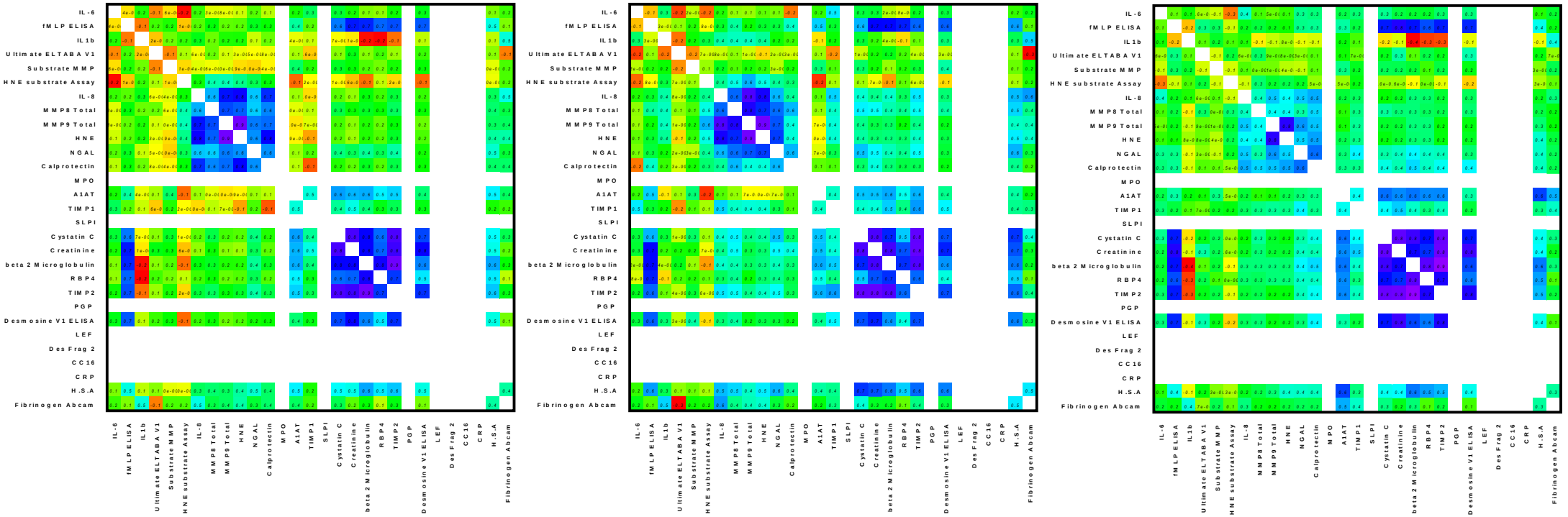


Figure 3.6. Heat map for biomarker correlation in CF stable samples (2) shown with all samples then stratified by gender. Scale of colour is shown on the right, purple with Spearman's r closer to '1' green for no correlation and towards red for a negative correlation. Spearman's r values shown on heatmap.

3.3.4 Gender specific urinary biomarkers

Biomarkers were analysed to determine influence of gender. Mann Whitney test was used to evaluate significance between the two groups. Sixteen of the biomarkers were found to be gender specific.

In females, higher levels of IL-8, MMP-9, HNE, Calprotectin and MPO were significant for all cohorts, COPD and CF. IL-1β, MMP-8, NGAL, and fibrinogen were found only to be significant in the CF cohorts.

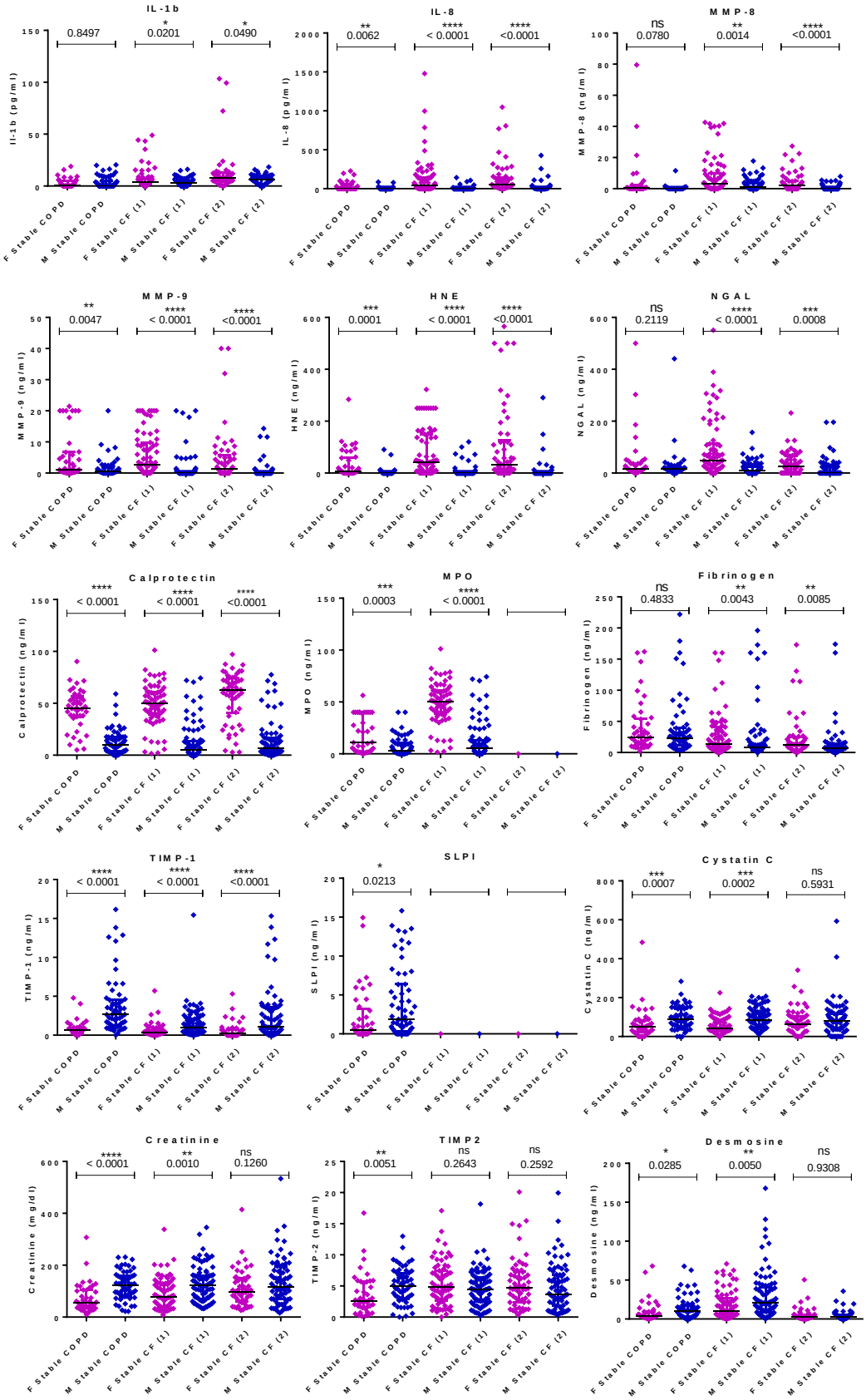
In males, higher levels of TIMP-1, SLPI, Cystatin C, Creatinine, TIMP-2, Desmosine, LEF and CC16 were found in the COPD cohort. Higher levels of TIMP-1 and Creatinine were found in both CF cohorts, whereas Cystatin C and Desmosine were only found to be significantly higher in one of the CF cohorts. CC16 was only tested in 1 of the cohorts but likely to be significant in both based on the p-value. SLPI was not tested in any of the CF cohorts.

In summary, there are strong gender specific biomarkers with p values that go below 0.0001 for some biomarkers. Females exhibit higher levels of IL-8, MMP-9, HNE, Calprotectin and MPO were obtained and in males, higher levels of TIMP-1, Creatinine and CC16 were found

3.3.5 Influence of age

To explore if there were any biomarkers associated with age, the CF cohort 1 was analysed and findings were confirmed with the Belfast CF data. The data were split into 2 groups, age group <18 (n = 78) with a median of 13yrs with an age range of 10yrs to 18yrs and age group >18yrs (n=79) with a median of 28yrs with an age range of 18-59yrs. Analysis into gender and age was also explored. There were 7 biomarkers that showed a significant difference $p < 0.05$ (unpaired t test) described in table 3.10.). These were MMP activity (Ultimate ELTABA), TIMP-2, Cystatin C, Ac-PGP, Desmosine, LEF and CC16. The only biomarker that appeared to increase with age was CC16. When sub dividing these into gender, biomarkers associated with females and age were Ultimate ELTABA, Ac-PGP, and LEF and those that were associated with males and age were IL-1β, TIMP-2, Cystatin C, Ac-PGP, desmosine and CC16.

Interestingly, if we to take the biomarkers where the gender difference in the CF cohort was not as significant compared to COPD i.e. TIMP-1, Calprotectin and CC16, and stratified gender differences by age, it is notable with aging (the more significant the result), this may explain why in COPD the gender difference is more significant (with less outliers) (figure 3.9).


IL-1b
Il-1b (pg/ml)
0.8497
*
0.0201
*
0.0490
IL-8
IL-8 (pg/ml)
**
0.0062
****
< 0.0001
****
<0.0001
MMP-8
MMP-8 (ng/ml)
ns
0.0780
**
0.0014
****
<0.0001
MMP-9
MMP-9 (ng/ml)
**
0.0047
****
< 0.0001
****
<0.0001
HNE
HNE (ng/ml)
***
0.0001
****
< 0.0001
****
<0.0001
NGAL
NGAL (ng/ml)
ns
0.2119
****
< 0.0001
***
0.0008
Calprotectin
Calprotectin (ng/ml)
****
< 0.0001
****
< 0.0001
****
<0.0001
MPO
MPO (ng/ml)
***
0.0003
****
< 0.0001
Fibrinogen
Fibrinogen (ng/ml)
ns
0.4833
**
0.0043
**
0.0085
TIMP-1
TIMP-1 (ng/ml)
****
< 0.0001
****
< 0.0001
****
<0.0001
SLPI
SLPI (ng/ml)
*
0.0213
Cystatin C
Cystatin C (ng/ml)
***
0.0007
***
0.0002
ns
0.5931
Creatinine
Creatinine (mg/dl)
****
< 0.0001
**
0.0010
ns
0.1260
TIMP2
TIMP-2 (ng/ml)
**
0.0051
ns
0.2643
ns
0.2592
Desmosine
Desmosine (ng/ml)
*
0.0285
**
0.0050
ns
0.9308
F Stable COPD
M Stable COPD
F Stable CF (1)
M Stable CF (1)
F Stable CF (2)
M Stable CF (2)

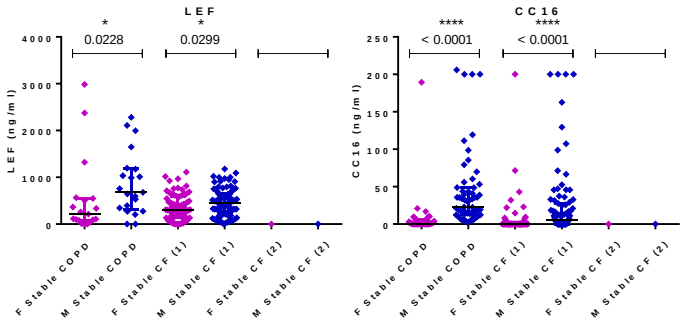


Figure 3.7. Detailed analysis of gender-biased biomarker values, showing spread and significance levels. Scatter plots showing the difference in each biomarker between females (F) and males (M) across 3 cohorts (COPD and CF). Median with interquartile range shown for each plot. Unpaired t tests displayed for each combination, >0.05 = not significant (ns), degree of significance indicated by *, 1 star being just significant to 4 stars being very significant.

Table 3.10. Summary table displaying biomarker levels with age and gender. Median and interquartile ranges and p values unpaired t-tests for selected biomarkers of interest. Groups are subdivided into gender and age (18yrs being the cut off). Significant p values <0.05 highlighted in bold.

Biomarker	**All data**			**Females**			**Males**		
	<18yr n = 78	>18yr n =79	P value	<18yr n = 36	>18yr n = 35	P value	<18yr n = 42	>18yr n = 44	P value
Il-1β	4.7 (1-7.2)	2.4 (0.3-4.8)	0.6558	4.7 (2.6-6.4)	4.1 (1.2-6.6)	0.4613	4.7 (0-7.7)	2.1 (0-4.4)	***0.0024***
Ultimate ELTABA	50.8 (23-91.7)	27.5 (9.7-52.5)	***0.0133***	36.7 (14.8-83.3)	16.3 (8.8-29.6)	***0.0242***	63 (36.1-95.7)	32.7 (13.4-63.7)	0.1716
TIMP-2	5.1 (3.3-7.5)	4.3 (1.8-6.2)	***0.0033***	4.9 (3.3-9.5)	4.7 (1.8-7.1)	0.1461	5.4 (3.2-7.3)	4 (1.6-5.7)	***0.0063***
Cystatin C	81 (37.7-126)	44.2 (28.1-93.6)	***0.0033***	46.7 (33-94.8)	34.2 (21.5-70.3)	0.0781	105.3 (43.4-153)	71.9 (36.1-103.3)	***0.0093***
Ac-PGP	1175 (790-1870)	861.6 (519.1-1284)	***0.0021***	1030 (692.2-1514)	759.9 (475-1162)	***0.011***	1492 (905.1-2296)	1044 (595.9-1508)	***0.0338***
Desmosine EIA	21.8 (8.7-45.8)	11.1 (3.7-24.8)	***0.0002***	12.7 (6.3-37)	6.1 (2.5-23.5)	0.0509	27.7 (13.7-48.4)	14.8 (4.9-25.8)	***0.001***
LEF	557.2 (314.5-777.6)	417.9 (203.7-689.2)	***0.0056***	528.9 (338.4-734.1)	357.7 (161.6-649.7)	***0.0360***	602.7 (311.1-866.7)	444.3 (242.9-760.2)	0.0688
CC16	0 (0-3.2)	5.7 (0-23.0)	***0.0368***	0 (0-0)	0 (0-4.5)	0.4847	0 (0-8.2)	14.5 (5.2-43.3)	***0.0478***

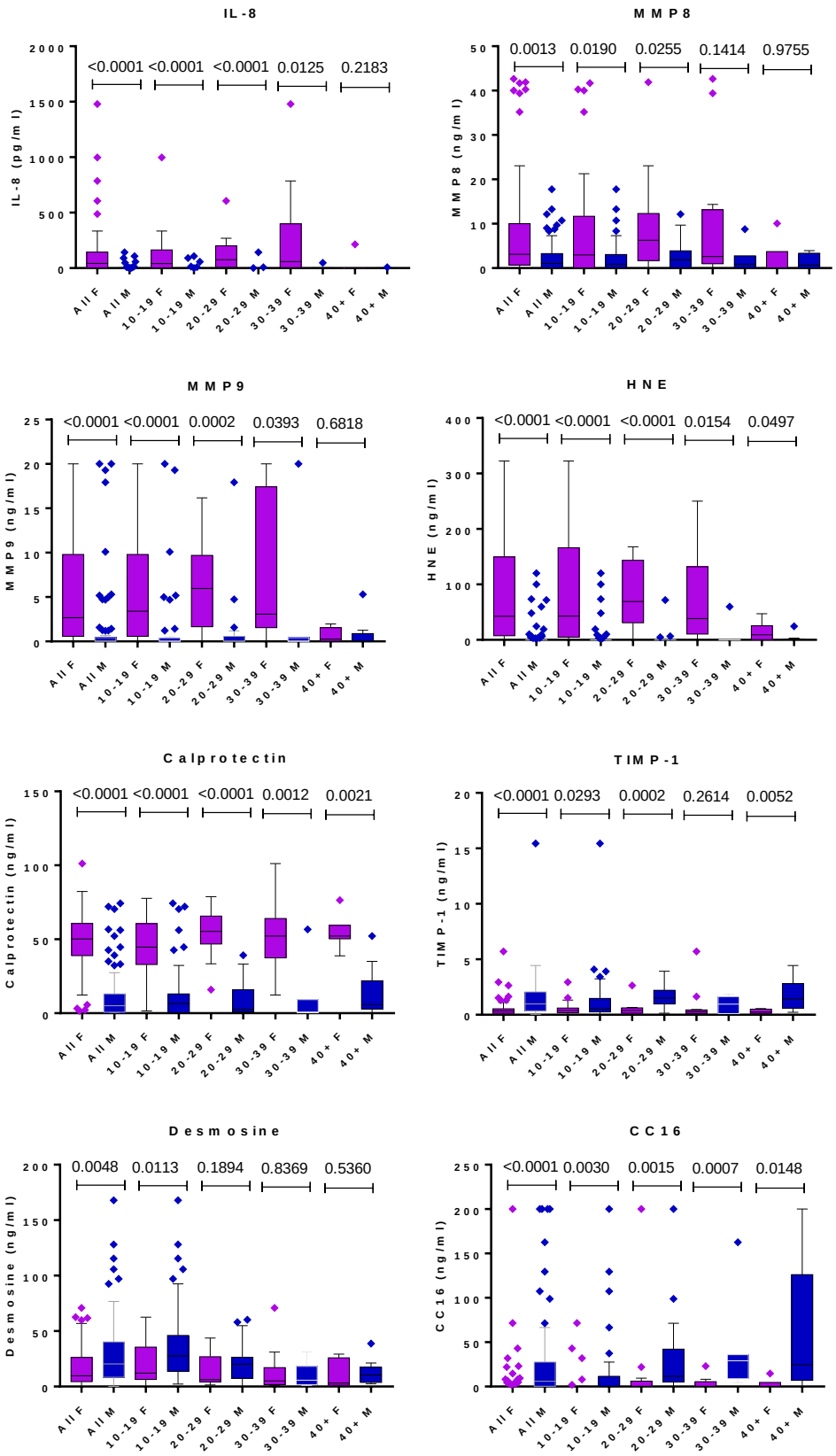


Figure 3.8. Box and Whiskers plot for stable CF (1) cohort stratified by age and gender. Non-parametric test Mann Whitney p values <0.05 deemed to be significant. Median and interquartile ranges shown.

3.3.6 Biomarkers and frequency of exacerbations

Repeated exacerbations of COPD are associated with a faster decline in lung function and poor health status. Currently the single best predictor of exacerbation is the patient's own history of exacerbations. To better predict and understand exacerbations, biomarkers associated with COPD exacerbations have been intensively investigated with limited success. Evaluated was the potential of a single biomarker or combination of urine biomarkers to characterize exacerbation status and improve the prediction of future COPD exacerbations. The donor group was divided into two subgroups of subjects, based on their apparent status as frequent or infrequent exacerbators. Patients were assigned according to the number of pulmonary exacerbations (PEx) experienced in year 1. The infrequent group consisted of subjects who experienced 1 or no PEx, and the frequent group comprised those individuals who had 2 or more in the first year (the maximum being a total of 8). These patients were either treated with oral corticosteroids (OCS) and/or antibiotics, either in hospital or at home. Table 3.3 presents the main demographical and clinical characteristics of the two groups at baseline. From the ECLIPSE data supplied to us, it was concluded that there were no significant differences in the baseline characteristics between infrequent and frequent exacerbators, except for the exacerbation frequency itself.

Urine samples donated by 98 COPD subjects enrolled in the ECLIPSE study were evaluated. Half of the donors had been identified as frequent exacerbators (n=49) and half as infrequent exacerbators (n=49) on the basis of their exacerbation rate in the first year of the study.

- Of the 49 subjects in the frequent exacerbation group, sixteen had $\leq$1 exacerbations prior to year one and 2-7 exacerbations in year 1.
- Of the 49 subjects in the infrequent exacerbation group, 1 had $\geq$1 exacerbations prior to year one.

3.3.6.1 Single biomarker analysis

At timepoint 1, the only biomarker showing promise was IL-1β with a p value of 0.02. The finding was confirmed in a second cohort from Birmingham cohort (chapter 4) where IL-1β was shown to be significantly higher in the frequent exacerbator group compared to the infrequent group. Results shown in figure 3.10.

3.3.6.2 Multiplex biomarker analysis

Combinations of markers giving the best discrimination between infrequent and frequent exacerbator groups were identified by logistic regression. Analysis using logistic regression allows for the examination of independent covariates (markers) in relation to a binary outcome. The analysis

started using a saturated model (all covariates included) with the number covariates reduced by exclusion based on their level of significance until only the significant ($p<0.05$) covariates remained. In this instance a backward elimination was more successful than a forward selection. Diagnostic accuracy was evaluated by AUC. A combination of 10 biomarkers was selected – Composite MMP activity, Pro MMP-9, TIMP-1, desmosine, fibrinogen, IL-6, IL-8, IL-1β, Cystatin C and A1AT with post-bronchodilator FEV1 %-predicted and SGRQ score (quality of life 50 item questionnaire). This panel differentiated the infrequent and frequent groups (specificity, 91.5%; sensitivity, 90.6%; AUC = 0.92), as shown in Figure 3.10, below.

The model correctly predicted 13 of the 16 subjects who converted from infrequent to frequent exacerbation, with a sensitivity of 81.3% and the one subject from frequent to infrequent state. These 13 subjects would have been missed on the basis of exacerbation history but were correctly identified by these biomarkers.

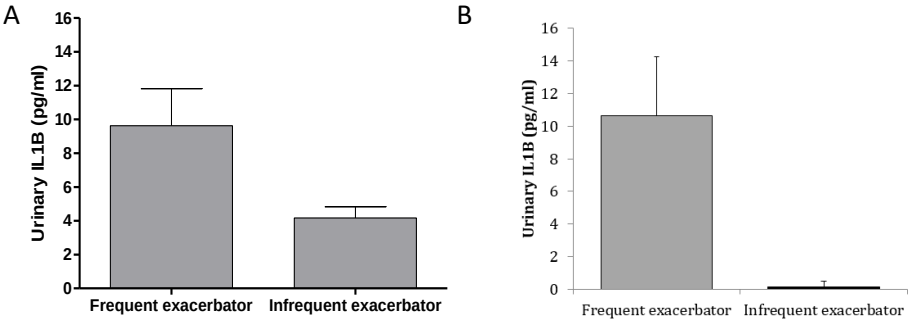


Figure 3.9. Urine marker IL-1β differing significantly between COPD subgroups in the stable state. The graph shows the difference in urinary IL1β between frequent and infrequent exacerbators a) COPD ECLIPSE samples (p-0.0156) and b) Birmingham COPD samples ($p<0.001$).

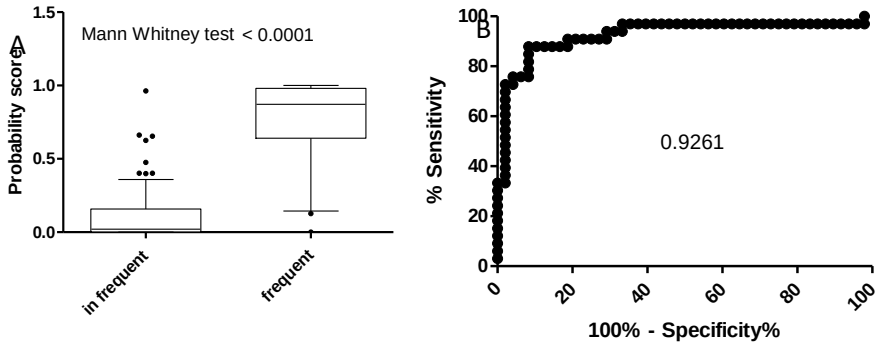


Figure 3.10. Statistical analysis of the data from 10 urinary biomarkers combined with FEV1% predicted. A) Box and whiskers plot, risk scores derived from the algorithm applied to all data B) ROC curve with AUC of 0.923 obtained in differentiation between infrequent and frequent exacerbations

3.4 Discussion

These results clearly show that the selected biomarkers can be measured in urine, have distinctive trends when associated with other biomarkers and there is strong evidence for individual thresholds. It is also apparent that with appropriate subgrouping selected biomarkers can define frequent and infrequent exacerbators.

Associations with low level inflammation found in stable COPD and CF

In relation to the biomarkers found in healthy samples, there were 15 biomarkers that were significantly different in either COPD or CF with a p value <0.05. There were 11 biomarkers associated with UTI, 9 of which overlapped with both COPD and CF thus leaving 2 biomarkers that were specific to UTI (HNE and RBP4). To note the other 9 biomarkers that overlapped with both COPD and CF were the proteases and signalling molecules and non-specific molecules (MMP-8, MMP-9 (both active and pro form), IL-1β, IL-6, IL-8, fibrinogen and HSA) which left just 5 markers specific to COPD/CF which comprised of all the protease inhibitors, a signalling molecule and a consequence molecule (Cystatin C, TIMP-1, A1AT, fMLP and desmosine). It can be concluded that there are biomarkers (n=15) that are associated with both stable COPD and CF in relation to healthy state and biomarkers (n=5) that remain unchanged. With regards to the specificity, it is clear that the signalling molecules are also associated with a UTI, however it is less clear regarding the protease and protease inhibitors. Although it appears from the data that proteases are more strongly associated with UTI in relation to the inhibitors, both protease and inhibitors are influenced by gender and the UTI cohort is entirely female whereas the other cohorts between 50-60% males.

Correlations: Cluster 1: IL-8, MMP-8, MMP-9, HNE, NGAL, Calprotectin and MPO (of which the greatest correlation was with MMP-9, HNE and MPO) had the strongest correlation in females and cluster 2: fMLP, Cystatin C, Creatinine, B2M, RBP4, TIMP-2 and desmosine (of which creatine and TIMP-2 gave the greatest correlation) had the strongest correlation in Males. This was demonstrated in 4 study cohorts – normal, stable COPD and stable CF. Group 1 consists of neutrophil released biomarkers and group 2 consists of protease inhibitors, degradation molecules and other molecules less defined. These defined correlations are strong with some of the markers exhibiting high Spearmans Rank r values > 0.7, tested in multiple cohorts to show repeatability, if the correlations are disrupted in more severe COPD states then it would increase the knowledge as to which biomarkers are involved and positioned in relevant biomarker pathways.

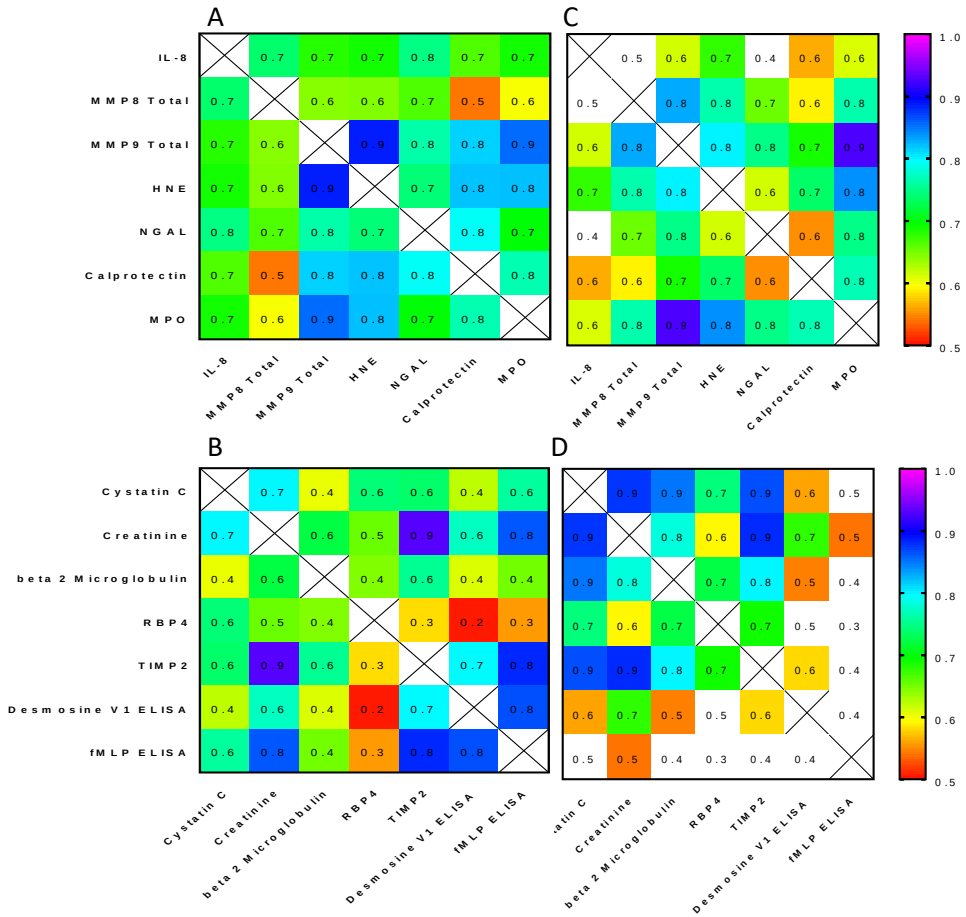


Figure 3.11. Correlations between biomarkers within clusters in healthy and stable COPD. A) healthy state cluster 1 B) Healthy state cluster 2 C) Stable COPD cluster 1 D) Stable COPD cluster 2. Spearmans rank r values shown with colour coded key ranging from red 0.5 to purple r =1.

Influence of gender: When stratifying the groups by gender the picture is further complicated by the fact that many of the markers were found to be gender-biased. This is relevant to findings reported in a previous paper on studies with the ECLIPSE cohort in which it was found that exacerbations were significantly more frequent in women with moderate COPD than men with moderate COPD. The results presented here are consistent with this reported gender bias in susceptibility to exacerbation, in that we have found higher levels of proteases and lower levels of inhibitors in female COPD. This biochemical imbalance is usually associated with inflammatory disorders and it is known what the consequence is of active proteases that are not inhibited. There have been some investigations focusing on gender specific markers in plasma and serum but results have been very limited, especially in association with COPD (147). There could be some links that could be explored further for example it was found in the ECLIPSE study that FEV1 decline, continued smoking and presence of emphysema were the strongest predictors of progression; CC16 was a potential biomarker for disease activity (decreased levels of CC16 related to more severe COPD) (148). The strongest predictors of emphysema

were continued smoking and female sex (149). In the urine testing that has been completed here it was found that there were marked gender differences with CC16, with lower levels in females when compared to males. Understanding how gender influences immunological mechanisms in health and disease and identifying gender-specific biomarkers would be invaluable in terms of targeted treatment.

Influence of Age:

There were 7 biomarkers that showed a significant difference $p < 0.05$ (unpaired t test) they were a mix of proteases, protease inhibitors, consequence molecules and CC16. There were not any differences observed with any of the signalling molecules. The only biomarker that appeared to increase with age was CC16. When sub dividing these into gender, biomarkers associated with females and age were Ultimate ELTABA, Ac-PGP, and LEF and those that were associated with males and age were IL-1β, TIMP-2, Cystatin C, Ac-PGP, desmosine and CC16. Further analysis by stratifying into smaller age groups did show better discrimination with ageing however, the numbers in these subgroups were limited.

Frequent and infrequent exacerbators: This study with urine samples from well-defined subjects in the ECLIPSE cohort enabled us to investigate and compare two different methods of identifying and predicting frequent and infrequent exacerbator status. The current and generally accepted method for prediction of exacerbation frequency is based on the actual number of exacerbation events in the previous year. Using this method of prediction at recruitment retrospectively gave a sensitivity of 67% and specificity of 98% (> 1 moderate/severe exacerbation in prior year). The urine algorithm developed modelling 10 biomarkers and 2 other non-biomarker tests applied to the samples collected at recruitment gave a substantial improvement, with sensitivity of 90% and specificity of 92% with the same subjects. This is the first-time urinary biomarkers in addition to clinical characteristics have been shown to predict frequency of exacerbations. This approach, if confirmed in larger cohorts, could be used to complement existing methods for monitoring disease activity and management of COPD exacerbations.

There was one single marker IL-1β that was able to differentiate with good significance between frequent and infrequent exacerbators in 2 different cohorts, ECLIPSE and samples from a Birmingham study (described in chapter 4) with p values of 0.0156 and <0.001 respectively.

These findings auger well for the wider use of urinary biomarkers in respiratory inflammatory disease or infections.

Chapter 4. Identification of biomarkers associated with COPD exacerbations

4.1 Introduction

The quest to identify a marker or a combination of markers associated with COPD exacerbations has been pursued for some time. Many groups have studied biomarkers in plasma, serum, sputum and bronchoalveolar lavage fluid (BAL fluid) to uncover markers that can not only predict an event but are also linked to disease severity and mortality. Repeated exacerbations are associated with a faster decline in lung function and it has been suggested that there may be a sub-group of patients that are more susceptible to exacerbations caused by persistently high levels of mediators that can be measured (23). One group found that IL-6, CRP and TNFα measurements were repeatable over a 12 month period in COPD patients and confirmed an association between IL-6 and CRP (CRP is regulated by IL-6) (150). Plasma CRP levels are increased in COPD patients and are associated with increased mortality as well as cardiovascular risk (151). TNFα has been shown to play a central role in the muscle wasting and weight loss seen in COPD patients (150). In another study, plasma CRP levels combined with a major exacerbation symptom such as dyspnoea, sputum volume, or sputum purulence was found to be useful in predicting exacerbation severity (152). CRP again was highlighted in another study where higher measurements in plasma were associated with greater perception of breathlessness during mobilisation. This led to the conclusion that CRP is an important predictor of future exacerbation and hospitalisation (153). Previous testing of the ECLIPSE blood samples led to the conclusion that elevations in white blood cell (WBC) count, CRP, IL-6, IL-8, fibrinogen and TNFα, if persistent, could be associated with worse clinical outcomes. Fibrinogen was associated with poor survival, risk of exacerbation and poor clinical outcome (23). This was also observed in a study looking at serum biomarkers in inflammation (IL-6, IL-8, IL-16 and TNFα), repair and injury (MMP-9, VEGF) and chemoattractants (PARC, MCP-3), but there was no significant increase with rising disease severity (154). In a study exploring recovery and recurrence at COPD exacerbation (155) it was found that the time-course of systemic inflammation following exacerbation was different between frequent and infrequent exacerbators, and that a high serum CRP concentration after 14 days following an exacerbation may be used as a predictor of recurrent exacerbations within 50 days. Conversely, a similar study evaluated plasma CRP and IL-6 levels over 3 years and found no difference in CRP concentrations but did find that IL-6 elevation was persistent, progressive and associated with worsening of symptoms and mortality (156).

Although clinical predictors of death have not been investigated in this thesis, a previous ECLIPSE study (157) has shown that a combination of WBC, IL-8, fibrinogen, CCL-18/PARC and SP-D measurements could significantly improve the ability to predict mortality in patients with COPD. A different marker not mentioned above was sputum IL-1β (14) (AUC, 0.89; 95% CI, 0.83-0.95). This mediator was also identified in the chapter 3 as a promising urinary biomarker. It was found in the

previous published study, that IL-1β, together with serum CXCL10 and peripheral eosinophil count, were biomarkers of bacteria-, virus-, or eosinophil-associated exacerbations of COPD (14). Other markers in sputum have been well studied, including MPO, elastase, leukotriene-B_4, IL-8, SLPI and CRP. SLPI was significantly lower in the frequent exacerbators. There have been numerous studies of biomarkers in BAL fluid, gathered through an invasive and unpleasant sampling technique. It was found that BAL fluid IL-8 was significantly higher in COPD patients with frequent exacerbation than infrequent (P=0.001) n=39. MPO, and TNFα were also measured and a significant difference was found with these markers when compared to controls.

Apart from urinary desmosine, no other urinary biomarker has been associated with COPD exacerbations. Desmosine and isodesmosine (by products of lung elastin degradation) have been found to be raised in exacerbations above the level found in the stable disease state (158).

Urine samples were obtained from 3 separate studies aimed to explore urinary biomarker profiles in stable, exacerbation and recovery states.

- QEH Birmingham, Samples were collected at QEH Birmingham at time of exacerbation then at subsequent days back to recovery – 7, 14 and 56.

- GSK AERIS – GSK provided sample sets of urine collected from a subgroup of COPD subjects participating in the AERIS study (the so-called “nasopharyngeal swab” cohort). Urine samples were provided from each planned monthly clinic visit during the first 12 months of the study for 35 patients. In addition, urine samples collected at the time of each unscheduled clinic visit for a COPD exacerbation was also provided (The study investigator made a clinical judgement that the subject was experiencing an exacerbation).

– Leicester BEAT-COPD study - blood and urine samples from COPD subjects were longitudinally collected at four visit types: namely stable state, exacerbation, two weeks post therapy and at recovery (six weeks post exacerbation visit).

4.2 Methods

4.2.1 Patient selection and assessment

For each cohort, the patient selection and assessment was varied, the reason for this was due to accessibly to clinical samples, all the samples were obtained from samples banks and it was not possible to specify inclusion and exclusion criteria or to contribute to the design of the clinical trials as they were all retrospective studies. Criteria were established based on the aims and objectives of each individual study. The details for each individual study are described below.

4.2.2 COPD subjects – QEH Birmingham

Patients with AECOPD admitted to 2 hospitals were approached from September 2012 to January 2014. Eligible patients had major symptom deterioration (sputum volume, sputum colour or dyspnoea) for ≥2 consecutive days and a documented clinical diagnosis of COPD. Exclusion criteria included lung cancer, interstitial lung disease, active pulmonary tuberculosis and pneumonia. The study was ethically approved (09/H1210/75) and informed consent taken within 24 hours of admission. Subjects underwent symptom and clinical assessment, completed the COPD assessment test (CAT), and had blood and a random urine sample (early morning) collected on admission (day 1) and at day 56. Urine was also collected at days 7 and 14. Symptom diary cards (Bronkotest®) were used, with the colour chart being used to define purulence of sputum, and post bronchodilator spirometry was performed at day 56.

4.2.3 COPD subjects – GSK AERIS study

Acute Exacerbation and Respiratory InfectionS in COPD (AERIS) was a prospective observational study funded by GSK. Participants were seen for an enrolment visit and then monthly for 2 years. In addition to these scheduled visits, all participants were seen in the clinic within 72 h (3 days) of onset of symptoms of AECOPD. AECOPD was defined as worsening of at least two major symptoms (dyspnoea, sputum volume and sputum purulence) or worsening of at least one major symptom and one minor symptom (wheeze, sore throat, cold (nasal discharge and/or nasal congestion), cough and fever (oral temperature >37.5°C) without other cause), considered clinically relevant at the site. Exacerbations were identified by means of electronic diary cards that participants completed daily. The data recorded daily in the electronic diary cards included self-performed peak flow measurement (peak expiratory flow (PEF) and FEV1), a series of morning questions to identify symptoms of exacerbations and the EXAcerbations of Chronic Pulmonary Disease Tool V.1.0 (EXACT-PRO) at bedtime. Data on patient-reported symptoms based on morning questions and on PEF/FEV1

were transmitted daily to the study clinic. Changes/worsening in these symptoms were monitored by the study staff and participants were contacted and invited to the clinic when an exacerbation was suspected.

Biological specimen collection. A wide range of biological specimens were collected from study participants, blood, sputum, nasopharyngeal swabs, breath and urine were collected. Urine samples were collected from all patients at study entry and at exacerbation and from a subcohort of 30 patients at monthly follow-up visits during the first year and at exacerbation.

Study procedures. In addition to the daily monitoring undertaken through the patient-completed electronic diary cards, a wide range of study procedures were performed at study entry, scheduled monthly visits and exacerbation visits (table 4.1).

4.2.4 COPD subjects – Leicester BEAT-COPD study

Samples (banked, frozen) were provided from a previous University of Leicester study (MRC funded BEAT-COPD (Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbations) study ISRCTN2422949).

Study details: From a two-staged single centre study, blood, sputum and urine samples from COPD subjects were longitudinally collected at four visit types: namely stable state (defined as being eight weeks free from an exacerbation visit), exacerbation (defined according to Anthonisen criteria [Anthonisen 2006] and healthcare utilisation), two weeks post therapy and at recovery (six weeks post exacerbation visit). Exacerbations were treated with oral corticosteroids and antibiotics according to guidelines or trial study design. Clinical data including demographics, symptoms, lung function, inflammatory profiling in blood and sputum, bacteriology including standard culture, qPCR for common pathogens and microbiomics, viruses by PCR and fungal culture were undertaken.

4.2.5 Laboratory methods

Blood samples were analysed for white cell count and C-reactive protein measurement as per usual care, and serum and plasma were isolated by centrifuge (10 minutes, 3000rpm) before storage at -80°C. Sputum samples were sent for standard laboratory microscopy, culture and sensitivity analysis where patients were able to produce a sample. Urine samples were stored at -80°C before transfer to Mologic Ltd. for testing.

4.2.6 Biomarker measurements

Urine samples were transferred to Mologic and stored at -80°C until analysis. The samples were analysed with the assays reported in Chapter 2 if available at the time. For the earlier studies, limited assays were undertaken as new assays were not introduced until a later date. The samples, once thawed, were tested on the same day.

4.2.7 Statistical analysis

All data were analysed using SPSS (version 21), GraphPad PRISM Version 7. Data normality was explored, and appropriate parametric or non-parametric tests chosen accordingly. Receiver-operator characteristic (ROC) analysis and Wilcoxon's signed rank test, Mann-Whitney or students t-test with significance levels $p<0.05$ was used to compare biomarker levels in different disease states, subgroups and gender. Logistic regression and decision tree analysis was used to develop predictive models, combining biomarkers that determined the outcome of exacerbation. Internal validation was addressed by dividing the cases into 80% training set and 20% test set. This process was repeated 5 times using assignment to training and validation sets by random number generation in SPSS.

4.3 Results

4.3.1 Patient characteristics

Combined patient characteristics are shown in table 4.1. Matched demographics were not possible across all three studies as the criteria were different for each study and CRF. There was not one criterion that stood out as being different except that the Leicester study recruited more males 65% compared to 54% for the other two studies. Comorbidities were variable but this information was not available for the Birmingham study so a complete comparison was not possible.

4.3.2 COPD subjects – QEH Birmingham

86 patients were consented to the study at the start of an exacerbation. A high proportion of patients re-exacerbated (n=13, based on their report or diary card) within the follow up period, thus a stable state 56 day sample was not available. Other losses to follow up included 4 patients who died, 2 who withdrew and 7 who failed to attend their day 56 appointment; this left 56 patients remaining in the study. 1 patient who had PiZZ AATD was excluded from analysis. Characteristics of the patients included in the final biomarker analyses (both day 0 and stable day 56 urine available) are shown in table 4.1. From exacerbation to recovery, CAT score improved (-6.8 (1.5); $p<0.0001$) whilst CRP fell in 54% patients and was static in the rest (median difference -1.0, $p=0.458$).

4.3.3 COPD subjects – GSK AERIS study

From the sub study of 37 participants, separated by gender, it was observed that males had a higher BMI, creatinine levels and procalcitonin whereas females had higher cholesterol, cholesterol HDL and platelets as shown by Mann-Whitney and unpaired t tests.

4.3.4 COPD subjects – Leicester BEAT-COPD study

One hundred fifty-six patients were enrolled; 145 (101 male, 70%) completed the first visit and 115 completed 12 months. For the urine analysis, 55 patients were selected, 66% were male, baseline clinical characteristics are shown in table 4.1. At baseline 0%, 23%, 22%, and 7% had GOLD I, II, III, and IV, respectively.

Table 4.1. Characteristics of the patients included in the paired sample analyses for all three studies. Data are shown as Number (%), mean (SD) or mean (SE*)

Criteria		Birmingham Cohort	AERIS	Leicester BEAT-COPD
		Baseline	Baseline	Baseline
		N= 56	N = 37	N = 55
Age yrs.	Mean (SE*) or (SD)	69.34 (1.39*)	69.16 (7.2)	72.8 (29.4)
Male	No (%)	31 (55%)	20 (54%)	36 (66%)
Smoking, pack-years	Mean (SE*) or (SD)	38	43.62 (18)	46 (4*)
Current smokers	No (%)	20 (36%)	9 (24%)	15 (27%)
BMI, kg/m2	Mean (SE*) or (SD)	-	27.02 (5.6)	24.0 (1.5*)
Frequent exacerbators (≥2 pa)	No (%)	43 (77%)	21 (57%)	-
mMRC Score	Mean (SE*) or (SD)	-	-	3.1 (0.9)
SGRQ-C Total Score	Mean (SE*) or (SD)	-	-	55.5 (18.9)
Exacerbations in year prior to recruitment	Mean	-	-	0.8
Emphysema	No (%)	32 (57%)	20 (54%)	-
Hypertension	No (%)	-	16 (43%)	-
Cardiovascular disease	No (%)	15 (27%)	7 (19%)	-
Hx Osteoporosis	No (%)	-	3 (8%)	-
Diabetes	No (%)	11 (20%)	3 (8%)	-
Physiology		-	-	-
FEV1 (L), post-BD	Mean (SE*) or (SD)	1.10	1.1 (0.4)	1.3 (0.5)
FVC (L), post-BD	Mean (SE*) or (SD)	-	2.8 (0.7)	2.6 (0.8)
FEV1/FVC, post-BD	Mean (SE*) or (SD)	-	45 (12.5)	51.1 (1.0*)
Oxygen sat	Mean (SE*) or (SD)	-	95.1 (2.1)	-
OLD GOLD Risk Index				
Mild [1]	No (%)	-	0	0
Moderate [2]	No (%)	-	15 (41%)	23 (42%)
Severe [3]	No (%)	-	17 (46%)	22 (40%)
Very severe [4]	No (%)	-	5 (14%)	7 (13%)
Inflammatory biomarkers				
White Blood Cell Count (X 106 /ml)	Mean (SE*) or (SD)	-	7.9 (1.7)	-
Serum creatinine (mg/dl)	Mean (SE*) or (SD)	82.17 (5.15*)	72 (19.2)	81.4

4.3.5 Individual Biomarker measurements

In this section, the individual biomarkers according to the disease state were reported.

- Biomarker profiles from exacerbation to recovery (QEH Birmingham)
- Biomarker profiles from stable to exacerbation (Leicester BEAT-COPD)
- Longitudinal profiles – Pre-exacerbation/exacerbation/post exacerbation (GSK AERIS)

4.3.5.1. Birmingham study

Only samples that had a matched exacerbation and recovery sample at day 56 were included in the analysis. Data from 56 patients were taken forward, however, not all biomarkers were measured for each sample, the number of samples tested are reported in table 4.2.

Using paired t-test analysis and Wilcoxon matched-pairs pair signed rank test, markers that were significantly different between exacerbations and recovery states were calculated. The p values are shown in Table 4.2, criteria of values <0.05 were deemed significant. There were six biomarkers that were significantly different from exacerbation to recovery states, in order of significance these were HSA, A1AT, TIMP-1, fibrinogen and RBP4. For cystatin C, the p value was 0.0507, this value was deemed to be significant.

4.3.5.2 Leicester study

In total 1216 urine samples were tested, of which, 427 sample were classified as stable, 168 as exacerbation samples, 89 as pre-exacerbation samples, 138 as 2-week recovery samples and 96 as 4-6-week recovery samples. From a total of 85 patients there were 168 PEx events, not all of them had a 2-week and 4-week recovery sample. Some of the patients also had other stable samples collected within the 1 year but some of these samples were not deemed to be 'stable' based on the close proximity collected to a reported exacerbation. From this cohort, 55 patients were identified with stable timepoints and enough collection points to establish a baseline. These patients and samples were taken forward for further analysis.

Using paired t-test analysis and Wilcoxon matched-pairs pair signed rank test, markers that were significantly different between stable and exacerbation states were calculated. The p values are shown in Table 4.3, and values <0.05 were deemed significant. There were thirteen biomarkers that were significantly different from stable to exacerbation states, in order of significance these were A1AT, creatinine, CRP, cystatin C, CHI3L1, fibrinogen, TIMP-2, calprotectin, NGAL, CC16, TIMP-1, MMP-9, RNASE-3.

4.3.5.3. GSK AERIS study

35 patients had samples collected over 1 year (a total of 454 samples were tested, 106 Exacerbation samples, 24 not recovered and 324 'stable').

71 exacerbation events were selected, on the basis that each event had a 'pre- exacerbation' and a 'post exacerbation' sample, allowing longitudinal tracking. It should be noted that not all pre or post samples were deemed to be stable or recovery samples respectively due to the close proximity to the date of when the exacerbation sample was collected.

- Pre-exacerbation sample was collected between 3-66 days before the exacerbation
- Post exacerbation sample was collected between 6 and 73 days after the exacerbation event

Using paired t-test analysis and Wilcoxon matched-pairs pair signed rank test, markers that were significantly different between exacerbations and pre-exacerbation and post-exacerbation were calculated. The markers were also normalised with creatinine. The median and interquartile ranges are shown in table 4.4 and the p values are shown in Table 4.5 with values <0.05 deemed significant.

Paired t tests with raw data (no normalisation). There were two biomarkers that were significantly different from pre-exacerbation to exacerbation states, in order of significance these were CRP and creatinine. There were five biomarkers that were significantly different from exacerbation to post-exacerbation, in order of significance these were CRP, Active MMP (as measured with Ultimate ELTABA*), calprotectin, Creatinine, A1AT.

Paired t tests with creatinine normalised data. There were three biomarkers that were significantly different from pre-exacerbation to exacerbation states, in order of significance these were CRP, LEF and calprotectin. There were five biomarkers that were significantly different from exacerbation to post-exacerbation, in order of significance these were fMLP, CRP, LEF, IL-6, IL-1β.

Wilcoxon matched-pairs signed rank test with raw data (no normalisation). There were two biomarkers that were significantly different from pre-exacerbation to exacerbation states, in order of significance these were CRP and A1AT. There were nine biomarkers that were significantly different from exacerbation to post-exacerbation, in order of significance these were CRP, A1AT, HSA, CC16, Ultimate ELTABA*, Creatinine, NGAL, fibrinogen and desmosine.

Wilcoxon matched-pairs signed rank test with creatinine normalised data. There was only one biomarker that was significantly different from pre-exacerbation to exacerbation states- CRP. There were two biomarkers that were significantly different from exacerbation to post exacerbation states, in order of significance these were CRP and fMLP*.

* Significance was also obtained with pre-exacerbation in relation to post exacerbation.

Table 4.2. Birmingham study; Biomarker results at exacerbation and recovery state. The table shows the results in exacerbation and recovery state for each urinary marker and the paired statistical test results for each marker between exacerbation and recovery state. Since most were non-normally distributed the data is shown as median (IQR). Significant p values <0.05 highlighted in bold

		Exacerbation		**Recovery**		**Paired t test**
	Unit	Number of values	Median (IQR)	Number of values	Median (IQR)	p value
IL-6	pg/ml	54	2.561 (0-7.665)	54	2.998 (0-8.647)	0.268868
fMLP ELISA	ng/ml	28	2.164 (0.4358 -7.287)	28	1.91 (0.5318-5.717)	0.859535
IL1b	pg/ml	51	1.137 (0-4.088)	51	2.765 (0.01-7.002)	0.789275
Siglec 8	ng/ml	-	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-	-
Ultimate ELTABA	ng/ml	39	347 (205-827)	36	444 (236-910)	0.477047
MMP Substrate assay	ng/ml	54	3.695 (0-13.6)	54	1.42 (0-10.12)	0.307046
HNE substrate Assay	ng/ml	19	83 (0-15830)	22	4506 (0-10685)	0.875767
IL-8	pg/ml	54	0.201 (0-34.86)	54	6.704 (0-48.93)	0.422378
MMP-8 Total	ng/ml	54	0.2545 (0-4.377)	50	0.195 (0.01425-6.385)	0.492476
MMP-9 Total	ng/ml	54	0.5735 (0.09825-4.845)	50	0.5605 (0.2518-8.284)	0.293608
HNE	ng/ml	54	13.99 (3.194-85.14)	50	15.62 (4.045-247)	0.281369
NGAL	ng/ml	54	33.63 (8.97-80.59)	50	29.71 (11.52-57.55)	0.771887
Calprotectin	ng/ml	53	26.9 (10-59)	49	37.6 (11-59.96)	0.124275
MPO	ng/ml	20	2.338 (0.9538-27.94)	18	10.41 (3.711-40)	0.075856
RNASE-3	ng/ml	-	-	-	-	-
A1AT	ng/ml	55	255.3 (27.6-759.2)	55	126.8 (15.52-326.4)	***0.019691***
TIMP-1	ng/ml	55	2.936 (1.435-8.312)	55	2.351 (0.762-5.293)	***0.025183***
SLPI	ng/ml	-	-	-	-	-
Cystatin C	ng/ml	54	89.75 (54.82-219.2)	54	88.16 (24.68-142.9)	***0.050729***
Creatinine	mg/dl	55	69.85 (40.7-105.4)	55	57.1 (33.8-104.4)	0.582562
beta 2 Microglobulin	ng/ml	53	281.9 (144.9-991.7)	49	147.2 (68.04-455.9)	0.129004
RBP4	ng/ml	51	196.5 (127.5-473.7)	51	147.8 (83.04-296.9)	***0.046883***
TIMP-2	ng/ml	55	4.194 (2.268-8.114)	55	3.342 (1.387-7.319)	0.178329
Ac-PGP	ng/ml	-	-	-	-	-
Desmosine V1 ELISA	ng/ml	54	20.45 (2.675-44.43)	53	17.41 (2.25-49.51)	0.994632
LEF	ng/ml	-	-	-	-	-
Desmosine fragments	ng/ml	-	-	-	-	-
CC16	ng/ml	-	-	-	-	-
CRP	ng/ml	-	-	-	-	-
Periostin	ng/ml	-	-	-	-	-
H.S.A	ng/ml	51	4194 (1270-15344)	50	2321 (667.6-8475)	***0.006548***
Fibrinogen Abcam	ng/ml	54	11.75 (4.423-30.55)	54	13.89 (5.281-58.39)	***0.034147***
sRAGE	ng/ml	-	-	-	-	-

Table 4.3. Leicester study; Biomarker results at stable and exacerbation state. The table shows the results in stable and exacerbation state for each urinary marker and the paired statistical test results for each marker between stable and exacerbation state. Since most were non-normally distributed the data is shown as median (IQR). Significant p values <0.05 highlighted in bold. For this data set, all '0' values were substituted with the Lower Limit Of Detection (LLOD) for each assay as indicated.

Biomarker assay	Unit	LLOD	Stable n=55	Exacerbation n=55	Paired t-test
IL-6	pg/ml	1.6263	1.63 (1.63-1.63	1.63 (1.63-3.86)	0.2991
fMLP ELISA	ng/ml	0.075	3.10 (0.08-7.29)	2.96 (0.08-10.64)	0.0657
IL1b	pg/ml	0.3697	24.74 (14.78-29.56)	24.89 (17.26-29.31)	0.3860
Siglec 8	ng/ml	0.78	179.90 (110.10-263.70)	222.60 (114.60-306.20)	0.2074
Chitinase 3 like protein	ng/ml	0.0007	0.03 (0.00-0.12)	0.06 (0.01-0.53)	***0.0055***
Ultimate ELTABA	ng/ml	3.9	65.23 (27.32-140.00)	77.74 (30.17-135.60)	0.7258
MMP Substrate assay	ng/ml	7.8125	7.81 (7.81-58.67)	7.81 (7.81-53.70)	0.8976
HNE substrate Assay	ng/ml	0.027	0.03 (0.03-0.03)	0.03 (0.03-0.03)	0.1483
IL-8	pg/ml	2.83	2.83 (2.83-2.83)	2.83 (2.83-2.83)	0.2791
MMP-8 Total	ng/ml	8.52	64.74 (8.52-193.70)	103.70 (8.52-734.70)	0.0557
MMP-9 Total	ng/ml	6.78	306.70 (38.61-1077	347.10 (53.10-2505)	***0.0409***
HNE	ng/ml	0.0695	0.64 (0.07-2.74)	0.88 (0.07-4.53)	0.0826
NGAL	ng/ml	0.0205	13.01 (5.88-24.89)	24.33 (6.88-41.53)	***0.0262***
Calprotectin	ng/ml	0.48	51.60 (0.48-253.60)	65.11 (0.48-349.10)	***0.0164***
MPO	pg/ml	10.37	4429 (1423-12386)	6328 (1152-24522)	0.0759
RNASE-3	pg/ml	16	16.00 (16.00-50.63)	16.00 (16.00-299.20)	***0.0465***
A1AT	ng/ml	0.164	44.14 (20.02-154.40)	123.80 (37.01-268.10)	***0.0001***
TIMP-1	pg/ml	14.305	1328 (424.20-3455)	1890 (530.90-5133)	***0.0361***
SLPI	ng/ml	0.1065	2.45 (0.11-8.78)	3.94 (0.69-11.16)	0.2236
Cystatin C	ng/ml	0.0002	31.90 (17.77-57.16)	70.11 (27.21-107.00)	***0.0025***
Creatinine	mg/dl	0.01	58.81 (29.71-93.38)	89.57 (40.16-128.10)	***0.0005***
beta 2 Microglobulin	ng/ml	0.02	16.34 (5.79-36.57)	49.69 (14.02-115.70)	0.0526
RBP4	pg/ml	11.72	48245 (18553-90095)	79464 (34107-167925)	0.5475
TIMP-2	pg/ml	1.5605	1899 (982.60-3519)	3121 (1415-5110)	***0.0136***
Ac-PGP	ng/ml	2.12	353.60 (207.70-652.20)	405.80 (213.20-632.80)	0.6569
Desmosine V1 ELISA	ng/ml	0.4095	37.54 (10.15-90.65)	41.67 (9.77-93.39)	0.0634
LEF	ng/ml	7.815	654 (354.80-1496)	1072 (356.20-2347)	0.0709
Desmosine fragments	ng/ml	0.41	1000 (1000-1000)	1000 (1000-1000)	0.1475
CC16	ng/ml	0.0101	17.60 (6.50-66.37)	33.90 (15.43-86.05)	***0.0284***
CRP	pg/ml	7.8125	7.81 (7.81-191.90)	107.70 (7.81-1268)	***0.0012***
Periostin	pg/ml	6.25	56.82 (6.25-139.70)	63.31 (6.25-165)	0.3202
H.S.A	ng/ml	1.25	2625 (1078-8423)	3399 (1923-10459)	0.6286
Fibrinogen Abcam	ng/ml	0.5	8.40 (3.93-34.57)	12.97 (6.50-37.37)	***0.0079***
sRAGE	ng/ml	0.01	0.03 (0.01-0.06)	0.03 (0.01-0.08)	0.1345

Table 4.4. AERIS study; Biomarker results at pre-exacerbation, exacerbation and post-exacerbation state. The table shows the results in all three states for each urinary marker. Since most were non-normally distributed the data is shown as median (IQR). Significant p values <0.05 highlighted in bold

Biomarker assay	**Unit**	**Median (IQR)**		
		Pre- exacerbation n=71	Exacerbation n=71	Post- exacerbation n=71
IL-6	pg/ml	0.06 (0-3.90)	0.59 (0-5.92)	0 (0-2.19)
fMLP ELISA	ng/ml	3.52 (1.77-5.79)	3.92 (2.20-6.50)	4.62 (2.43-6.77)
IL1b	pg/ml	2.11 (0-5.96)	3.14 (0-6.09)	2.68 (0-6.01)
Siglec 8	ng/ml	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-
Ultimate ELTABA	ng/ml	16.63 (0-46.63)	24.36 (0-48.08)	11.22 (0-34.55)
MMP Substrate assay	ng/ml	0 (0-2.41)	0 (0-2.109)	0 (0-2.28)
HNE substrate Assay	ng/ml	0 (0-0)	0 (0-0)	0 (0-0)
IL-8	pg/ml	2.823 (0-33.87)	2.67 (0-35.72)	1.453 (0-26.8)
MMP-8 Total	ng/ml	0 (0-1.89)	0.343 (0-1.709)	0 (0-1.22)
MMP-9 Total	ng/ml	1.185 (0-5.089)	0.92 (0-4.24)	0.706 (0-3.54)
HNE	ng/ml	3.99 (0-16.06)	3.538 (0-16.93)	1.366 (0-12.38)
NGAL	ng/ml	17.10 (5.47-46.68)	22.54 (4.05-56.31)	10.39 (3.77-24.89)
Calprotectin	ng/ml	35.67 (14.54-55.81)	36.03 (15.93-61.54)	33.16 (11.14-48.68)
MPO	ng/ml	9.57 (2.23-34.85)	6.93 (1.53-40.00)	4.69 (1.19-19.18)
RNASE-3	ng/ml	-	-	-
A1AT	ng/ml	70.14 (12.37-144.50)	106.70 (20.82-381.30)	61.10 (12.86-162.80)
TIMP-1	ng/ml	1.57 (0.71-4.19)	1.71 (0.71-4.65)	1.57 (0.70-3.65)
SLPI	ng/ml	-	-	-
Cystatin C	ng/ml	62.79 (32.64-100.30)	71.03 (46.06-121.60)	61.45 (32.90-92.94)
Creatinine	mg/dl	91.72 (51.96-130.90)	106.50 (72.00-161.70)	72.95 (53.97-118.80)
beta 2 Microglobulin	ng/ml	55.33 (28.03-100.80)	67.03 (36.80-128.30)	52.05 (26.03-80.21)
RBP4	ng/ml	94.70 (62.92-128.20)	103.10 (64.96-144.60)	94.49 (53.00-138.50)
TIMP-2	ng/ml	4.47 (1.93-6.79)	4.59 (2.68-7.50)	3.46 (2.08-6.59)
Ac-PGP	ng/ml	2425 (1068-4678)	3213 (1714-7168)	2350 (1027-5411)
Desmosine V1 ELISA	ng/ml	10.69 (2.48-21.66)	11.62 (4.36-28.21)	5.94 (2.79-18.68)
LEF	ng/ml	707 (301-1124)	663 (394-1039)	692 (410-1180)
Desmosine fragments	ng/ml	677.2 (199-1000)	748.9 (278.1-1000)	581.8 (187.2-1000)
CC16	ng/ml	5.97 (0-30.6)	10.84 (0-80.9)	8.762 (0-26.98)
CRP	ng/ml	211.5 (31.2-723.4)	1004 (149-3997)	237.6 (31.2-1012)
Periostin	ng/ml	-	-	-
H.S.A	ng/ml	2259 (747.7-5845)	2266 (868.5-8286)	1285 (527.5-4649)
Fibrinogen Abcam	ng/ml	18.78 (9.19-63.12)	24.42 (12.48-60.7)	18.97 (9.005-50.42)
sRAGE	ng/ml	-	-	-

Table 4.5. AERIS study; Pre-exacerbation vs exacerbation, exacerbation vs post exacerbation and pre-exacerbation vs. post exacerbation comparisons (p-values). No creatinine ratio vs. creatinine ratio and 2 different statistical tests performed – student paired t-test and Wilcoxon matched-pairs signed rank tests. Significant p values <0.05 highlighted in bold

Biomarker assay	**Unit**	**Paired t-test**						**Wilcoxon matched-pairs signed rank test**					
		No creatinine ratio			Creatinine ratio			No Creatinine ratio			Creatinine ratio		
		Pre-PEx to PEx	PEx to post PEx	Pre-PEx to Post-PEx	Pre-PEx to PEx	PEx to post PEx	Pre-PEx to Post-PEx	Pre- PEx to PEx	PEx to post PEx	Pre-PEx to Post-PEx	Pre-PEx to PEx	PEx to post PEx	Pre-PEx to Post-PEx
IL-6	pg/ml	0.9548	0.0680	0.2502	0.4522	***0.0248***	0.1894	0.2961	0.0153	0.1539	0.2804	0.0306	0.1442
fMLP ELISA	ng/ml	0.4592	0.6127	0.3101	0.2033	***0.0014***	0.4551	0.4032	0.5699	0.3625	0.0593	***<0.0001***	***0.0057***
IL1b	pg/ml	0.6224	0.7802	0.5617	0.3468	***0.0444***	0.5262	0.1914	0.9718	0.6433	0.8747	0.0632	0.2904
Siglec 8	ng/ml	-	-	-	-	-	-	-	-	-	-	-	-
Chitinase 3 like protein	ng/ml	-	-	-	-	-	-	-	-	-	-	-	-
Ultimate ELTABA	ng/ml	0.3564	***0.0080***	***0.0401***	0.8997	0.0628	0.1110	0.3819	***0.0143***	***0.0462***	0.4167	0.1561	0.274
MMP Substrate assay	ng/ml	0.2437	0.1751	0.7265	0.7103	0.5291	0.3965	0.7093	0.2688	0.6981	0.9886	0.795	0.3529
HNE substrate Assay	ng/ml	0.5548	0.2148	0.4679	0.2285	0.0907	0.7747	0.7209	0.8264	0.5562	0.3529	0.2069	0.4575
IL-8	pg/ml	0.6400	0.9822	0.6935	0.3161	0.4512	0.4710	0.8602	0.2276	0.6509	0.6288	>0.9999	0.907
MMP-8 Total	ng/ml	0.5384	0.3200	0.7168	0.1817	0.1648	0.9172	0.7038	0.5773	0.2427	0.234	0.9381	0.3377
MMP-9 Total	ng/ml	0.9308	0.7547	0.6617	0.2324	0.4615	0.5492	0.7687	0.5846	0.1501	0.2306	0.956	0.3001
HNE	ng/ml	0.1691	0.2131	0.7549	0.0890	0.1331	0.9088	0.4873	0.6353	0.0543	0.1969	0.732	0.1452
NGAL	ng/ml	0.6075	0.1385	0.5221	0.2114	0.5410	0.5684	0.7726	***0.02***	0.0705	0.3317	0.292	***0.0234***
Calprotectin	ng/ml	0.5310	***0.0284***	0.1723	***0.0361***	0.1351	0.4935	0.8711	0.0564	0.2097	0.1162	0.7049	0.5925
MPO	ng/ml	0.8231	0.1639	0.0727	0.0839	0.8475	0.1157	0.5765	0.3815	0.0784	0.1475	0.9589	0.1637
RNASE-3	ng/ml	-	-	-	-	-	-	-	-	-	-	-	-
A1AT	ng/ml	0.0998	***0.0496***	0.8936	0.8069	0.5920	0.5346	***0.0069***	***0.0045***	0.6168	0.1131	0.0509	0.9444
TIMP-1	ng/ml	0.5207	0.1000	0.3787	0.5620	0.6891	0.8500	0.9846	0.3215	0.2769	0.5804	0.822	>0.9999
SLPI	ng/ml	-	-	-	-	-	-	-	-	-	-	-	-
Cystatin C	ng/ml	0.0935	0.7411	0.4538	0.6885	0.5284	0.7684	0.2555	0.0689	0.5786	0.7956	0.1363	0.4633

Creatinine	mg/dl	***0.0446***	***0.0475***	0.9740	-	-	-	0.079	***0.0177***	0.6384	-	-	-
beta 2 Microglobulin	ng/ml	0.1987	0.5890	0.7372	0.2977	0.4869	0.8627	0.0661	0.0201	0.5884	0.7956	0.1363	0.4633
RBP4	ng/ml	0.0556	0.6915	0.3757	0.7761	0.8772	0.8973	0.1216	0.0916	0.9252	0.9796	0.6022	0.7262
TIMP-2	ng/ml	0.2670	0.1909	0.7500	0.3221	0.1901	0.8070	0.4501	0.2115	0.4198	0.4779	0.2406	0.6933
Ac-PGP	ng/ml	0.1519	0.1373	0.8272	0.4598	0.4624	0.9061	0.161	0.0829	0.9796	0.6345	0.6417	0.8176
Desmosine V1 ELISA	ng/ml	0.4496	0.1845	0.4381	0.4007	0.7739	0.3824	0.5516	***0.0322***	0.052	0.8891	0.1332	0.1071
LEF	ng/ml	0.7985	0.9592	0.8367	***0.0323***	***0.0123***	0.7875	0.65	0.9478	0.6507	0.1243	0.0751	0.8577
Desmosine fragments	ng/ml	0.5995	0.4921	0.8314	0.0536	0.2007	0.1841	0.5793	0.4203	0.8132	0.0714	0.2338	0.7606
CC16	ng/ml	0.0836	0.0121	0.3680	0.4831	0.3010	0.5773	0.1288	***0.007***	0.7628	0.3041	0.2312	0.6254
CRP	ng/ml	***0.0001***	***0.0027***	0.3509	***0.0001***	***0.0019***	0.3301	***<0.0001***	***0.0013***	0.6038	***<0.0001***	***0.0004***	0.3814
Periostin	ng/ml	-	-	-	-	-	-	-	-	-	-	-	-
H.S.A	ng/ml	0.1351	0.0774	0.8065	0.1648	0.7747	0.0989	0.3029	***0.0069***	0.3944	0.688	0.5215	0.0448
Fibrinogen Abcam	ng/ml	0.4198	0.3667	0.7458	0.1488	0.4578	0.4002	0.3952	***0.0214***	0.5402	0.6922	0.6062	0.3194
sRAGE	ng/ml	-	-	-	-	-	-	-	-	-	-	-	-

4.3.6 Multiplex biomarker analysis of exacerbations

4.3.6.1 QEH Birmingham study

An exacerbation and recovery sample was available for 55 patients and for each sample there were up to 23 biomarker measurements. For the multiple biomarker analysis, entering the raw concentration values for each state (exacerbation and recovery) into logistic regression analysis was not successful. This was expected as it was established from the analysis performed on stable samples in chapter 3 that individual threshold levels of biomarkers was essential and that trying to select population thresholds would be challenging due to the different baseline values that exists between people. For the single biomarker analysis, there were indications that certain biomarkers were able to differentiate between the different states with p values <0.05, these were HSA, A1AT, TIMP-1, fibrinogen, RBP4 and cystatin C. For the multiple biomarker analysis, a different method was used to analyse the data which involved using biomarker ''change'' from one state to the other and then adding biomarkers in a stepwise order until the optimal sensitivity was obtained.

In order to determine whether biomarker concentrations changed in the transition from exacerbation to recovery (day 56), the percentage difference was first calculated. The biomarker that identified a change greater than 10% for the majority of the 55 patients was TIMP-1 (36 cases, 65%), the next biomarker that could be added to this panel that identified a change greater than 10% from the remaining 35% of the group was Cystatin C, the additional 9 cases brought the percentage to 82%, the third biomarker, A1AT added an additional 5 cases which together amounted to 90%. This is illustrated in Figure 4.1. Single biomarkers provided no clear patterns. However, 3 urinary markers selected from each of the 3 pathophysiological pathways (NE driven, MMP driven, endothelial/renal dysfunction), namely A1AT, TIMP-1 and cystatin C, combined, it was possible to detect recovery in 90% cases. All three biomarkers individually showed a >10% decrease from exacerbation to recovery.

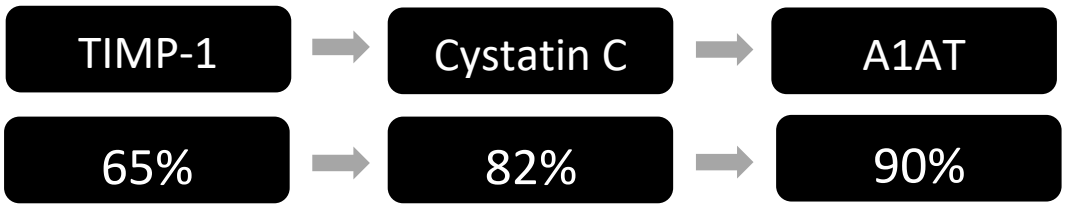


Figure 4.1: Birmingham study; Multiple urinary biomarker panel for AECOPD. The flow diagram shows the proportion of patients whose TIMP-1 fell with recovery. If patients whose Cystatin C fell were added, the proportion identified rose to 82%, and addition of a fall in A1AT improved this to 90%.

4.3.6.2 GSK AERIS study

No single marker was found to universally correlate with disease or its progression, but urinary CRP came very close. With the single biomarker analysis, CRP was significantly different pre-exacerbation in relation to exacerbation (p=0.0001) and exacerbation in relation to recovery (p=0.0027). As already identified, individual threshold values are critically important, as baseline values vary from patient to patient. Similar to the analysis done for the exacerbation – recovery samples in section 4.3.1.1, percentage change values were calculated from pre-exacerbation to exacerbation and then exacerbation to post exacerbation. CRP was the one biomarker that gave a greater than 10% change for the greatest number of cases out of 71. The remaining biomarkers were then examined to determine which added the most value and then a final biomarker was selected to create a panel of three biomarkers for identification of exacerbation and a panel of three biomarkers for identification of recovery state.

In summary, a combination of 3 markers was able to collectively group 94% of the exacerbation events into the exacerbation group relative to those in a stable group, and 93% in the recovery group post exacerbation i.e. increase at PEx and decrease at recovery. Urinary CRP and desmosine were common markers see figure 4.2a and figure 4.2b.

- Focussing on 'identifying the exacerbation event', CRP alone increased from baseline to exacerbation (>10% increase) for 44 of the 71 events, equating to 62% of the patient population, yet when combined with IL1b this was increased to 77.5% and further still to 87.3% with the addition of Desmosine.
- Focussing on 'identifying the recovery state', CRP alone decreased from exacerbation to recovery (>10% decrease) for 43 of the 71 exacerbation events, equating to 60.6%, yet when combined with Desmosine this was increased to 84.5% and to 90.1% with the addition of Fibrinogen.

This does not take into account the status of the stable sample i.e. pre-exacerbation and post-exacerbation, as some of the samples were collected less than a week before or after the event, as described in section 4.3.5.3, the minimum collection day prior to exacerbation was 3 days and post exacerbation was 6 days.

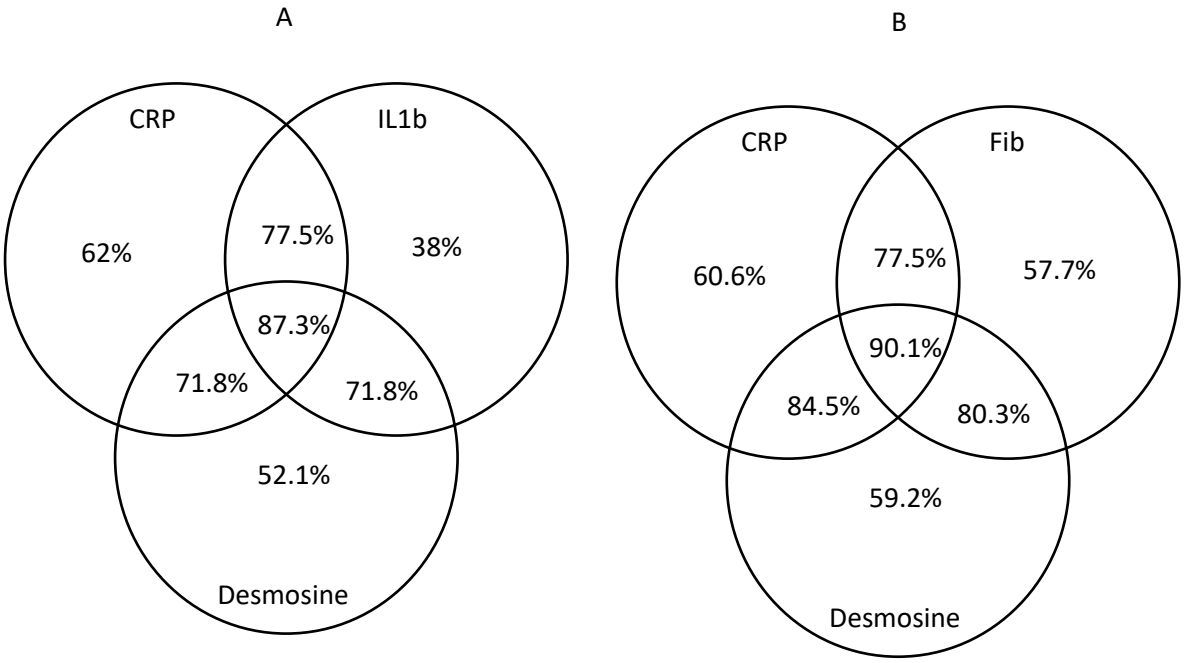


Figure 4.2. Diagrammatic representation of the value of each biomarker alone or in combination with other biomarkers. A) proportion of patients (%) with a 10% increase from pre- exacerbation to exacerbation. B) proportion of patients (%) with a 10% decrease from exacerbation to post-exacerbation.

4.3.6.3 Leicester COPD study- Multi-Marker assessment with baseline measurements (stable to exacerbation)

The approach taken for the statistical analysis closely resembled how the test would be used in practice which is to learn and track the biomarker profile that prevails during stable phases of the disease and determine whether the stable profile has shifted to an exacerbation profile by looking for a change in the biomarker levels (figure 4.3).

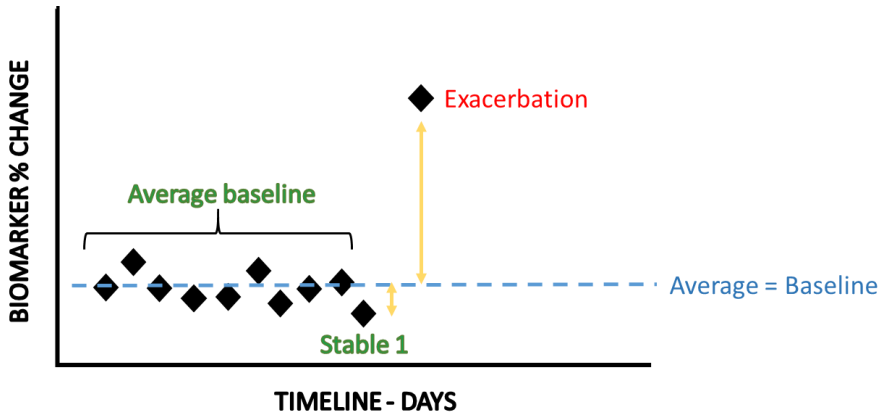


Figure 4.3. Example of how the analysis fits in with the proposed use of the test. An average of frequently tested samples to calculate the baseline and increase to exacerbation state.

For this analysis, one stable (S1) and one exacerbation sample (E1) were selected from each patient and an average of the remaining stable samples was used as the baseline (BL) sample. The percentage change of S1 and E1 was calculated from the baseline sample. The stable and exacerbation samples % change values were analysed for each biomarker for each patient using a variety of statistical methods to determine the combination of biomarkers that could differentiate between the stable and exacerbation states.

The distribution of the continuous variables was studied using histograms, values of skewness and kurtosis, and normality was tested by the Kolmogorov–Smirnov test. Paired t test and Wilcoxon matched-pairs signed rank test were used to compare quantitative data in the two groups. ROC analysis was used to study the accuracy of the various diagnostic tests and logistic regression to find the best combination of biomarkers. P values<0.05 were considered to be statistically significant. Statistical analyses were carried out through the use of computer IBM software SPSS 21 (Chicago, IL, USA), GraphPad Prism 5 and in R.

1. The data were analysed with all data and male and female separately
2. Paired t tests were performed using fold change of the log format, <0.05 was deemed significant

3. Those mediators which showed good discriminatory power at univariate level were taken forward for ROC analysis (fold change data)
4. Those with individual AUC of <0.4 and >0.6 were deemed significant
5. Logistic regression analysis was performed with % change values for stable vs. baseline and exacerbation vs. baseline with the selected markers to determine the best combination of markers

The criteria for selecting the biomarkers for logistic regression analysis was a significant parametric pairwise t-test ($p \leq 0.05$) and a ROCAUC ≥ 0.59 or ≤ 0.41 (see table 4.6). The biomarkers that met these criteria and that were taken forward for further analysis were IL-6, CHI3L1, MMP-8, NGAL, A1AT (ELISA and LF), TIMP-1, Cystatin C, Creatinine, B2M Abcam, RBP4, TIMP-2, Desmosine (V2 ELISA), CC16, CRP, Fibrinogen.

A backward stepwise regression was used, starting with all variables (all 16 from the list above) included the model. It then removed the least significant variable, that is, the one with the highest p-value, at each step, until all variables had been added. By scrutinising the overall fit of the model, variables were automatically removed until the optimum model was found.

Using logistic regression modelling, 5 biomarkers were selected that gave the best sensitivity and specificity. The combination of desmosine (V2 ELISA), CC16, CRP, MMP-8 and A1AT (LF) gave an AUC of 0.84 (95% confidence interval 0.76-0.92). With a cut off of 0.3959, sensitivity of 80% and specificity of 81.82 was obtained and a PPV of 81.48% and NPV of 80.36%.

Further analysis subdividing the groups by gender identified a further panel of biomarkers for incorporation in the multiplex panel.

For the females (n=19), the combination of fibrinogen, desmosine V2, CC16, TIMP-2 and MMP-8 gave an AUC of 0.83 (95% confidence interval 0.69 to 0.96). At an optimal cut-off of 0.408 sensitivity and specificity was 84.21 and 73.68 respectively and PPV of 76.19%and NPV 82.35%.

For the males (n=36), the combination of desmosine (V2 ELISA), CRP, MMP-8 and A1AT (LF) gave an AUC of 0.88 (95% confidence interval 0.79 to 0.96). At an optimal cut-off of 0.3887, the sensitivity and specificity were 83.3 and 80.6 respectively with a PPV of 81.48% and NPV of 80.36%. A second model with 6 biomarkers, fMLP (LF), CC16, desmosine (V2 ELISA), CRP, MMP-8 and A1AT (LF) (Figures not shown) gave an AUC of 0.89 (95% confidence interval 0.81-0.97). At an optimal cut-off of 0.3836 the sensitivity and specificity were 83.33% and 77.78% respectively with a PPV of 78.95 and NPV of 82.35.

Table 4.6. BEAT-COPD study; stable vs. exacerbation fold change values from baseline. Paired t test using log transformed data and ROC (AUC) for each analyte. Significant p values <0.05 highlighted in bold

Biomarker assay	Combined M+F		Females only		Males only	
	Paired t-test	AUC	Paired t-test	AUC	Paired t-test	AUC
IL-6	***0.0325***	**0.6025**	0.7403	0.4792	***0.0143***	***0.6601***
fMLP ELISA	0.4919	0.5407	0.8394	0.4820	0.2539	0.5748
IL1beta	0.5715	0.5045	0.1200	***0.6177***	0.5504	0.4460
Siglec 8	0.7582	0.5319	0.1387	***0.6039***	0.6083	0.4985
Chitinase 3 like 1	***0.0139***	***0.6172***	0.3288	0.5706	***0.0149***	***0.6435***
Ultimate ELTABA	0.7495	0.5327	0.2321	***0.6579***	0.2486	0.4776
Substrate MMP	0.6176	0.4855	***0.0385***	0.5776	0.1113	0.4352
HNE substrate Assay	0.2244	0.4970	0.4842	0.5623	0.1758	0.4637
IL8	0.0965	0.5529	0.0850	***0.6150***	0.4841	0.5251
MMP-8	***0.0170***	***0.6003***	***0.0477***	***0.6607***	0.1562	0.5714
MMP-9	0.1203	0.5481	0.5900	0.5526	0.1235	0.5459
HNE	0.0546	0.5945	0.2968	0.5748	0.1093	***0.6069***
NGAL	***0.0072***	***0.6169***	0.4778	0.5263	***0.0046***	***0.6551***
Calprotectin	0.3195	0.5681	0.5967	0.5263	0.1099	0.5853
MPO	0.5272	0.5380	0.2873	0.5983	0.9655	0.5181
RNASE3	***0.0339***	0.5848	***0.0033***	***0.7161***	0.4720	0.5093
A1AT ELISA	***0.0000***	***0.7240***	0.1030	***0.6316***	***0.0000***	***0.7685***
A1AT LF	***0.0001***	***0.7630***	***0.0088***	***0.7618***	***0.0057***	***0.7708***
TIMP-1	***0.0325***	***0.6446***	0.5620	0.5693	***0.0118***	***0.6779***
SLPI	***0.0268***	0.5917	0.2541	0.5693	0.0513	0.5914
Cystatin C	***0.0098***	***0.6747***	0.9160	0.5582	***0.0086***	***0.7157***
Creatinine	***0.0009***	***0.6460***	0.8226	0.5208	***0.0001***	***0.7037***
B2M Abcam	***0.0000***	***0.7398***	0.1786	***0.6399***	***0.0000***	***0.7847***
RBP4	0.1030	***0.6777***	0.7724	0.5762	***0.0008***	***0.7400***
TIMP-2	***0.0287***	***0.6271***	0.9889	0.5235	***0.0059***	***0.6775***
Ac-PGP	0.4728	0.5246	0.7481	0.4432	0.2325	0.5748
Desmosine V1 ELISA	0.2934	0.5656	0.8227	0.5291	0.2461	0.5826
Desmosine V2 ELISA	***0.0386***	***0.6210***	0.1452	0.5845	0.1022	***0.6424***
LEF	0.1934	***0.6003***	0.6122	0.5125	0.2361	***0.6350***
Desmosine Fragments ELISA	***0.0068***	0.4036	0.6134	0.5069	***0.0034***	***0.3665***
CC16	***0.0025***	***0.6405***	***0.0600***	***0.6302***	***0.0085***	***0.6451***
CRP	***0.0002***	***0.6463***	0.0873	***0.6302***	***0.0006***	***0.6578***
Periostin	0.7441	0.5145	0.1366	0.4169	0.1773	0.5856
Human Serum albumin	0.1174	0.5736	0.2202	0.5983	0.3282	0.5440
Fibrinogen Abcam	***0.0019***	***0.6380***	***0.0043***	***0.6620***	***0.0483***	***0.6296***
sRAGE	0.1492	0.5739	0.5511	0.4571	0.0540	***0.6335***

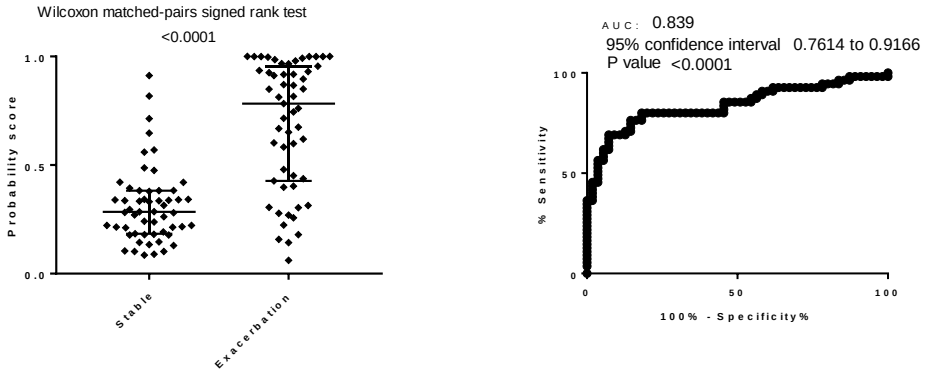


Figure 4.4. BEAT-COPD study; All data n=55 (male and female combined), discrimination between stable and exacerbation. (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values)

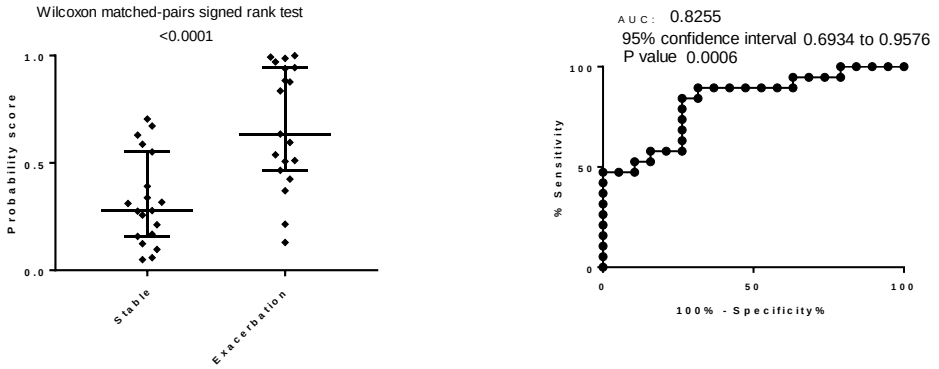


Figure 4.5. BEAT-COPD study; Female data n= 19, discrimination between stable and exacerbation. (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values)

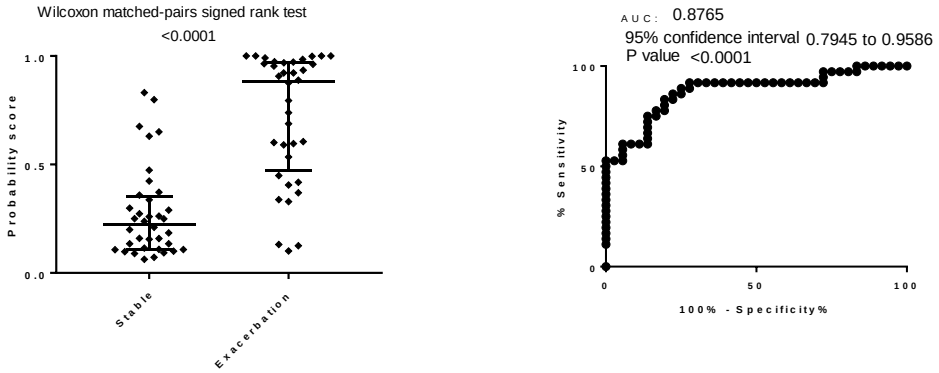


Figure 4.6. BEAT-COPD study; Male only n=36, discrimination between stable and exacerbation. (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values)

4.4 Discussion

In order to consolidate all these results together in one simple table, the most promising biomarkers were taken forward for discussion (table 4.7). Firstly, the biomarkers that showed significance between different disease states relating to stable, exacerbation and recovery were explored. Based on this analysis there were clear candidate markers, these were A1AT, and CRP which were significant across all groups, although CRP was not tested on the Birmingham cohort. Other candidate biomarkers were: TIMP-1, Cystatin C, Human serum albumin, Fibrinogen, NGAL, Calprotectin and CC16. The type of statistical analysis used in this case, only took into account population thresholds values but it has been established from the analysis performed on stable samples that individual threshold levels exist as a result of gender, severity of disease and even age as described in chapter 3 and likely to also be influenced by medication and comorbidities although this has not been proven.

The second analysis considered the percentage change from the stable and/or recovery sample. Biomarkers that showed a greater than 10% increase from stable state compared to exacerbation state or a greater than 10% reduction from exacerbation state compared to a recovered state were taken forward for multiplex biomarker analysis (table 4.7). This allowed for both individual thresholds and gender differences. Again, there were clear candidate markers, these being A1AT, RBP4 and B2M. Other candidate biomarkers were: TIMP-1, cystatin C, HSA, fibrinogen, CRP and desmosine. Combining both types of analysis, the four strongest biomarkers were CRP, A1AT, RBP4 and B2M.

The third study is probably the most relevant analysis. This took into account a near as possible baseline value for each patient by calculating a baseline based on 2 or more stable samples (for each biomarker), and then calculating the percentage change from the baseline for both a different stable sample and exacerbation sample. The differences between the stable and exacerbation % change values for each biomarker was then analysed using paired t tests and ROC analysis. When selecting biomarkers from this list that were significant, this brought in line other potential biomarkers: TIMP-1, Cystatin C, Fibrinogen, CC16 with a secondary list of NGAL, TIMP-2 and desmosine. With 11 biomarkers in the final refined list of biomarkers from a total of 34 biomarkers this was then narrowed down to 10 biomarkers for the point of care test development using logistic regression analysis. An additional consideration was the need to develop assays that were practical as it known that lateral flow is not as sensitive as ELISA. The inclusion of desmosine, although shown to be promising for various types of analysis, it seemed to be variable between the different assays, V1, V2 and the lateral flow, therefore, this was eliminated from the final panel. Finally, although a Cystatin

LF was available (already developed at Mologic), it was at decided at risk that fMLP was included in the final 10 biomarker panel, this was based on previous studies, potentially an early marker that would be useful for the prediction of an exacerbation and the only signalling molecule. The final 10 biomarkers selected based on all studies were: A1AT, TIMP-1, TIMP-2, CRP, Fibrinogen, fMLP, CC16, NGAL, RBP4 and B2M.

The creatinine result cannot be ignored, it was significant in most studies. The use of creatine ratio has been explored and it has not added value to the result in terms of reducing the variation. Creatinine is difficult to measure accurately at PoC and in addition, it has been shown to be highly variable in relation to gender, age, body mass, demographics and renal malfunction (159). In addition, it is noted that this is a spot sampling result rather than a more challenging 24 hour collection.

Different biomarkers may be required for different genders or at least a different statistical model could be developed to allow for gender. To refine the decision aid it is essential that a true baseline value is obtained from more frequent sampling. This will reduce the variability in the stable state and allow the use of a rolling mean taking in to account the within subject variability giving greater statistical power to detect the onset of the exacerbation event. The findings to date are promising and give encouragement that the likelihood of identifying clinically meaningful biomarkers is high.

Table 4.7. Summary of results from all retrospective studies. P values <0.05 deemed significant. Paired t-tests were used unless indicated by * which is a Wilcoxon matched-pairs signed rank test. Creatinine ratio not used.

	Population levels of biomarkers				**% change from stable or recovery sample (% that had >10% change)**			**% change from baseline**
	Leicester study Stable to PEx	GSK Aeris study		Birmingham study PEx to recovery	Leicester Stable to PEx	GSK Aeris study	Birmingham study PEx to recovery	Leicester Stable to PEx (including baseline)
		Pre - PEX	PEX - post			Pre - PEX		
A1AT	0.0001	0.0069*	0.0496 0.0045*	0.019691 0.0379*	72.7	54.9	57.1	<0.0001
TIMP-1	0.0361			0.025183 0.0122*	60		65.95	0.0325
Cystatin C	0.0025			0.050729 0.0101*	61.8		59.6	0.0098
RBP4				0.046883 0.0049*	69.1	52.1	68.6	
Human serum albumin			0.0069*	0.006548 0.0102*	50.9		61.7	
Fibrinogen			0.0214*	0.034147	70.9	53.5		0.0019
Chitinase 3 like protein	0.0055		Not tested				Not tested	0.0139
MMP-9 Total	0.0409				52.7			
NGAL	0.0262		0.02*		54.5			0.0072
Calprotectin	0.0164		0.0284					
RNASE-3	0.0465							
Creatinine	0.0005	0.0446	0.0475		61.8	54.9	52.7	0.0009
TIMP-2	0.0136				60			0.0287
CC16	0.0284		0.0121 0.007*	Not tested	61.8		Not tested	0.0025
CRP	0.0012	0.0001 <0.0001*	0.0027 0.0013*	Not tested	54.5	62.0	Not tested	0.0002
IL6			0.0153*					0.0325
Desmosine			0.0322*		54.5 (V2)	52.1		0.0386 (V2)
fMLPLF					60			
B2M				0.0227*	76.4	56.3	61.2	<0.0001
MMP-8								0.0170

Assessing the final 10 biomarkers on the Leicester BEAT-COPD study gave an AUC of 0.84 (95% confidence interval 0.76 to 0.92). At an optimal cut-off of 0.4065, the sensitivity and specificity were 80% and 76.36 respectively with a PPV of 77.19% and NPV of 79.25%.

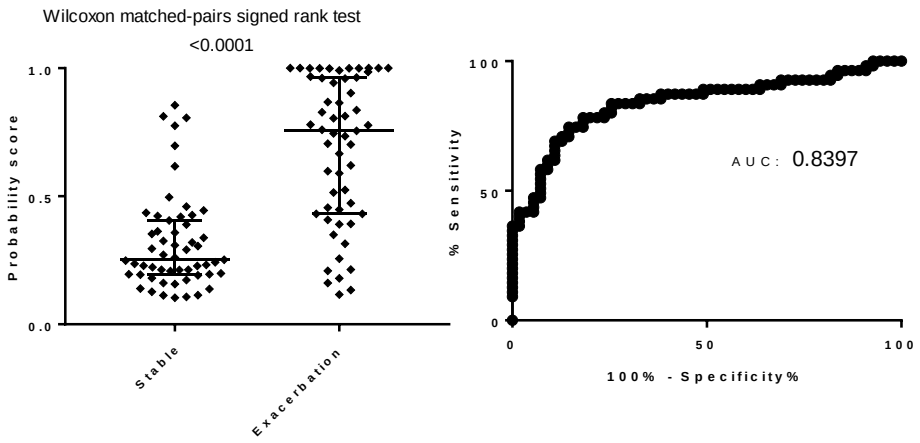


Figure 4.7. BEAT-COPD study; Combined Male and females n=55 with final 10 selected biomarkers. (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve with AUC shown

The outcomes from each of these studies have demonstrated the relevance of urinary biomarkers to COPD exacerbations, and their potential in the stable state to pick out individuals whose prognosis may be worse, such as those with frequent exacerbations or emphysema. The main aims were to explore biomarkers of pathophysiology of COPD measurable in the urine. it was initially thought that there might be some individuals in whom elastin degradation and the action of HNE would be marked whilst in others MMP driven processes might be more important, for these reasons, a multimarker panel consisting of 10 biomarkers has been selected for further investigations. There are 3 pathophysiological pathways in which these biomarkers are involved in this context; MMP driven pathway: TIMP-1, TIMP-2, NGAL and HNE driven pathway: A1AT.

Chapter 5. Development of a point of care lateral flow test to measure biomarkers of interest in urine

5.1 Introduction

Key requirements for the exacerbation alert test (named 'Headstart') in order to measure specific biomarkers in urine are that it must to be simple to use, rapid and cost-effective. The test system will be designed to comprise of a disposable test cassette, similar to a pregnancy test that can measure up to 5 analytes in the urine sample, together with a very compact opto-electronic reader. When the test cassette is placed in the reader, a timer will start, and a reading automatically taken after a set time of 10 minutes. The reader will interpret the concentration of the biomarkers in the sample and then save the result to memory. The reader will be Bluetooth enabled so that it will be able to transmit the data to a remote server for processing via an appropriate algorithm (the results of which will be transmitted back to the patient or Health Care Practitioner, as appropriate. Reports back to the patient will be in the form of very simple instructions regarding medication, further testing or seeking medical advice, as appropriate.

5.1.1 Lateral flow technology

The "Clearblue ®" pregnancy test is the most widely known product that is based on lateral flow technology. This is a simple to use rapid, point of care test that can be bought "over the counter" with a visible line indicating a positive result for hCG and an absence of a line indicating a negative result. A lateral flow test can be qualitive such as the pregnancy test with a yes/no result, or semi quantitative, where a low, medium, high result is indicated or quantitative, where a reader is required in order to measure the intensity of the line and convert it to a concentration value using a standard calibration curve. A typical lateral flow comprises of 4 key components as shown in figure 5.1. The ''sample pad'' receives the test sample, distributes the sample on to the test strip uniformly. It may contain chemicals to modify the sample composition and may also act as a filter. The "conjugate pad" holds the detector reagent i.e. antibodies conjugated to gold particles, in a dry state, does not interfere with detector reagent stability and releases the detector reagent quickly, consistently and quantitatively to provide uniform transfer of detector reagent to the nitrocellulose membrane. The membrane is the surface used to immobilise the capture reagents, upon which the immunocomplexes forms (i.e. signals) and controls the overall flow rate of the system. The "absorbent pad" serves as the "sink" for the sample, it determines the total sample volume that can be processed and typically is not chemically altered. All components are laminated onto a backing card and enclosed inside a plastic housing which contains sample ports for addition of the sample. There are three main formats as described in more detail in figure 5.2- 5.4. A sandwich assay is commonly used where a multi-epitope analyte is available and a competitive and inhibition assay where a single epitope analyte is only available.

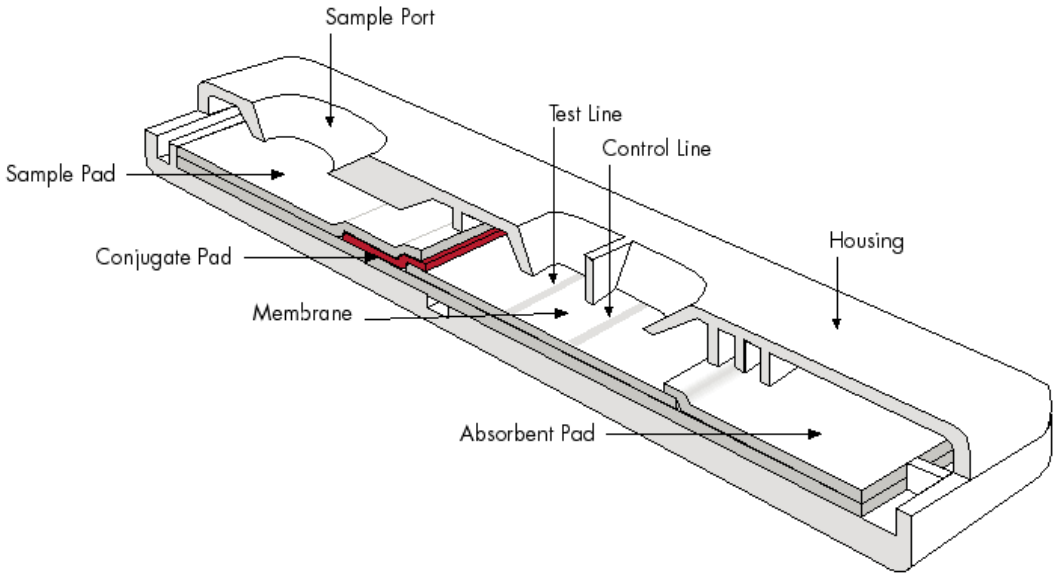


Figure 5.1. A typical lateral flow assay. A strip is enclosed in a plastic housing with a sample port for sample addition and a read window where the result can be interpreted by the user in the form of test lines and control lines (courtesy of Millipore)

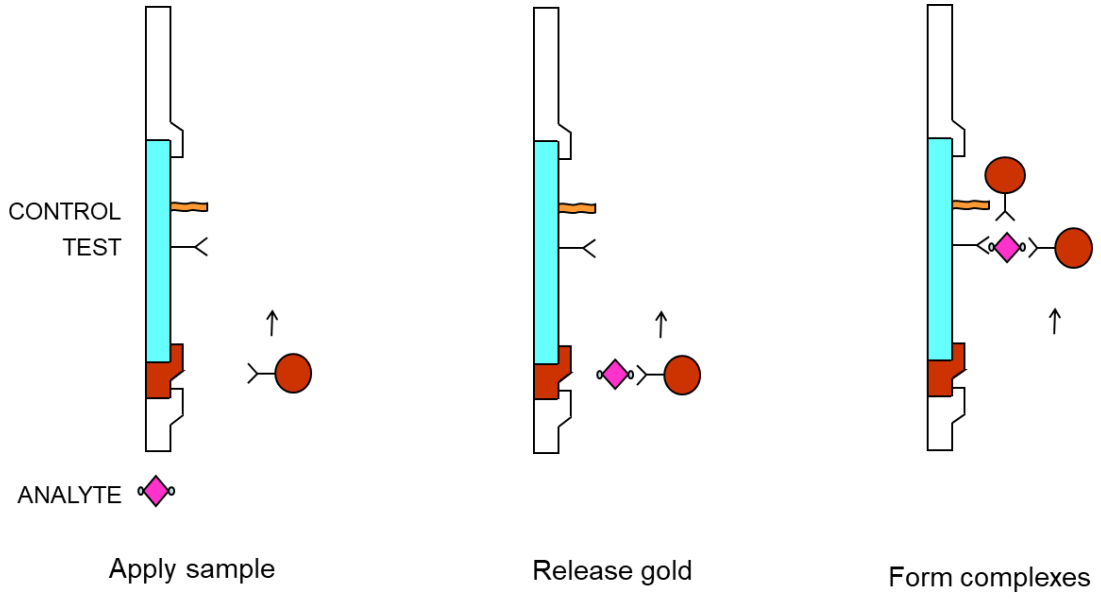


Figure 5.2. A sandwich assay format. The analyte in the sample forms a complex with the primary antibody-detector conjugate i.e. gold which then forms a complex with a secondary antibody immobilised on the nitrocellulose membrane forming a visible line. A control line in this example is an anti-species to the primary antibody.

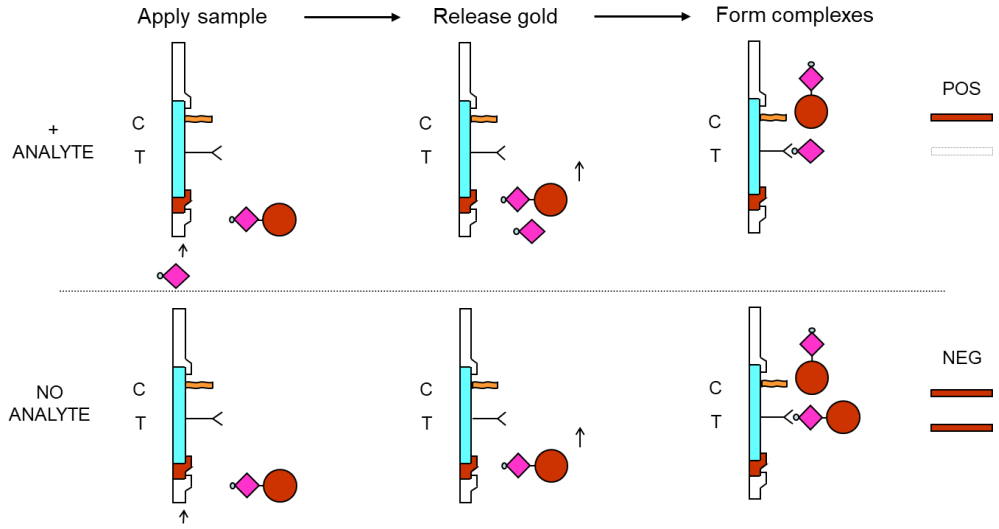


Figure 5.3. A competitive assay format. The analyte in the sample competes with the analyte-detector conjugate i.e. gold with the antibody immobilised on the nitrocellulose membrane. With unlabelled analyte bound to the antibody test line, there is an absence of a visible line.

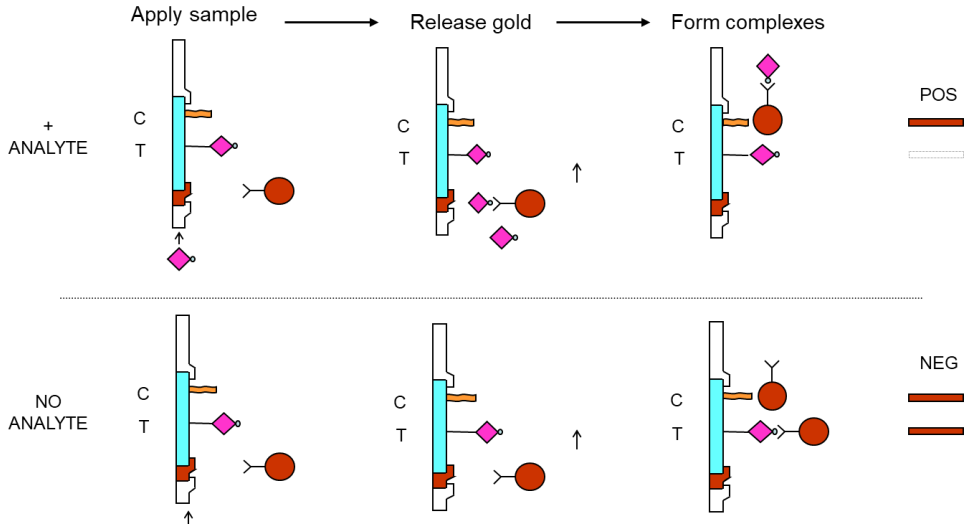
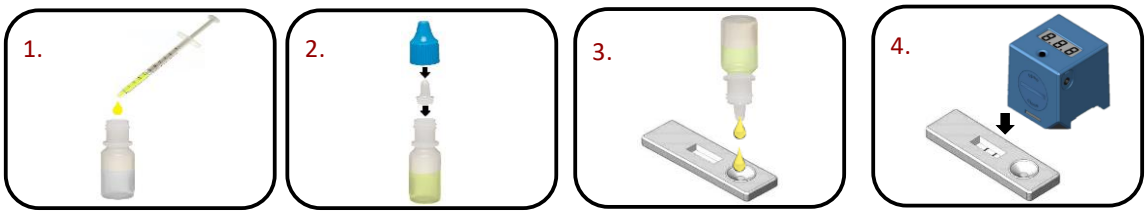


Figure 5.4. An inhibition assay format. The analyte in the sample inhibits binding of the detector reagent at the test line by forming a complex with the antibody-detector conjugate i.e. gold preventing it from binding to the analyte immobilised on the nitrocellulose membrane. With no antibody- detector conjugate bound to the test line, there is an absence of a visible line.

5.1.2 Next generation development of the test device

The first version of the multiplex urine device developed required a sample processing step and manual addition of the sample to the device (figure 5.5). Such a test system was evaluated with cystic fibrosis patients in their own homes. The biomarkers selected for incorporation into this device were A1AT, TIMP-1, TIMP-2 and CRP based on previous studies outlined in this thesis. This patient group was a younger population, who were generally very motivated and capable of undertaking self-testing manipulations. It was predicted that a test for people with COPD needed to be simpler and more user-friendly, avoiding additional steps required to perform the test. The new version developed incorporated a sample wick which could be directly placed in the urine flow and then the whole cassette placed directly into the reader (figure 5.6). The reader system selected was the cube reader (OpTricon GmbH, Berlin, Germany), this is a simple lateral flow reader that serves as an 'electronic' eye obtaining exact readings. The OD readings obtained from the reader ranged from 0-300 unit where a reading above '10' units was observed as a visual read line on the test.

The transition to the new version was envisioned to be challenging, since as well as a modification to the test strip dimensions itself, the chemistry and reagents also required substantial re-optimisation. A new plastic injection-moulding had to be developed specifically to house the new strip and sample wick. Finally, the new test had to be validated and verified in-house to ensure that specifications were met. During development, the assays were first developed as single assays and then multiplexed (Headstart V2). An interim analysis with COPD patients was undertaken to determine the usability and acceptability of the complete test system. As there were 10 biomarkers to be measured, they were separated into 2 tests each consisting of 5 assays (Headstart V3). The assays were subjected to multiple evaluations before they were deemed to be acceptable including comparisons to the reference assays and testing with frozen urine samples to ensure the assays were within the correct dynamic range.



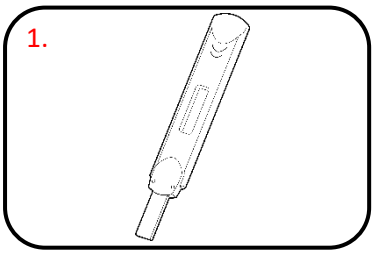
Add urine sample (1ml) to dropper bottle with fixed amount of buffer

Insert lid and shake gently

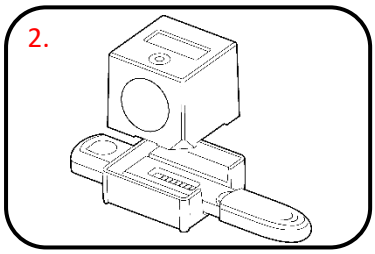
Addition of 2 drops to device

Insert into reader and result displayed in 10 minutes

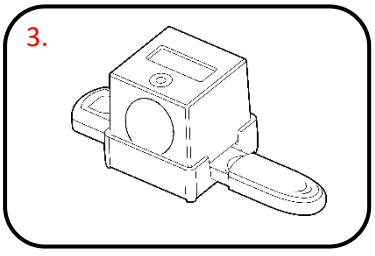
Figure 5.5. Headstart V1 procedure. The urine sample prior to application to the test device requires a 1 in 10 dilution in a sample buffer supplied in dropper bottle.



Remove a test device from the foil pouch. Hold the sample wick pointing downwards in either the urine stream OR collected urine sample for 10 seconds then remove



Replace cap. The pink liquid will start to migrate up the test strip. The green line will be replaced with a red line – the control line. Insert into the adaptor and place the cube reader on top



Press button on reader to start automatic timer of 10 minutes.

Figure 5.6. Headstart V2/V3 procedure. The device containing sampling wick is added directly to the urine without any sample processing.

5.2 Methods

5.2.1 Development of new cassette moulding

Stages of development involved design of cassette, evaluation of prototype assessing flow characteristics, performance characteristics and manufacturing aspects. A few iterations were assessed before finalising the design, this was evaluated using 3D printed cassettes. The tool was developed and first prototypes were again assessed and further modifications were required before finalising the design resulting in a small-scale manufacture (figure 5.7). The test strip was optimised with urine samples both in terms of dipping the cassette in a urine sample and midstream testing to ensure compatibility and ensure that that was no flooding of the test strip and that the flow was reproducible and consistent.

5.2.2 Development of Headstart V2 prototype tests

As previously mentioned, the first version test required a sample dilution and although an evaluation by CF patients in a parallel home monitoring study was successful, it was desirable to modify the test to make it more user-friendly for COPD patients. The previous test required the patient to perform 4 steps including dilution of the sample into a dropper bottle.

The second version of the Headstart test was designed to be similar to a pregnancy test in which the cassette contained a sample wick to be placed directly in the urine stream. In addition to the new cassette mounding, the chemistry of the test also required further development and optimization, as the previous version used a 1 in 5 sample dilution compared to neat urine required for the current version. Challenges that had to be addressed were matrix issues (which occur when using neat sample) and desensitisation of each of the assays. There were 10 biomarkers selected from chapter 4 to take forward. As each test device could only house 5 assays, they had to be built into 2 separate test devices (a and b), the decision on which device each assay was placed was based on cross reactivity testing.

The strip architecture needed to be optimised in order to fit into the new cassettes. The changes are shown in table 5.1 and illustrated in figure 5.8. Non-critical modifications were required with regards to the dimensional changes. The backing card, nitrocellulose and conjugate pad needed to be longer in order to allow for more assays to fit into the same strip. The largest change involved 1) using a faster nitrocellulose membrane due to the longer length of the strip (from 25mm to 40mm) to allow sufficient flow along the strip and 2) switching out the blood separator pad (which was not specifically needed for this test) to a wick that acted as the interface between the urine sample and the test strip.

Details of the assay development are not included in this thesis as the work undertaken by the team at Mologic was very extensive. For the test line and detector reagents for each of the 10 assays, evaluation of 3 different antibodies/antigens was undertaken for both capture and detector reagent. For each detector reagent, the gold conjugate was optimised in terms of antibody-loading, conjugation buffer formulation and pH. Once conjugated, the gold conjugates were assessed in "wet assays" with the different capture reagents on the NC before assessing in a "dry assay" in various gold drying buffers and at different OD's. The best pairings were selected in combination with the other assays to determine if there was any cross reactivity. This determined which of the 2 test devices they were best positioned (a or b). Optimisation included assessing effect of drying temperatures and drying times for both the nitrocellulose and the gold pad as well as assessing different line positions on the NC. This was important as assays which were too sensitive required the "capture line" closer to the sampling end and assays that required more sensitivity required it to be positioned further away as the flow of the analyte is slower further along the strip thus allowing more time for the test to develop.

There were a number of techniques that were used to desensitise some of the assays which was the most challenging aspect of development. Small improvements were made such as line positioning and changing the NC speed as described above, but the three methods that had the largest effect and were all incorporated were:

- Addition of a pre- absorbance line that was hidden from view just below the window of the test device, this removed some of the free analyte as long as the gold was in excess and allowed the optimum amount to flow past and bind to the test line
- Introducing free antibody to the system was used to sequester "excess" analyte in the sample, this complex then competed with the analyte/gold labelled antibody to bind to the test line.
- A competition or inhibition format was generally less sensitive than a sandwich assay.

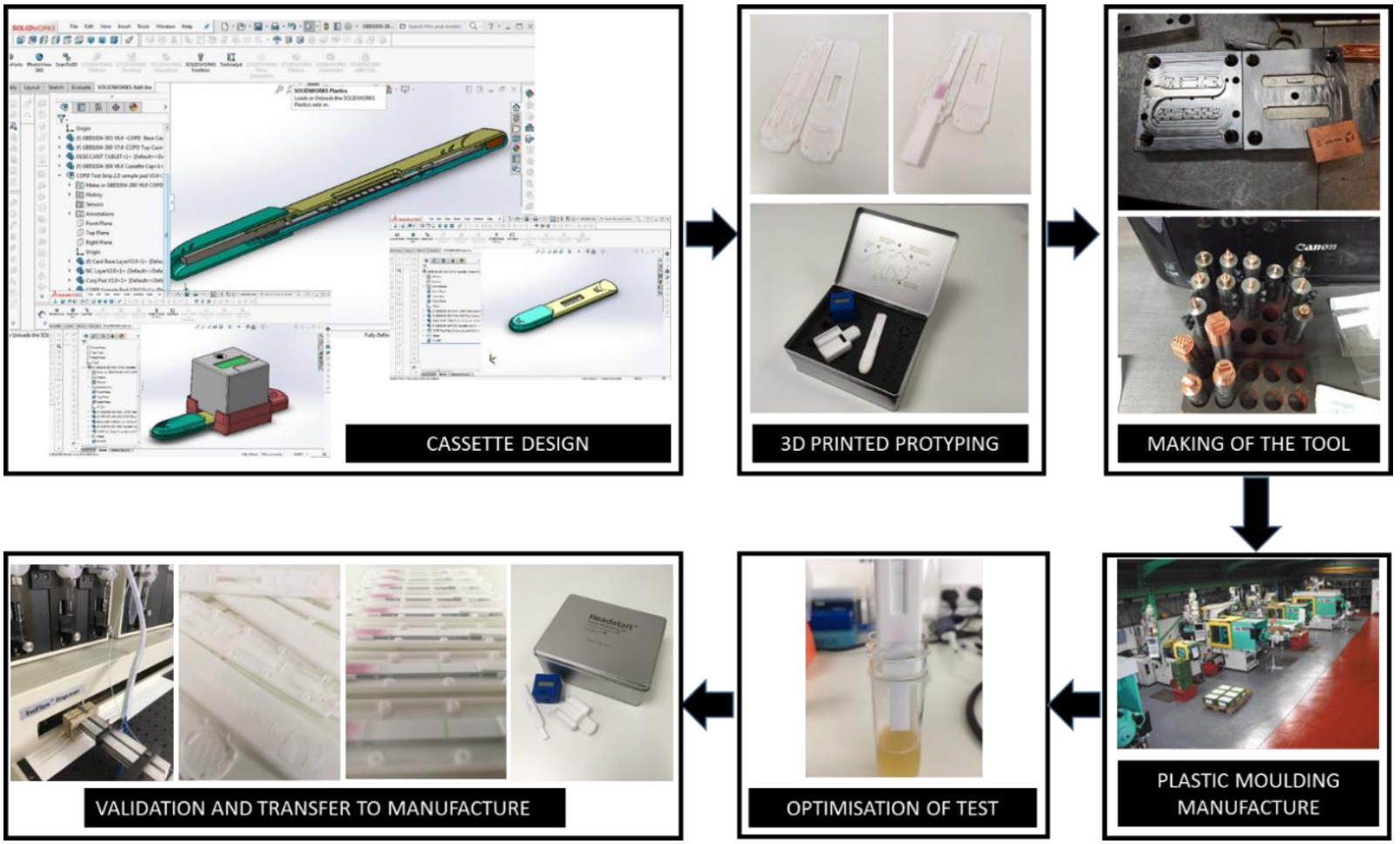


Figure 5.7. Development of plastic housing from point of concept to manufacturing. 3D printed devices were evaluated before committing to the tool development. Which required further refinements before freezing the design and transfer to manufacture.

Table 5.1. Component change from version 1 (enclosed in a single well housing where by the sample is manually added to the test device) and version 2 (with an added wick where the sample is directly applied by dipping into a urine sample or midstream urine)

	Version 1	**Version 2**
Backing card	60mm G&L backing card	80mm Lohmann Backing card
Nitrocellulose membrane	25mm Sartorius CN140	40mm Sartorius CN095
Conjugate pad	17mm Millipore G041	27mm Millipore 8951
Absorbent pad	22mm Ahlstrom Grade 222	22mm Ahlstrom Grade 222
Sample pad	10mm FR-1 blood separator pad	35 x 8 x 2.5mm Essentra wick
Plastic devices	1 well Forsite diagnostic (base and top)	Custom made, Mologic (base, top and lid)
Desiccant pouch	1g Brownell	1g Brownell
Foil pouch	Riverside	Riverside

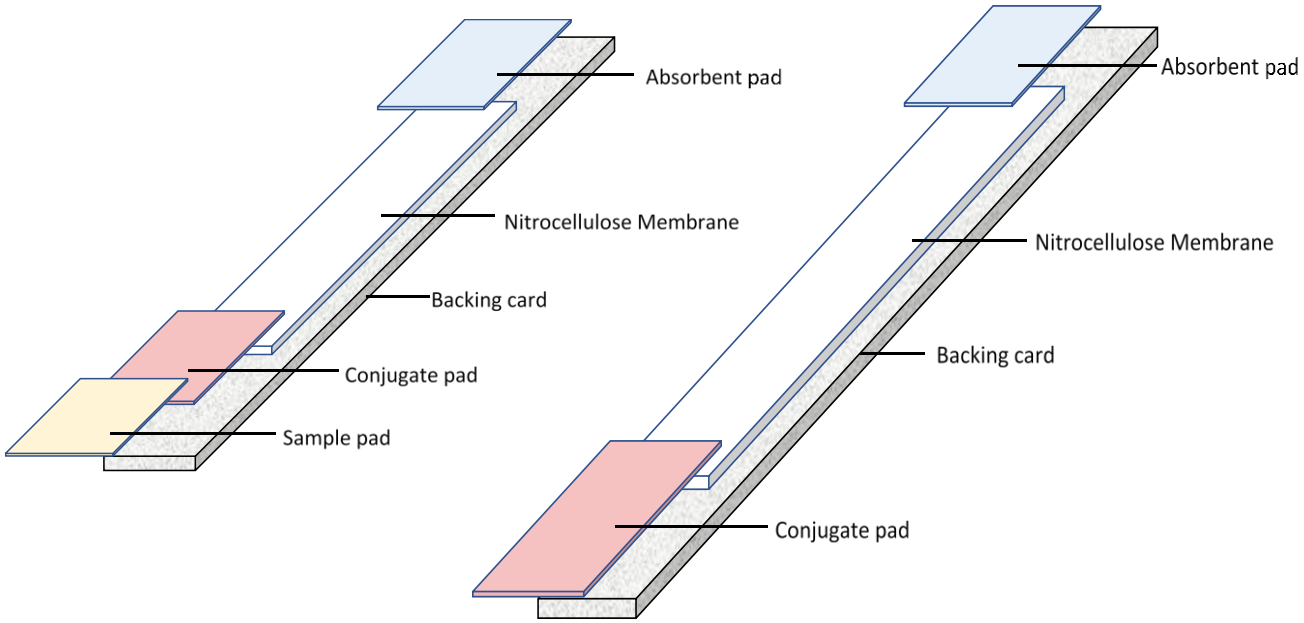


Figure 5.8. Visual representation of the different strip architecture. Version 1 being a shorter strip and with a sample pad and version 2, a longer strip with a wick added during assembly.

5.2.3 Development of final Headstart V3 prototype tests (a and b)

The challenge of optimising all 10 assays with a neat urine sample is highlighted by assessing the sample dilution required for the ELISAs. These were (in order of dilution factor), RBP4 1 in 2000, B2M and NGAL 1 in 100, A1AT 1 in 20, TIMP-1, TIMP-2, CC16 and CRP 1 in 10, fMLP and Fibrinogen 1 in 2.

Headstart assay (A) consisted of 5 biomarkers, A1AT, TIMP-2, NGAL, Fibrinogen and CRP. All 5 were sandwich assays however, there was an additional pre-absorbent line for A1AT and free antibody addition for the NGAL assay which was refined to give just the required assay range as otherwise the assay had a strong "high-dose hook" effect with high levels of NGAL. A diagrammatic representation can be seen in figure 5.11, with a comparison to version 1 in figure 5.10.

For the NC, all capture lines (not control line) were prepared at 1mg/ml in PBS 1% sucrose. Anti - A1AT sheep polyclonal pre-absorbent line was plotted at 3mm, anti-A1AT BSA fab was plotted at 7mm, anti-TIMP-2 sheep polyclonal was plotted at 10mm, anti-NGAL BSA fab was plotted at 13mm, anti-Fibrinogen sheep antibody at 16mm, anti-CRP was plotted at 19mm and BSA biotin was plotted at 2mg/ml with 10% green food dye in the control at 22mm. All on 40mm CN95 nitrocellulose at a deposition rate of 0.05µl/mm. Materials were dried in the Hedinair drier at 60ºC at 10mm speed and heat sealed in a foil pouch with 5 x 1g desiccant and cured at 37°C incubator for 18hrs.

Antibodies were conjugated to gold particles individually and then were added together to make up the final OD of 5 for A1AT, TIMP-2, NGAL, Fibrinogen, OD10 for CRP and OD2 for anti-biotin gold conjugate in a gold drying buffer (consisting of a tween 20, sucrose and BSA) + 15µg/ml final of free anti-NGAL BSA fab. The final conjugate mix was sprayed onto 8951 glass fibre conjugate pad (22mm, equivalent to GFDX) at a deposition rate of 0.8µl/ml. Materials were dried in the Hedinair drier at 60ºC at 5mm speed and stored in a pouch with 5 x 1g desiccant.

Headstart assay (B) consisted of 5 biomarkers, RBP4, CC16, B2M, TIMP-1 and fMLP, that, with the exception of TIMP-1, all were inhibition assays. A diagrammatic representation can be seen in figure 5.12.

For the NC, all capture lines were prepared in PBS 1% sucrose. RBP4 was plotted at 1.5mg/ml at 7mm, CC16 was plotted at 0.5mg/ml at 10mm, B2M was plotted at 0.5mg/ml at 13mm, anti-TIMP-1 BSA fab at 1mg/ml at 16mm, Ovalbumin-fMLP was plotted at 1mg/ml 19mm and BSA biotin was plotted at 2mg/ml with 10% orange food dye in the control at 22mm. All on 40mm CN95 nitrocellulose at a deposition rate of 0.05µl/mm. Materials were dried in the Hedinair drier at 60ºC at 10mm speed and heat sealed in a foil pouch with 5 x 1g desiccant and cured at 37°C incubator for 18hrs.

Antibodies were conjugated to gold particles individually and then were added together to make up the final OD of 5 for TIMP-1 and B2M, OD10 for RBP4, CC16 and fMLP and OD2 for anti-biotin gold conjugate in gold drying buffer. The final conjugate mix was sprayed onto 8951 glass fibre conjugate pad (22mm, equivalent to GFDX) at a deposition rate of 0.8µl/ml. Materials were dried in the Hedinair drier at 60ºC at 5mm speed and stored in a pouch with 5 x 1g desiccant.

For lamination, onto 80mm backing card, the base of the NC was placed at 25mm height, the base of the conjugate pad was lined up flush with the base of the backing card. The absorbent pad lines up flush to the top of the backing card forming a 7mm overlap with the top of the NC. The strips were cut into 5mm wide strips. Each strip was placed into the cassette housing with a wick and sealed shut using a specific device closing machine and a cap was placed (green for device 'a' and orange for device 'b') before sealing in individual foil pouches with 1g desiccant (figure 5.9).

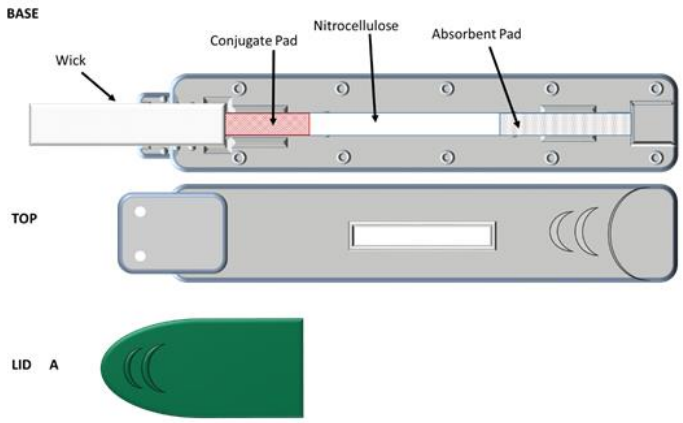


Figure 5.9. Diagrammatic representation of assembly process for test device version 2 with a green lid for A and an orange lid for B. Manual addition of strip and wick but machine closed to ensure equal distribution of pressure.

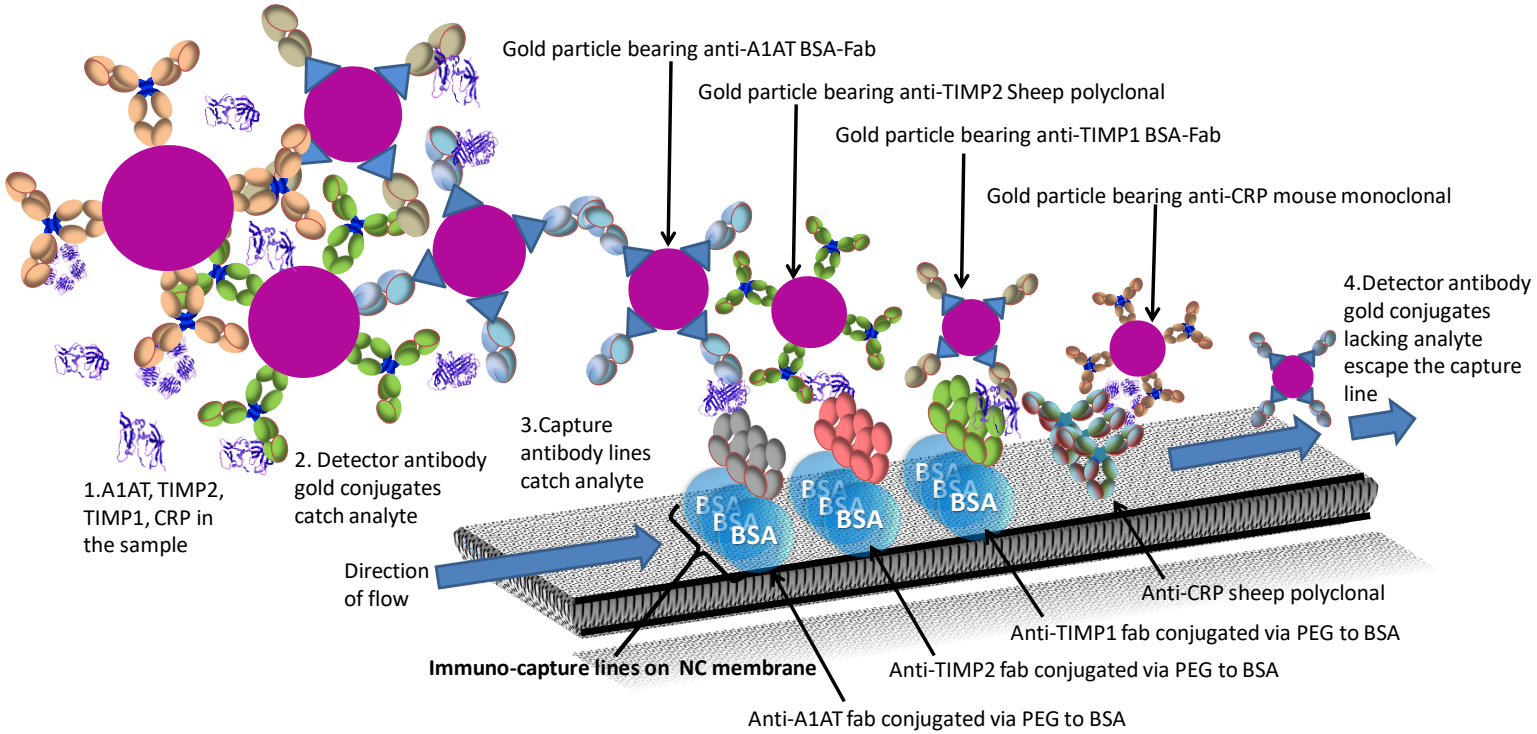


Figure 5.10. Diagrammatic representation of the first version test that measured 4 biomarkers, A1AT, TIMP-2, TIMP-1 and CRP in a 1 in 5 dilution urine sample. The sample added to the test device, first reconstitutes the gold from the conjugate pad and releases the 4 gold conjugates. In the presence of analyte in the sample, the analyte is recognised and forms a complex with the antibody on the gold conjugate and then migrates up the strip and is captured on the antibody immobilised on the nitrocellulose forming a red line. In the absence of analyte, the sandwich is not formed resulting in an absence of a line. The control line not shown consists of an BSA biotin on the capture line and an anti-biotin gold conjugate that when complexed indicate if the test is run correctly.

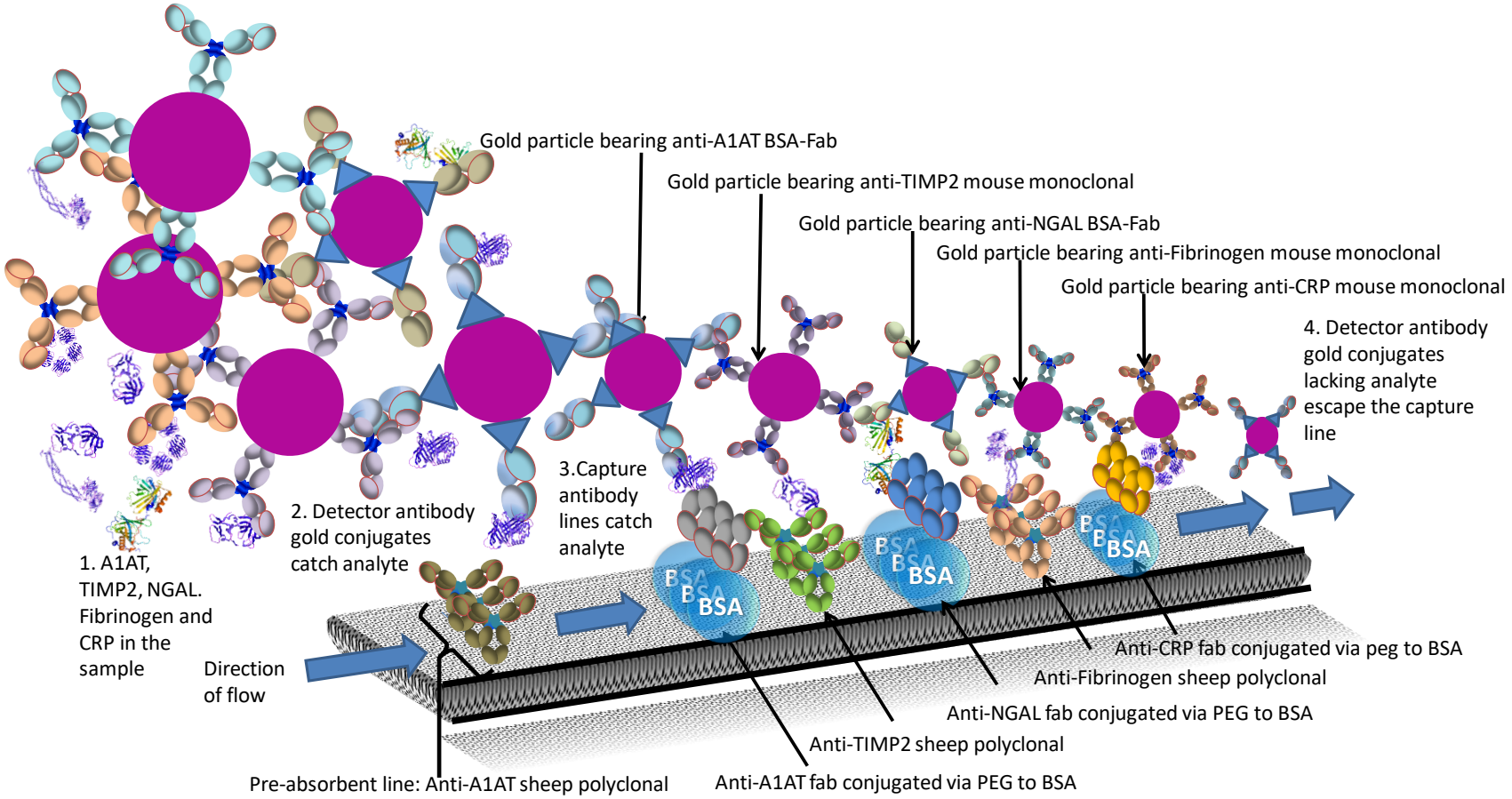


Figure 5.11. Diagrammatic representation of the second version test (a) that measured 5 biomarkers, A1AT, TIMP-2, NGAL, Fibrinogen and CRP in a neat urine sample. The sample added to the test device, first reconstitutes the gold from the conjugate pad and releases the 5 gold conjugates (and free NGAL not shown). In the presence of analyte in the sample, the analyte is recognised and forms a complex with the antibody on the gold conjugate and then migrates up the strip and is captured on the antibody immobilised on the nitrocellulose forming a red line. In the absence of analyte, the sandwich is not formed resulting in an absence of a line. For the A1AT assay, a pre-absorbent line is used to de-sensitise the assay. The control line not shown consists of an BSA biotin on the capture line and an anti-biotin gold conjugate that when complexed indicate if the test is run correctly

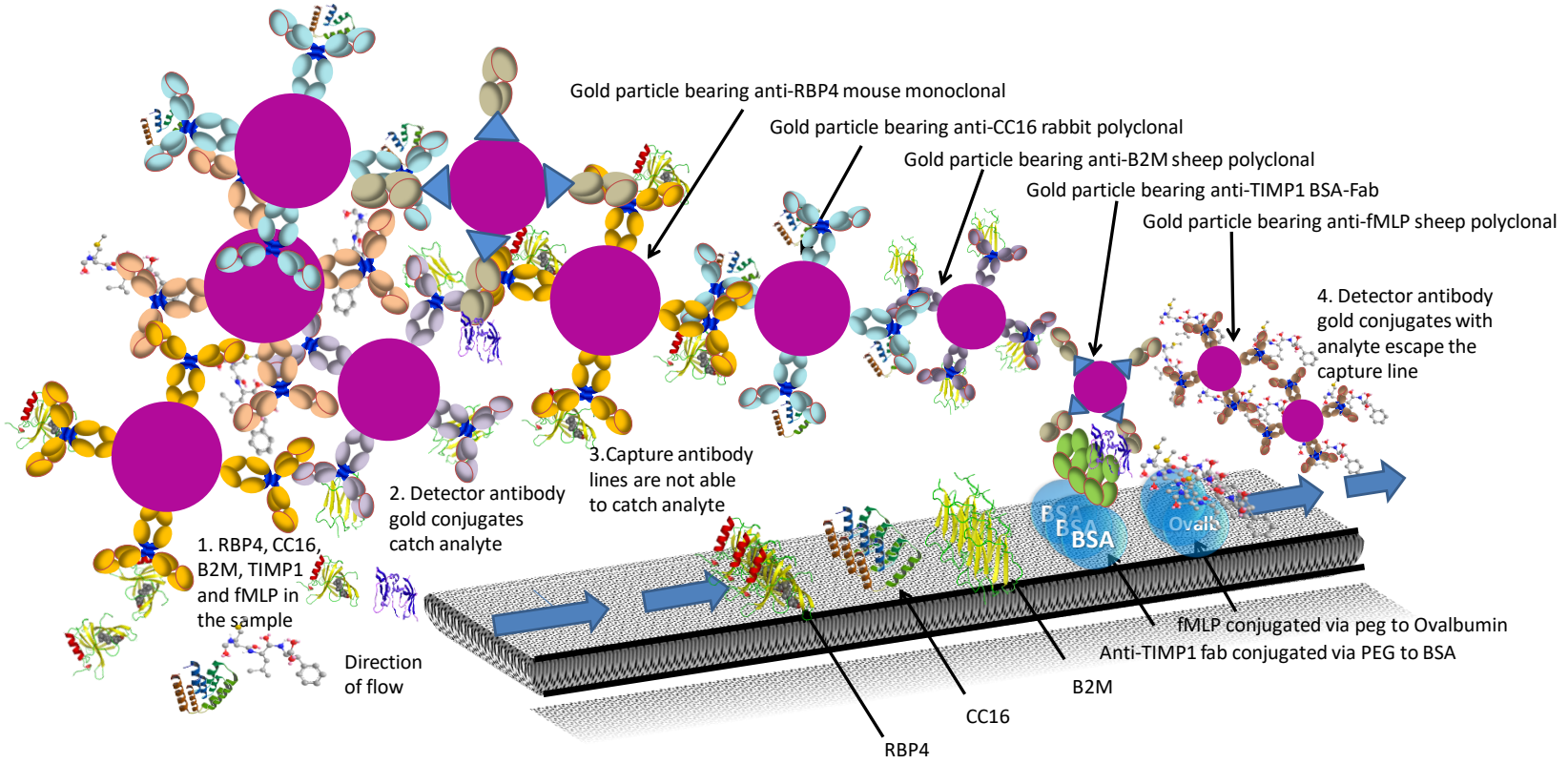


Figure 5.12. Diagrammatic representation of the second version test (b) that measured 5 biomarkers, RBP4, CC16, B2M, TIMP-1 and fMLP in a neat urine sample. The sample added to the test device, first reconstitutes the gold from the conjugate pad and releases the 5 gold conjugates. For the 1 sandwich assay, In the presence of analyte in the sample, the analyte is recognised and forms a complex with the antibody on the gold conjugate and then migrates up the strip and is captured on the antibody immobilised on the nitrocellulose forming a red line. In the absence of analyte, the sandwich is not formed resulting in an absence of a line. for the 4 inhibition assays, the complex is formed as previously described with the analyte and detector antibody gold conjugate but as a result, the complex is no longer able to bind to the capture lines hence a line is not formed. In the absence of the analyte, the gold conjugate will bind directly to the test line producing a red line. The control line not shown consists of an BSA biotin on the capture line and an anti-biotin gold conjugate that when complexed indicate if the test is run correctly.

5.2.4 Materials/reagents

- Buffer PBST- 1%BSA (10mM phosphate buffered saline pH7.5, supplemented with 1% (w/v) BSA)
- Antigens for Multiplex A: A1AT (Merck Calbiochem, 178251), TIMP-2, (Kent university, Canterbury), NGAL (Alere San Diego, RFPR017674), Fibrinogen (BBI, 132-3), CRP (Lee Biosolutions, 140-11R)
- Antigens Multiplex B: RBP4 (BBI, P124-1), CC16 (Novoprotein CU06), B2M (BBI, P122-1), TIMP-1 (Novoprotein, C456), fMLP (Sigma, 47729)
- Fresh urine samples collected from healthy controls
- Headstart devices a and b (batch SR130717)
- ELISAs for all 10 biomarkers (as described in chapter 2)

5.2.5 Equipment

- Cube reader; Cube/device adaptor; Cable (OpTricon)
- RFID card wide control + Area under the curve (AUC)
- Pipettes
- Universals/Bijou/2 ml Eppendorf tubes
- Timer
- Plate shaker/plate washer

5.2.6 Testing buffer standards

Standard testing was performed as follows: i) devices were removed from the pouches immediately before testing; ii) caps were removed and device was placed on a flat surface; iii) 10 minute timer was initiated; iv) With the device laid on a surface, 650µL of each standard was pipetted on to the region of the wick closest to the conjugate pad. v) the device was read after 10 minutes using a cube reader. vi) once reading was complete, the devices were discarded.

Standards for Multiplex A & B were made according to table 5.2.

Devices were taken from the beginning, middle and end of the batch, 9 devices in total and the criteria for the standard curve range was determined by the accuracy and CV's of the replicates. The standard curves were compared to standard curves obtained from the ELISA's to determine whether the range of the assays were acceptable.

5.2.7 Headstart Usability study at Leicester

The current Headstart version was evaluated by 10 patients at Leicester Hospital to evaluate key parameters such as usability/ease of use, frequency of testing, data transfer, safety, practicalities and recommendations/improvements. The study had ethics approval (REC Ref: 08/H0406/189) – An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation. Each patient undertook daily tests for approximately 30 days. At the end of the study a short questionnaire was completed by each patient. Three non-COPD volunteers also participated in the short usability study.

5.2.8 Statistical analysis

Conversion of cube values from the standards to concentration values used 'my assay' an online data analysis programme. All data were analysed using Graphpad PRISM Version 7.

Table 5.2. Standards used for standard testing. The top standard was prepared at shown concentrations for all analytes from the stock concentrations and then diluted 1 in 2.5 in the standard dilution buffer PBST 1% BSA.

ng/ml	A1AT	T2	NGAL	FIB	CRP	RBP4	CC16	B2M	T1	fMLP
1	1000	200	500	1000	25	500	2500	500	100	50
2	400	80	200	400	10	200	1000	200	40	20
3	160	32	80	160	4	80	400	80	16	8
4	64	12.8	32	64	1.6	32	160	32	6.4	3.2
5	25.6	5.12	12.8	25.6	0.64	12.8	64	12.8	2.56	1.28
6	10.24	2.048	5.12	10.24	0.256	5.12	25.6	5.12	1.024	0.512
7	4.096	0.8192	2.048	4.096	0.1024	2.048	10.24	2.048	0.4096	0.2048
8	0	0	0	0	0	0	0	0	0	0

5.3 Results

5.3.1 Headstart Usability study at Leicester

There were no adverse issues recorded with using the test device itself. Statistics showed that from a planned number of 273 tests, 1.1 % of tests were missed, 7% of tests were run incorrectly (control line failed to develop) with an overall success rate of 91.9%. In parallel, three non-COPD volunteers also participated. In this case the statistics showed that from a planned number of 91 tests, 7.7 % of tests were missed, 1.1% of tests were run incorrectly (control line failed to develop) with an overall success rate of 91.2%.

Results from the patient questionnaire, regarding frequency of the testing, confirmed that: 60% would be happy to perform daily testing; 30% said preferred once a week; and 10% said they would take the test only when they were worried. The majority (90%) of patients indicated that having to take the Headstart test for the rest of their life would not be a burden. None of the participants required any help to collect the urine samples and all confirmed that it was easy to collect the sample. With regards to the connectivity, 70% of the patients said they would be able to use a smart phone. Of the 30% who were not keen, 67% had no tablet/phone and 33% had no internet. Other feedback highlighted issues surrounding the size of the cube reader button, as well as its automatic timing function which proved difficult to initiate.

5.3.2 Standard curves and assay range

The standard curves run on the multiplex devices are graphically represented in figure 5.13. The average was taken from the 9 replicates and entered into 'my assay' programme. Shown in tables 5.3 – 5.7 are the details for each assay with calculated averages, SD and %CV from 9 replicates. The backfit is the returned concentration calculated by the standard curve and the accuracy is how close that estimated concentration was compared to the expected concentration. The criteria was: accuracy +/- 20% (80-120) for at least 6 of the 7 standards; %CV < 20% and r^2 >0.95. The final assay ranges for both the multiplex LF devices and ELISA are shown in table 5.8.

Table 5.3. A1AT and TIMP-2. The average, SD, %CV, accuracy cube from 9 replicates shown. The r^2 was 0.9993 and 0.9995 for A1AT and TIMP-2 respectively

Conc	Average	SD	%CV	Backfit	Accuracy
1000	164	5.8	3.5	1024	102.4
400	129.1	9.7	6.6	388.9	97.23
160	117.5	6.5	5.4	158.3	98.96
64	72	10.5	11.4	67.91	106.1
25.6	58.2	3.2	5.5	23.58	92.09
10.24	32.7	3.8	10.2	10.88	106.3
4.096	16.5	2.9	15.6	4.011	97.93

Conc	Average	SD	%CV	Backfit	Accuracy
100	182.3	5.6	3.1	103.3	103.3
40	152.3	5.9	3.9	38.03	95.06
16	118.2	7.5	6.3	16.54	103.4
6.4	78.9	2.5	3.2	6.491	101.4
2.56	47.5	2.6	5.5	2.373	92.69
1.024	32.8	1.4	4.2	1.097	107.1
0.4096	22.9	2.4	10.5	0.411	100.4

Table 5.4. NGAL and Fib. The average, SD, %CV, accuracy cube from 9 replicates shown. The r^2 was 0.9995 and 0.9998 for NGAL and Fibrinogen respectively

Conc	Average	SD	%CV	Backfit	Accuracy
250	165.3	0.5	0.3	250.7	100.3
100	118.1	4.3	3.6	99.23	99.23
40	76.3	3.2	4.2	39.85	99.63
16	48.5	2.1	4.4	17.19	107.4
6.4	26.8	1.7	6.2	5.433	84.89
2.56	19.4	1.3	6.9	2.48	96.87
1.024	15.8	2.0	12.7	1.3	***127***

Conc	Average	SD	%CV	Backfit	Accuracy
1000	116.9	5.1	4.4	1006	100.6
400	88.7	3.4	3.9	394.8	98.7
160	59.2	6.2	10.5	162	101.3
64	35.9	1.7	4.8	65.3	102
25.6	21.2	1.4	6.6	22.77	88.96
10.24	16.4	1.6	9.9	11.21	109.5
4.096	13.4	1.6	12.3	4.498	109.8

Table 5.5. CRP and RBP4. The average, SD, %CV, accuracy cube from 9 replicates shown. The r^2 was 0.9998 and 0.9988 for CRP and RBP4 respectively

Conc	Average	SD	%CV	Backfit	Accuracy
25	244.1	4.7	1.9	25.3	101.2
10	219.1	5.0	2.3	9.969	99.69
4	178.0	6.4	3.6	3.927	98.17
1.6	129.2	2.7	2.1	1.656	103.5
0.64	75.8	2.9	3.8	0.6137	95.88
0.256	43.3	0.2	0.4	0.2642	103.2
0.1024	21.6	0.5	2.3	0.1014	99.06

Conc	Average	SD	%CV	Backfit	Accuracy
1000	8.6	0.6	6.8	785.9	***78.59***
400	12.5	1.8	14.1	478.9	119.7
160	31.0	6.5	***21.1***	159.7	99.81
64	62.7	5.9	9.4	60.96	95.25
25.6	94.0	9.7	10.3	27.49	107.4
10.24	125.6	18.2	14.5	9.261	90.44
4.096	136.5	3.0	2.2	4.415	107.8

Table 5.6. CC16 and B2M. The average, SD, %CV, accuracy cube from 9 replicates shown. The r^2 was 1 and 0.9997 for CC16 and B2M respectively

Conc	Average	SD	%CV	Backfit	Accuracy
1000	58.1	3.9	6.8	992.5	99.25
400	76.5	8.2	10.7	403.1	100.8
160	115.0	16.4	14.3	159.2	99.47
64	161.7	4.8	3.0	64.28	100.4
25.6	194.6	4.5	2.3	25.62	100.1
10.24	209.6	11.9	5.7	9.931	96.98
4.096	214.4	1.5	0.7	4.344	106.1

Conc	Average	SD	%CV	Backfit	Accuracy
2000	15.5	1.1	7.4	1784	89.22
800	24.0	0.6	2.4	873.3	109.2
320	45.7	6.6	14.4	319.7	99.91
128	77.6	2.8	3.6	126.6	98.94
51.2	115.0	2.3	2.0	50.3	98.24
20.48	144.9	10.0	6.9	21.51	105
8.192	168.1	1.8	1.1	7.847	95.79

Table 5.7. TIMP-2 and fMLP. The average, SD, %CV, accuracy cube from 9 replicates shown. The r^2 was 0.9992 and 0.9994 for TIMP-2 and fMLP respectively

Conc	Average	SD	%CV	Backfit	Accuracy
200	238.8	2.9	1.2	203	101.5
80	218.3	7.1	3.3	82.3	102.9
32	181.2	12.0	6.6	29.53	92.27
12.8	144.9	7.8	5.4	13.84	108.2
5.12	91.8	6.4	6.9	4.97	97.07
2.048	51.2	4.4	8.5	2.006	97.94
0.8192	23.2	1.3	5.4	0.8405	102.6

Conc	Average	SD	%CV	Backfit	Accuracy
100	29.5	0.6	1.9	78.26	***78.26***
40	32.3	3.5	10.8	42.26	105.6
16	39.9	1.4	3.6	17.92	112
6.4	59.9	1.1	1.8	5.982	93.47
2.56	83.2	1.3	1.6	2.61	102
1.024	110.4	4.9	4.4	1.029	100.5
0.4096	129.7	4.3	3.3	0.4059	99.1

Table 5.8. Assay range for biomarkers in urine samples. The samples were run neat in the multiplex lateral flow device but diluted for the ELISA, the assay range for the ELISA was recalculated taking into account the dilution factor and the range listed as minimum – maximum (actual range).

	Multiplex LF		ELISA				
	Assay range (min)	**Assay range (max)**	**Assay range (min)**	**Assay range (max)**	**ELISA dilution**	**Assay range (min) Actual**	**Assay range (max) Actual**
A1AT	**4ng/ml**	**1000ng/ml**	**0.3ng/ml**	**80ng/ml**	**20**	**7ng/ml**	**1600ng/ml**
TIMP-2	**0.4ng/ml**	**100ng/ml**	**0.03ng/ml**	**2ng/ml**	**10**	**0.3ng/ml**	**20ng/ml**
NGAL	**1ng/ml**	**250ng/ml**	**0.08ng/ml**	**5ng/ml**	**100**	**8ng/ml**	**500ng/ml**
Fibrinogen	**4ng/ml**	**1000ng/ml**	**0.6ng/ml**	**40ng/ml**	**2**	**1ng/ml**	**80ng/ml**
CRP	**0.1ng/ml**	**25ng/ml**	**0.02ng/ml**	**1ng/ml**	**10**	**0.2ng/ml**	**10ng/ml**
RBP4	**4ng/ml**	**1000ng/ml**	**0.02ng/ml**	**1.5ng/ml**	**2000**	**47ng/ml**	**3000ng/ml**
CC16	**4ng/ml**	**1000ng/ml**	**0.03ng/ml**	**2ng/ml**	**10**	**0.3ng/ml**	**20ng/ml**
B2M	**8ng/ml**	**2000ng/ml**	**0.01ng/ml**	**50ng/ml**	**100**	**1ng/ml**	**5000ng/ml**
TIMP-1	**0.8ng/ml**	**200ng/ml**	**0.03ng/ml**	**2ng/ml**	**10**	**0.3ng/ml**	**20ng/ml**
fMLP	**0.4ng/ml**	**100ng/ml**	**0.8ng/ml**	**50ng/ml**	**2**	**2ng/ml**	**100ng/ml**

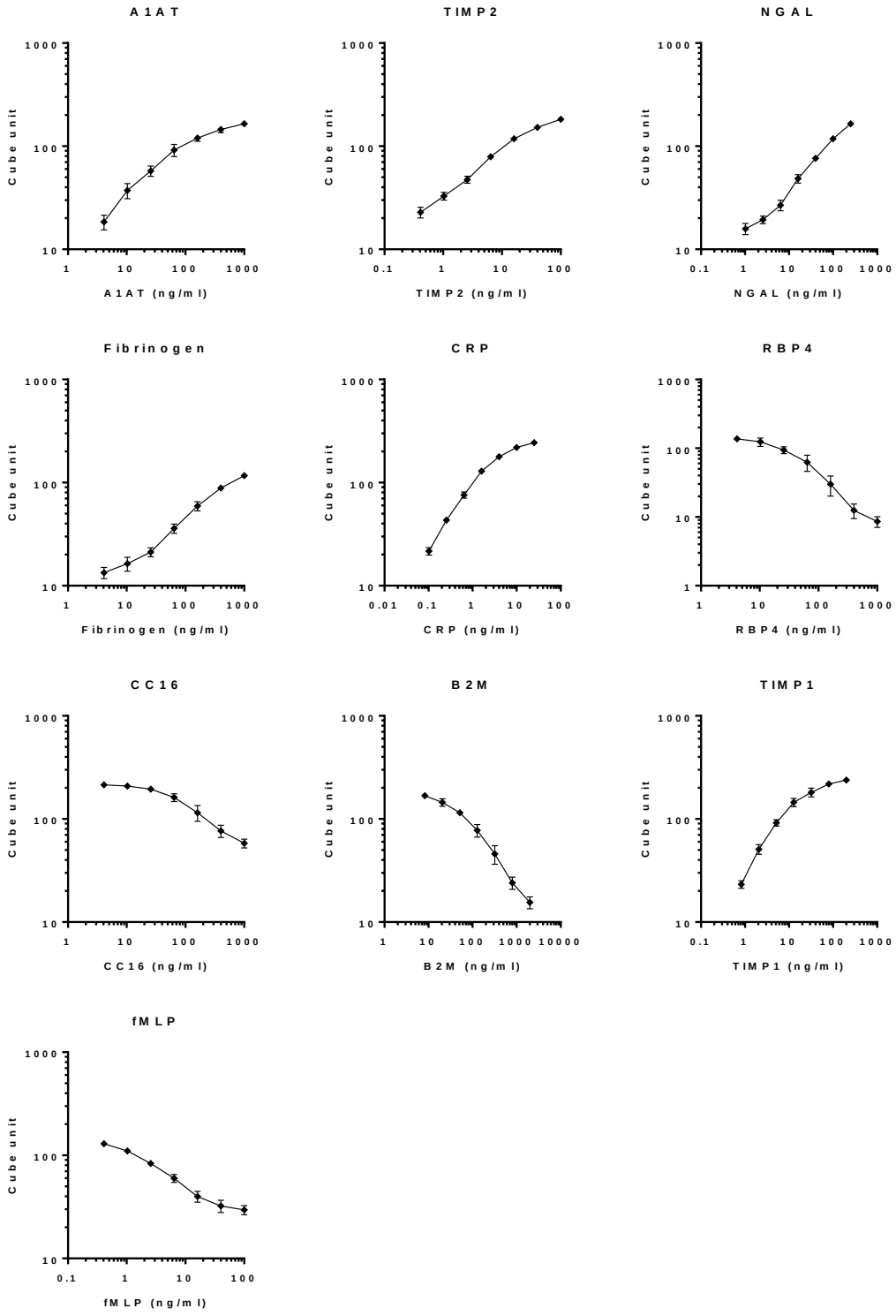


Figure 5.13. Graphical representation of the standard curves for each of the assays within the multiplex assay. Nine replicates for each standard were run, the mean and SD are presented in each of the graphs for each standard. The 'cube unit' is the reader value obtained by reading the colour intensity of the line on the lateral flow strips.

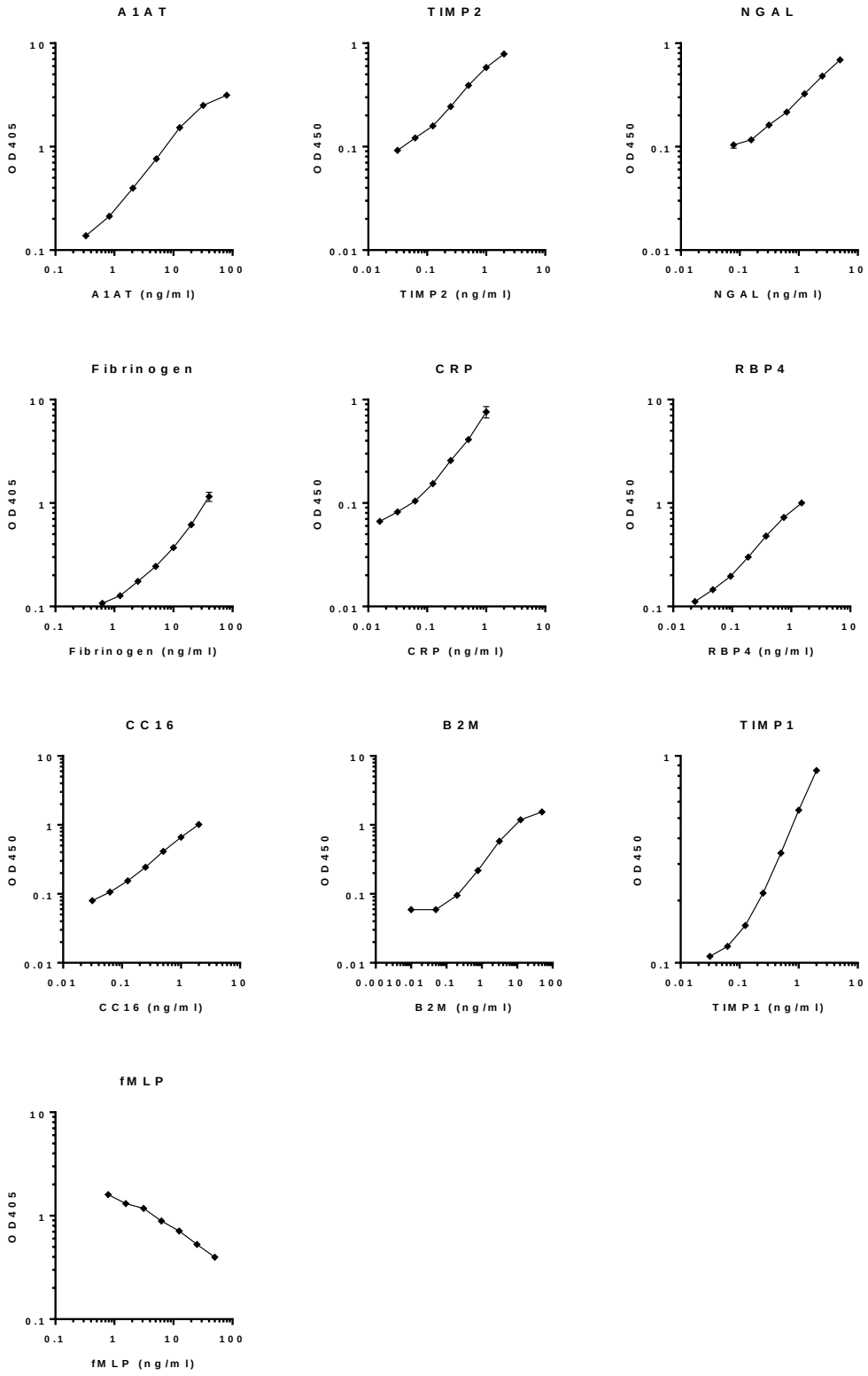


Figure 5.14. Graphical representation of the standard curves for each ELISA. Two replicates for each standard were run, the mean and SD are presented in each of the graphs for each standard.

5.4 Discussion

As described in this chapter, 2 multiplex assays (a and b) have been successfully developed. Multiplex 'a' consisted of 5 biomarkers, A1AT, TIMP-2, NGAL, Fibrinogen and CRP and Multiplex 'b' also consisted of 5 biomarkers, RBP4, CC16, B2M, TIMP-1 and fMLP. The assays in multiplex ''a'' were all sandwich assays, however, 2 of the assays that were too sensitive required further manipulation. The A1AT required an additional pre-absorbent line to remove excess A1AT and the NGAL assay required the addition of free antibody into the system which had the same effect. Three of the 5 assays in multiplex ''b'' were inhibition assays, format of which was required to de-sensitise the assays. fMLP had to be an inhibition or competition assay due to the small size of the analyte. The test system was subjected to a small usability feedback survey with 10 people with COPD and 3 non-COPD users, who trialled the system for 1 month. Feedback from the volunteers highlighted issues surrounding the size of the cube reader button, as well as its automatic timing function which proved difficult to initiate as a result of multiple steps. The changes would be addressed by making modifications to the button and the reader firmware adapted to simplify the process of running the test by removing the choice of reading the test without the timer. It was concluded that after implementing these minor changes the technology platform would be deemed acceptable for the patients to use with no added burden to their already challenging lifestyle.

The assays within the multiplex devices correlated with the reference ELISA assays with urine samples that covered the expected range r^2>0.75. To achieve the same dynamic range for all assays to those obtained from the reference assays was more challenging as a) there was no allowance for sample dilution for the lateral flow assays compared to the laboratory assays (where a sample dilution step was possible), b) It was also important to take into account the large range of levels of each biomarker found in the samples that was challenging even for the ELISAs. Observed levels throughout all the testing showed that frequently, there were samples that fell outside of the ELISA standard curves, below and above. In principle the aim was to get as large a dynamic range as possible for each of the assays. A summary of the performance of each assay is discussed below:

- The A1AT assay had a good standard curve, CVs were all below 20% and the accuracy between 80-120% for all the standards. The dynamic range of the assay was not as large for the multiplex assay (4-1000ng/ml) compared to the ELISA range (7-1600ng/ml). Previous testing has suggested that higher levels of A1AT would be found in fresh samples which would result in a high dose hook effect.
- The performance of the TIMP-2 assay was above expectation, the CVs and accuracy were within the specifications set and the assay range was similar to the ELISA.

- The NGAL assay had a good standard curve, the accuracy of the bottom standard was out of specification in this instance (127%), however, removal of the lowest standard from the calibration curve would not be detrimental to the assay as similar to the A1AT assay it is slightly too sensitive (1-250ng/ml) compared to the ELISA (8-500ng/ml).
- The Fibrinogen assay had a good standard curve that met all specifications, however, it did appear to lack sensitivity (4-1000ng/ml) when compared to the ELISA (1-80ng/ml). This is reflected by the correlation with the ELISA with urine samples, it confirms that better sensitivity would be beneficial based on frozen sample testing.
- The performance of the CRP assay met all specifications, the CVs and accuracy were within the specifications set and the assay range was an improvement in relation to the standard curve obtained by ELISA.
- The RBP4 assay, similar to the A1AT and NGAL assay was too sensitive and non-optimal at the higher range (4-1000ng/ml compared to 47-3000ng/ml for the ELISA). The levels of RBP4 are not expected to present in urine at the higher range based on previous sample testing with frozen samples. It would again be beneficial to further de-sensitise the assay with a wider range should the assay be taken forward after evaluation with fresh samples.
- The CC16 assay had a good standard curve and met all specifications (accuracy and %CV), however, it did lack sensitivity (4-1000ng/ml) in relation to the ELISA standard curve (0.3-20ng/ml), the resulting range is deemed acceptable based on levels found in previous samples tested to date.
- The B2M assay had a good standard curve for the optimised range set and met all specifications, however, it did lack sensitivity (8-2000ng/ml) when compared to the ELISA (1-5000ng/ml), similar to CC16 above, the resulting range is deemed acceptable based on levels found in previous samples tested to date.
- The performance of the TIMP-1 and fMLP assay meets all specifications set and the assay range an improvement to those obtained by ELISA.

Out of the 10 assays, 5 of the assays required no known improvements and performed as expected. Three of the assays lacked sensitivity – fibrinogen, CRP, CC16 and two assays- A1AT and RBP4 were too sensitive. It was unknown if the challenges identified during the testing performed to date on frozen sample analysis would be repeatable in fresh urine samples. The final tests were deemed to be acceptable for further evaluation with fresh urine samples and in the setting for which they have been designed for use. The performance of the test will be challenged in further studies.

Chapter 6. Verification and validation of the point of care multiplex lateral flow tests

6.1 Introduction

For laboratory testing and development of the multiplex assays, the samples used were all frozen urine samples and, as the samples were limited, a set volume of sample was added per device (650µl) which was the minimum volume of sample that could be used for the device to be used effectively. It was important to understand the potential implications of using these methods when testing in the laboratory environment as compared to how the devices would be used in "real-life" situations. For the planned trial (chapter 7), the two test devices developed in chapter 6 were to be used by patients for home monitoring of COPD exacerbations (observational study). The devices were to be used one at a time by dipping them into urine, in a jug, and setting the measuring instrument (the cube) to read the device after 10 minutes. Ideally the devices would be removed from their pouches immediately before testing, dipped in urine and placed on the cube reader adaptor to be read 10 minutes later, with the cube programmed with an automatic timer. outside of a trial setting, the devices should be suitable for mid-stream testing.

The following aspects of the use of the device were considered:

1) Evaluation of different methods of applying the sample to the test devices including pipetting vs dipping - Dipping the device into the sample could lead to variation as the test could either be dipped into the sample at an angle for 5-10 seconds or it could be performed upright. Visually the indication that the device has started to run is the presence of the red gold conjugate flowing in the device window and this criterion was used to assess the different methods.

2) Blood spiking - It is possible that some samples could also contain potential interfering substances such as blood. In order to see if blood had any effect on the assays, samples with and without spiked blood were tested.

3) Cross reactivity – Each device consisted of five different assays, cross reactivity studies were performed on the devices to understand if any of the assays cross reacted with each other in the presence of each analyte.

4) Sample stability study - stability studies were performed to assess whether samples were stable during transport and upon freeze-thawing. Verification of this was required as the patients from the planned clinical trial were going to send weekly urine samples by mail to the lab for further analysis. Once received, the samples would be tested, aliquoted and frozen at -80°C. In order to determine if these processes had any effect on the samples, fresh urine samples were initially tested and then subjected to a transport and freeze-thawing study to evaluate any changes in results.

5) Samples tested within a 24hr period - in the planned clinical study, the volunteer would be running 2 tests from one collected urine sample sequentially. This experiment was designed to determine how stable the urine sample was after collection over 24hrs.

6) Line development/Measurement time - the test was designed for the result to be read after 10 minutes, the reader has an automatic timer of 10 minutes. However, the time at which the device is run and then placed in the reader cannot be controlled. Accordingly, it is important to determine whether any deviation from the protocol in terms of delay in reading is likely to affect the test result.

7) Batch reproducibility - volunteers were likely to receive multiple, and potentially different, batches during the trial and therefore, as part of validation it was necessary to ensure that batch-to-batch variability was minimal and that the QC criteria was appropriate to identify any batches that were likely to be unreliable.

6.2. Methods

6.2.1 Materials/reagents

- Buffer PBST- 1%BSA (10mM phosphate buffered saline pH7.5, supplemented with 1% (w/v) BSA)
- Antigens for Multiplex A: A1AT (Merck Calbiochem, 178251), TIMP-2, (Kent university, Canterbury), NGAL (Alere San Diego, RFPR017674), Fibrinogen (BBI, 132-3), CRP (Lee Biosolutions, 140-11R)
- Antigens Multiplex B: RBP4 (BBI, P124-1), CC16 (Novoprotein CU06), B2M (BBI, P122-1), TIMP-1 (Novoprotein, C456), fMLP (Sigma, 47729)
- Fresh urine and blood samples collected from healthy controls
- Headstart devices a and b (batch AD210317, AD240517, AD030417)
- ELISAs for all 10 biomarkers (as described in chapter 2)
- Multistix 10 SG Reagent Strips (Siemens)

6.2.2 Equipment

- Cube reader; Cube/device adaptor; Cable (OpTricon)
- RFID card wide control + Area under the curve (AUC)
- Pipettes
- Universals/Bijou/2ml Eppendorf tubes
- Timer
- Plate shaker/plate washer

6.2.3 Testing buffer standards - pipette method

Standard testing was performed as follows: i) devices were removed from the pouches immediately before testing; ii) caps were removed and device was placed on a flat surface; iii) 10 minute timer was initiated; iv) With the device laid on a surface, 650µL of each standard was pipetted on to the region of the wick closest to the conjugate pad. v) the device was read after 10 minutes using a cube reader. vi) once reading was complete, the devices were discarded.

Standards for Multiplex A & B were made according to table 6.1.

6.2.4 Testing urine samples

Pipette method: as described above for the buffer standard testing.

Dipping method – at a ''slant'': device was dipped into the urine sample at a slant for approximately 45° for 15 seconds so that the wick was immersed and then laid flat on a surface. The device was then read after 10 minutes using the cube reader as already described in section 6.2.3.

Dipping method – ''upright'': device was dipped into the urine sample held upright so that the wick was immersed and removed once the gold conjugate solution was visible in the device read window. The device was then laid flat on a surface and read after 10 minutes using the cube reader as already described in section 6.2.3.

Table 6.1. Standards used for standard testing. The top standard was prepared at shown concentrations for all analytes from the stock concentrations and then diluted 1 in 2.5 in the standard dilution buffer PBST 1% BSA.

ng/ml	A1AT	T2	NGAL	FIB	CRP	RBP4	CC16	B2M	T1	fMLP
1	1000	200	500	1000	25	500	2500	500	100	50
2	400	80	200	400	10	200	1000	200	40	20
3	160	32	80	160	4	80	400	80	16	8
4	64	12.8	32	64	1.6	32	160	32	6.4	3.2
5	25.6	5.12	12.8	25.6	0.64	12.8	64	12.8	2.56	1.28
6	10.24	2.048	5.12	10.24	0.256	5.12	25.6	5.12	1.024	0.512
7	4.096	0.8192	2.048	4.096	0.1024	2.048	10.24	2.048	0.4096	0.2048
8	0	0	0	0	0	0	0	0	0	0

6.2.5 Testing dipping variation

In order to test for variability due to dipping variation, the devices (Batch AD210317) were tested with fresh samples using the following methods: Dipping the device in the sample at a slant for 15seconds; dipping upright with the wick fully immersed into the sample until the device starts running and pipetting 650µL of sample on the wick. Urine samples were collected from 21 healthy volunteers, and 5 replicates were tested.

Results were taken from the 5 replicates for each sample for each assay and each method and the mean, SD and %CV calculated. Graphs were produced using GraphPad Prism Version 7.

6.2.6 Blood spiking testing

According to the literature, the Multistix Test can detect between 150 and 620 µg/l of free haemoglobin. Normal blood contains 15 g/dl (150,000,000 µg/l). EDTA-treated blood was diluted 1/1,000,000 in 3 healthy urines (1/1000 in H_2O followed by 1/1000 in urine) and applied to the blood reagent pad of a Multistix (Yellow: no blood present, patchy green: intact blood present (moderate), green: haemolysed blood present large).

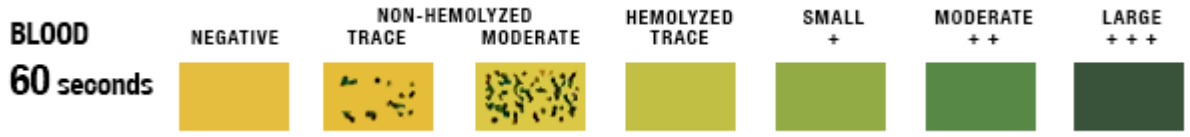


Figure 6.1. Multistix results regarding blood detection in urine.

In order to establish the maximum amount of blood that the Multistix can detect, 3 healthy urines were spiked with the blood diluted 1/100,000 and applied to the blood reagent pad of a Multistix to confirm the spiking. Once the samples were spiked with optimal volumes of blood, devices were tested with the samples in the same way as in testing urine samples (batch AD240617).

6.2.7 Cross-reactivity testing

This was undertaken to establish if there was cross-reactivity between the biomarkers in both multiplex devices with the top standards of each assay (the most extreme condition).

Testing was performed using triplicate devices. The standards used for testing were formulated according to table 6.1 and the combinations of standards prepared for this testing are shown in table 6.2. The batch of devices used was AD240517. A combination where the specific standard was missing yet a positive result was obtained for that assay would indicate that there was cross-

reactivity. The experiment was designed so that should there be cross reactivity then the cause would be identified from a secondary analysis of the data.

Table 6.2. Standards used for cross-reactivity testing. TS stands for Top Standard, ✓ indicates the presence of the antigen and X indicates the absence of that antigen on the standard mix.

	Headstart A						Headstart B				
Assay	**A1AT**	**T2**	**NGAL**	**FIB**	**CRP**	**Assay**	**RBP4**	**CC16**	**B2M**	**T1**	**fMLP**
TS	✓	✓	✓	✓	✓	**TS**	✓	✓	✓	✓	✓
A1AT	X	✓	✓	✓	✓	**RBP4**	X	✓	✓	✓	✓
T2	✓	X	✓	✓	✓	**CC16**	✓	X	✓	✓	✓
NGAL	✓	✓	X	✓	✓	**B2M**	✓	✓	X	✓	✓
FIB	✓	✓	✓	X	✓	**T1**	✓	✓	✓	X	✓
CRP	✓	✓	✓	✓	X	**FMLP**	✓	✓	✓	✓	X

6.2.8 Transport /Stability Study

The objective of the transport study was to collect urines from healthy individuals, pool them together, determine high and medium levels of each marker by spiking and testing on both devices at specific timepoints after the pooled samples had been exposed to the different conditions. The conditions tested were: ambient storage, transport storage (car with TinyTag datalogger to track the temperature), and freeze-thaw at -80°C storage. In order to perform this testing, the pooled samples were first tested with the device to determine initial levels of the markers. Based on these results the pooled samples were split into 10 aliquots and 5 were spiked with multiplex A antigens (A1- A5) and the remaining 5 with multiplex B antigens (B1-B5) according to table 6.4. Following spiking, each set of samples for A & B was tested on both of the respective multiplex devices to obtain initial timepoint reference values. Sample testing was performed according to 6.2.4 using the pipetting method.

In order to facilitate the study each pooled sample (A1-A5 and B1-B5) were aliquoted as follows:

- For ambient storage study: 15ml in a universal container
- For transport study (with TinyTag datalogger): 15ml in a universal container
- For freeze / thaw study, -80°C Storage: 15ml in a universal container
- 50 aliquots of 1.5ml in 2ml tubes – to be used as urine QC test for HSV3 batches, -80°C Storage

Each aliquot was tested at different timepoints (1, 4, 6, 8, 12, 15 days) for each study. For each timepoint, 2 replicates were tested.

Table 6.3. Summary of the spiking of the pooled samples. Five spiked solutions for both A and B multiplexes. Spiking was by adding each antigen to the polled urine samples directly from the antigen stock according to the respective dilutions.

	A1		A2		A3		A4		A5	
	Dilution	**µL**	**Dilution**	**µL**	**Dilution**	**µL**	**Dilution**	**µL**	**Dilution**	**µL**
A1AT	1/1000	150	1/2000	75	1/8000	18.75	0	0	0	0
T2	0	0	1/6000	25	1/12000	12.5	1/24000	6.25	0	0
Fib	1/2000	75	0	0	1/500	300	1/1000	150	0	0
NGAL	1/8000	18.75	1/16000	9.4	0	0	1/4000	37.5	0	0
CRP	1/40000	37.5	1/80000	18.75	0	0	0	0	1/20000	75
	B1		**B2**		**B3**		**B4**		**B5**	
	Dilution	**µL**	**Dilution**	**µL**	**Dilution**	**µL**	**Dilution**	**µL**	**Dilution**	**µL**
RBP4	1/2000	75	1/4000	37.5	1/8000	18.75	0	0	0	0
CC16	0	0	1/500	300	1/1000	150	1/2000	75	0	0
B2M	1/4000	37.5	0	0	1/1000	150	1/2000	75	0	0
T1	1/4000	37.5	1/8000	18.75	0	0	1/2000	75	0	0
fMLP	1/2000	75	1/4000	37.5	0	0	0	0	1/1000	150

6.2.9 Comparison of fresh and frozen samples

A total of 40 fresh urine samples that were a combination of COPD and CF samples (sent by post to Mologic >1 day old) were tested with both the multiplex lateral flow tests and the ELISAs. The samples were aliquoted into small vials and frozen at -80°C, the fresh samples were stored at 2-8°C. The frozen samples were thawed and the fresh samples were equilibrated to room temperature before testing within 24 hrs of receipt of samples. The batch of test devices used was batch AD240517. Cube reader results were converted to concentration values for analysis.

The statistical analysis used to compare results from fresh samples and frozen samples with both ELISA and LF was r^2, Spearman's rank test and Bland Altman plots all using GraphPad Prism V7.

6.2.10 Stability of urine throughout the day

In order to test the urine variation throughout the day, the devices (Batch AD030417) were tested with fresh samples (UD 28, 32, 37, 40, 45, 46) in duplicate (for each device A & B) at different timepoints: 12:45-0h, 13:45-1h, 14:45-2h, 15:45-3h, 16:45-4h, 9:00-16h, 12:45-24h. The profiles were plotted using Graphpad Prism Version 7.

6.2.11 Testing Line development/measurement time

To test line development, standards with high, medium, low and zero (negative) concentrations of antigens were tested in duplicate on devices (Batch AD030417). Standard testing was made using the pipetting method. The cube readings were made as soon as the device starts, reading every 10 seconds for 30 minutes. The Autoclicker software was used together with the Cube reading software using 180 repetitions and sleeping time of 3333 million milliseconds. The devices were discarded once reading was complete. The standard concentrations used for testing are displayed in table 6.5.

The results were plotted on graphs with the 10 minute timepoint highlighted. The graphs shown were produced in excel.

Table 6.4. Standards used for Line development test.

ng/ml	A1AT	T2	NGAL	FIB	CRP	RBP4	CC16	B2M	T1	fMLP
High	1000	200	500	1000	25	500	2500	500	100	50
Medium	64	12.8	32	64	1.6	32	160	32	6.4	3.2
Low	4.096	0.8192	2.048	4.096	0.1024	2.048	10.24	2.048	0.4096	0.2048
Negative	0	0	0	0	0	0	0	0	0	0

6.2.12 Batch to batch reproducibility

Three batches were tested by one operator, 5 replicates per batch, with a complete standard curve in a randomised order. Pipette method was used for running the assay. Standards were prepared, and the same preparation was tested on all three batches (batch 1, SR180917, batch 2 SR260917, batch 3 SR031017) Analysis involved assessing correlations between each batch with a desired outcome of an r^2 greater than 0.95.

6.3 Results

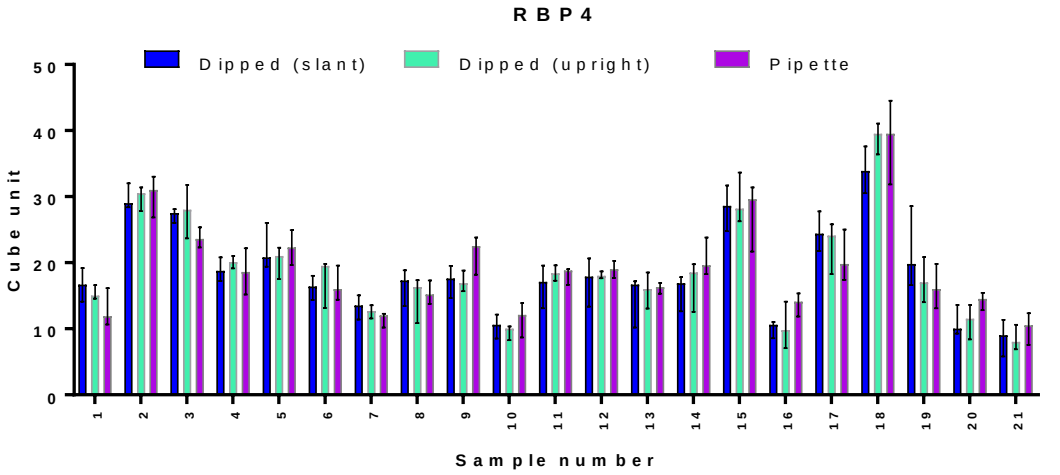
6.3.1 Testing dipping variation

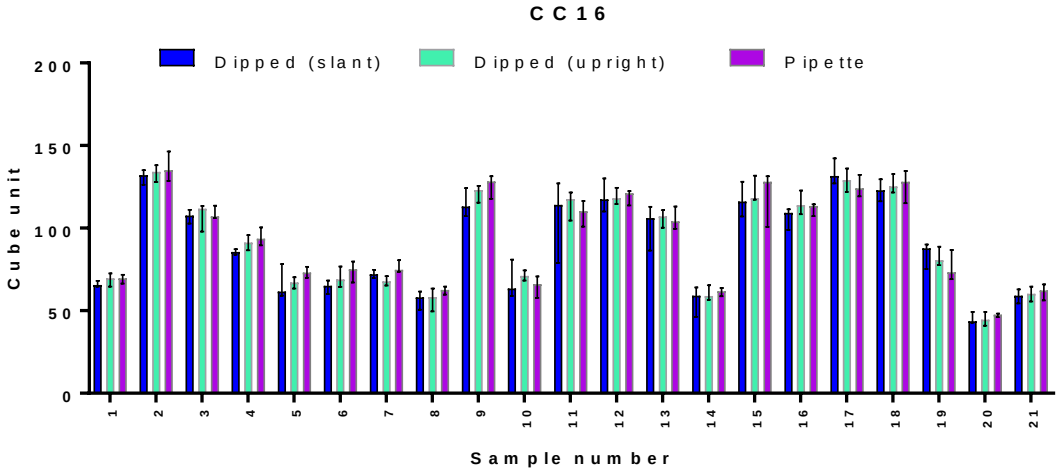
Three methods of applying the sample to the test device were evaluated, the first 2 methods for dipping the device and the third method for the standard laboratory testing of applying a set volume of sample to the device using a pipette.

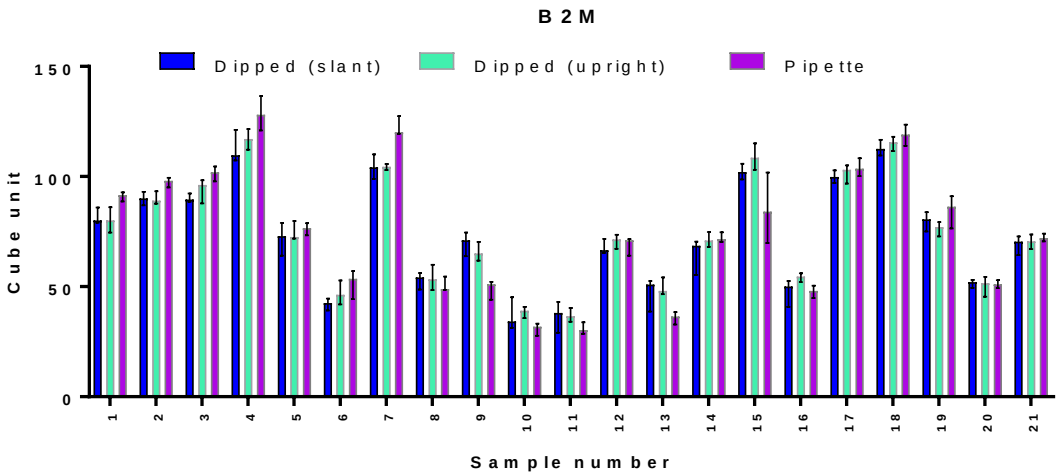
In principle according to the graphs below in figure 6.2, all results for all three methods fitted within the normal variation within the test. There was not one method that stood out as being different. Variation was assessed using % CV and identifying the proportion of samples that had higher than 20% CV for each assay.

- For the RBP4 assay, method 1, 8/21 samples gave a CV higher than 20% (38%), method 2, 6/21 samples gave a CV higher than 20% (29%), and method 3, 5/21 samples gave a CV higher than 20% (24%).
- For the CC16 assay, method 1, 1/21 samples gave a CV higher than 20% (5%), method 2, 0/21 samples gave a CV higher than 20% (0%), and method 3, 0/21 samples gave a CV higher than 20% (0%).
- For the B2M assay, method 1, 2/21 samples gave a CV higher than 20% (10%), method 2, 0/21 samples gave a CV higher than 20% (0%), and method 3, 1/21 samples gave a CV higher than 20% (5%).
- For the TIMP-1 assay, method 1, 4/21 samples gave a CV higher than 20% (19%), method 2, 2/21 samples gave a CV higher than 20% (10%), and method 3, 3/21 samples gave a CV higher than 20% (14%).
- For the fMLP assay, method 1, 9/21 samples gave a CV higher than 20% (43%), method 2, 3/21 samples gave a CV higher than 20% (14%), and method 3, 2/21 samples gave a CV higher than 20% (10%).

The general trend was that method 1 gave slightly worse performance than the others and method 3 gave the least variability.


RBP4
Dipped (slant)
Dipped (upright)
Pipette
Cube unit
0
10
20
30
40
50
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Sample number


CC16
Dipped (slant)
Dipped (upright)
Pipette
Cube unit
0
50
100
150
200
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
Sample number


B2M
Dipped (slant)
Dipped (upright)
Pipette
Cube unit
0
50
100
150
1
2
3
4
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6
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11
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19
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21
Sample number

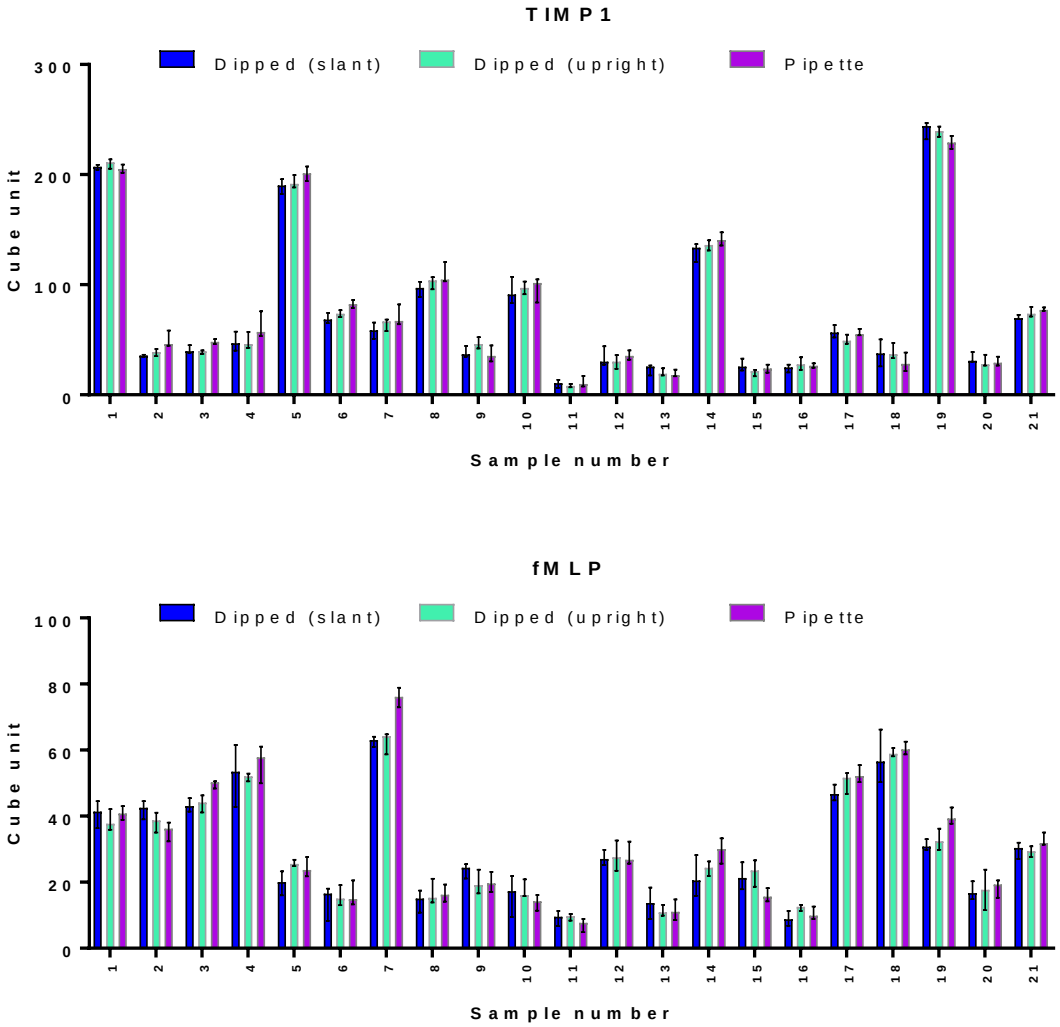


Figure 6.2. Graphical representation of the variation of each assay (cube units) for the samples tested with 3 different methods: dipping devices at a slant for 15s, dipping upright until it starts and pipetting 650 µL; Each column cluster represents the different samples tested, each column the 3 different methods tested, Y-axis cube units. Median and interquartile range shown.

Table 6.5. The average, %CV for 3 different method of sample application for RBP4, CC16 and B2M (dipping with a slant, dipping upright till the sample has run, and pipette application). Highlighted in bold are the results out of specification.

Sample ID	RBP4						CC16						B2M					
	Dipping (slant)		Dipping (upright)		Pipette		Dipping (slant)		Dipping (upright)		Pipette		Dipping (slant)		Dipping (upright)		Pipette	
	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV
1	16.7	16.6	15.5	9.6	13.1	***23.3***	66.1	3.4	68.8	7.3	69.2	4.1	82.0	4.9	80.2	7.8	90.9	3.0
2	30.0	7.9	29.8	7.5	30.1	10.4	130.9	3.6	133.2	5.0	137.0	6.7	90.0	4.2	90.2	4.3	97.4	2.6
3	27.1	4.2	27.8	15.6	23.8	7.1	107.0	5.1	106.9	8.2	109.2	5.5	90.2	2.2	93.7	5.8	101.3	3.5
4	18.9	9.8	20.1	4.9	18.7	19.4	85.6	2.0	91.1	6.2	94.7	5.7	112.7	7.1	116.9	4.1	128.6	6.7
5	22.3	16.6	20.1	16.1	22.3	13.7	67.3	16.3	66.8	6.4	73.2	5.2	71.8	13.6	75.1	5.9	76.1	4.2
6	16.2	11.6	17.1	***22.8***	16.8	16.6	64.4	6.5	70.2	9.2	73.8	8.8	42.0	8.2	47.2	13.1	51.2	13.4
7	13.3	15.6	12.6	10.6	11.4	10.8	72.2	4.0	68.0	4.7	76.6	4.9	104.4	6.7	104.3	1.5	122.8	3.6
8	16.4	***22.2***	14.5	***25.1***	15.5	14.7	56.5	11.9	56.9	12.7	62.3	4.6	52.8	8.0	54.0	13.0	51.0	6.4
9	17.2	14.6	17.2	11.0	21.3	16.9	115.2	8.2	121.0	4.7	125.3	5.9	69.5	8.5	65.8	7.1	48.7	9.0
10	10.4	18.7	9.4	13.4	11.5	***23.1***	68.6	16.6	71.3	5.1	64.6	13.1	37.4	20.2	38.4	7.3	30.7	9.9
11	16.5	***23.6***	18.4	7.4	18.0	7.1	105.2	***26.5***	113.9	7.8	109.0	8.6	36.4	***23.3***	37.0	8.4	31.0	9.1
12	17.2	***23.4***	18.1	3.4	19.0	8.0	119.6	11.2	119.2	4.3	118.6	4.6	68.1	5.1	70.6	5.4	68.4	5.8
13	14.3	***27.3***	15.8	17.3	16.2	5.2	100.9	17.7	105.9	5.4	105.9	7.2	46.6	**22.4**	49.9	8.1	35.8	10.4
14	15.6	***23.0***	16.6	***23.4***	20.7	14.0	55.9	16.4	60.6	8.1	61.3	4.6	64.0	12.6	71.3	5.3	72.3	3.4
15	28.3	13.2	29.6	17.9	27.2	***23.0***	117.2	8.9	123.2	7.9	118.4	14.0	102.2	3.7	108.9	6.5	85.4	***21.1***
16	9.9	17.5	10.4	***36.0***	13.7	17.6	105.9	7.8	115.2	7.3	111.4	4.8	47.3	13.1	54.1	5.0	47.7	6.3
17	24.7	13.0	22.4	18.4	20.9	19.8	134.0	6.2	129.0	6.2	125.4	5.4	99.9	4.0	101.3	4.3	104.1	4.9
18	34.0	13.0	38.9	6.6	38.4	18.4	122.9	6.6	126.7	4.8	125.4	8.5	113.0	3.2	114.9	3.1	118.8	4.7
19	22.0	***32.5***	17.3	20.9	16.4	***24.8***	83.7	9.4	82.6	7.8	76.9	12.2	79.6	5.5	76.2	5.6	84.2	9.9
20	11.1	***21.7***	11.1	***25.2***	14.2	10.0	45.5	7.6	45.0	9.3	47.2	2.7	51.4	4.3	50.2	10.3	51.1	4.3
21	8.7	***34.1***	8.6	***22.4***	10.1	***30.0***	58.7	8.3	60.1	8.4	61.3	8.2	68.9	6.3	70.4	6.3	72.2	2.6

Table 6.6. The average, %CV for 3 different methods of sample application for TIMP-1 and fMLP (dipping with a slant, dipping upright till the sample has run, and pipette application). The results out of specification are highlighted in bold type.

Sample ID	TIMP-1						fMLP					
	Dipping (slant)		Dipping (upright)		Pipette		Dipping (slant)		Dipping (upright)		Pipette	
	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV	Average	%CV
1	206.7	1.1	209.8	2.3	205.3	2.2	40.6	10.5	38.7	8.8	40.9	5.4
2	35.3	3.6	38.5	9.1	50.4	20.3	41.9	7.9	38.2	9.9	35.4	11.1
3	41.2	9.1	39.0	5.4	48.3	4.7	43.3	4.9	43.8	6.2	49.6	2.3
4	48.1	18.8	49.0	15.2	63.1	20.5	52.6	18.7	51.7	2.6	55.9	11.0
5	189.2	3.9	193.5	2.9	200.8	3.6	19.7	***24.3***	25.8	4.8	24.5	13.1
6	69.5	7.1	73.6	4.7	82.5	4.9	13.8	39.4	15.9	20.1	16.5	22.5
7	58.2	13.1	63.8	10.1	71.9	14.8	62.6	3.4	62.2	6.7	75.9	4.5
8	96.0	7.8	101.8	6.3	110.3	8.5	14.3	***24.9***	17.0	***22.3***	16.6	16.3
9	38.9	14.3	46.9	14.6	37.2	***22.6***	23.5	12.1	20.0	***23.6***	20.0	20.1
10	94.4	13.0	97.0	7.2	95.8	11.9	16.0	***41.9***	17.9	20.0	13.8	18.7
11	10.1	***48.4***	8.2	***21.7***	11.8	***41.4***	9.1	***26.0***	9.4	11.5	7.0	***31.1***
12	34.7	33.0	29.9	21.0	35.9	12.3	27.4	9.2	27.9	20.0	28.5	14.5
13	22.9	***27.4***	20.5	16.9	19.5	19.5	13.7	***40.3***	11.4	15.4	11.5	***28.2***
14	129.6	6.9	135.8	4.1	141.4	5.2	21.7	***32.8***	24.1	9.9	29.6	13.8
15	27.1	***22.9***	20.0	16.3	23.7	17.5	21.8	***23.0***	22.8	19.6	16.1	13.2
16	24.0	15.4	28.2	***26.3***	26.6	8.6	9.0	***31.1***	12.3	8.0	10.6	18.1
17	57.5	10.0	50.2	8.3	56.5	7.1	47.1	5.8	50.2	6.7	52.7	5.1
18	38.0	***36.3***	39.5	19.0	29.6	***34.8***	57.9	14.4	59.3	2.7	60.5	3.5
19	240.4	3.4	239.1	2.4	229.2	2.7	31.3	7.1	32.9	11.0	40.0	7.1
20	33.7	17.5	30.7	18.5	30.2	15.9	17.4	***21.2***	17.7	***40.7***	18.2	15.6
21	70.5	3.3	75.1	6.7	77.7	2.3	29.7	8.7	29.3	5.5	32.9	6.7

5.3.2 Blood spiking testing

The Multistix results suggested that 1/100,000 dilution of blood in urine was the most suitable dilution for assessing the potential interference on the tests by the presence of blood.

According to the data the majority of the assays were unaffected by the addition of blood. There were two samples that appeared to give a higher signal for the spiked blood sample in comparison to the unspiked samples, these mainly had an impact on the assays in device b: B2M, CC16 and TIMP-1.

The spiked samples gave significantly higher signals in relation to the unspiked samples for these three assays which were non-specific as these were a mix of sandwich and inhibition assay therefore no pattern was observed.

The assays that were not affected overall were A1AT, TIMP-2, NGAL, fibrinogen, CRP, RBP4 and fMLP.

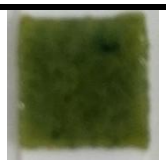
Sample	Unspiked urine	Spiked Urine	
		1/100,000,000 EDTA blood	1/100,000 EDTA blood
1			
2			
3			

Figure 6.3. Optimisation of blood spiking for Multistix testing. Multistix results obtained before and after spiking three of the healthy urine samples with EDTA treated blood at different concentrations. The remaining samples used for testing were also spiked and tested on Multistix and all presented a green colour after spiking (Data not shown).

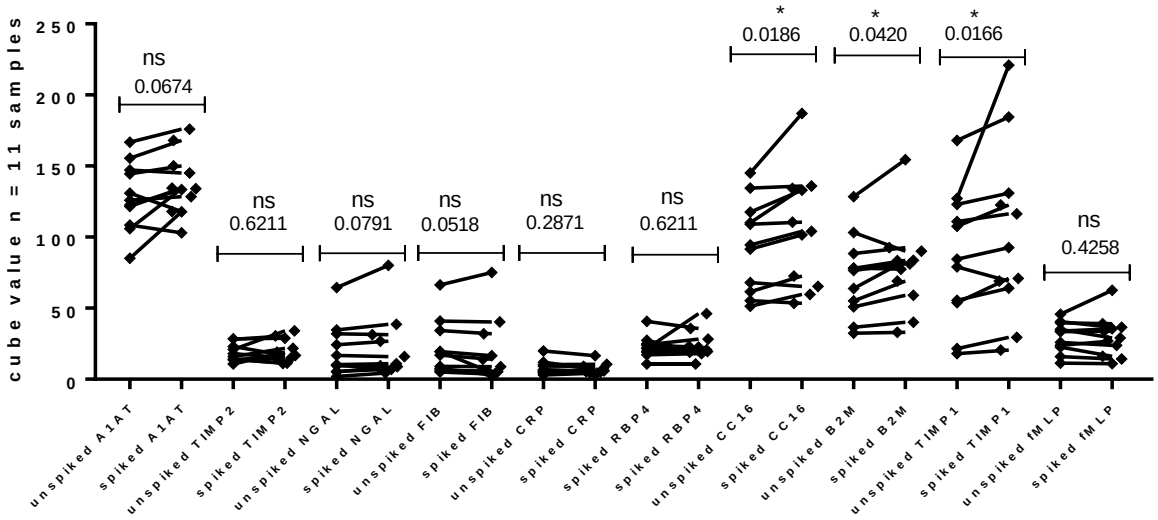


Figure 6.4. Pre- and post-spike of blood (1/100,000 blood) into 10 urine samples for each individual assay. Wilcoxon matched-pairs signed rank test used to determine significance between each group, p value <0.05 was deemed significant.

6.3.3 Cross-reactivity testing

The aim of this cross-reactivity testing was to determine if there was any cross reactivity between the markers in the multiplex assays. The devices were tested with a mixed antigen solution that included the top standard of the respective multiplex assays on that device, with top standards of all the assays minus its respective standard and a buffer only sample where all antigens were absent.

Regarding cross reactivity testing on the **sandwich assays**, the expected cube unit values of an assay on the multiplex should be low in the absence of the corresponding antigen from the standard tested. If the cube units for said assay were not low it would mean that something other than the antibody for a specific antigen was binding to said antibody, *i.e.* if A1AT was missing from the mixed antigen solution, the A1AT line should have a low line intensity on the A1AT assay, otherwise it would indicate that there was cross reactivity with another assay on the multiplex. In analysing the data for Multiplex A (figure 6.5), it was clear that in the absence of each antigen in the respective assay the cube units were low, which was the expected result for sandwich assays.

Regarding cross reactivity testing on the **inhibition assays** (all assays on multiplex B except TIMP-1), the expected cube unit values of an assay on the multiplex should be high in the absence of the corresponding antigen from the standard tested. If the cube units for said assay were not as high as normal it would mean that something other than the antibody specific antigen was binding to said antibody, *i.e.* if RBP4 was missing from the mixed antigen solution, the RBP4 line should have a high line intensity, otherwise it would indicate that there was cross reactivity with another assay on the multiplex, as something is binding to the anti-RBP4 antibody conjugated to the gold nanoparticles. In analysing the data for Multiplex B (figure 6.6), it was clear that in the absence of each antigen the respective assay cube units were high, which is the expected result for the inhibition assays. Regarding CC16, the reason why the difference between the presence and absence of the antigen was not as significant when compared to the other inhibition assays, was because the top standard used for the CC16 assay was not high enough to completely inhibit the anti CC16 antibody from binding to the test line.

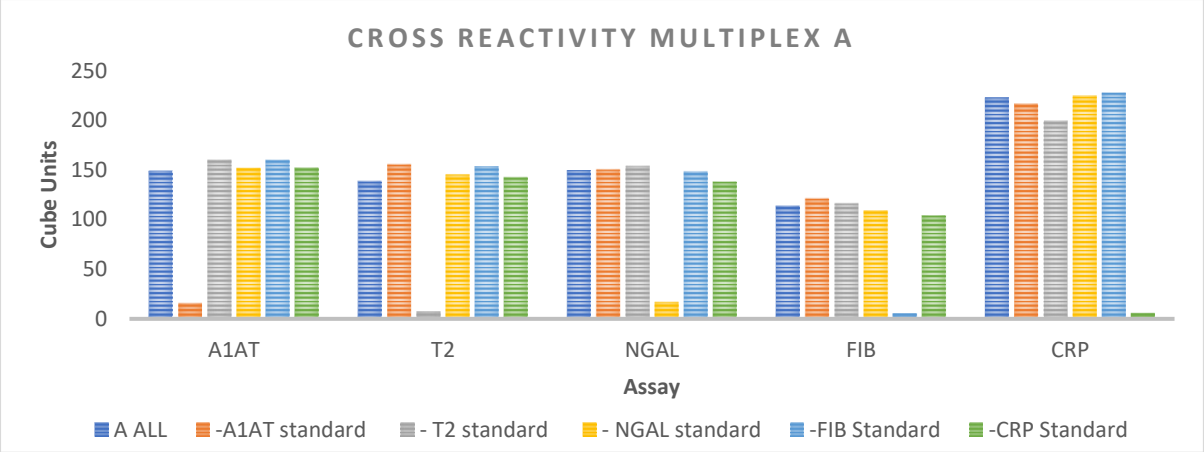


Figure 6.5. Graphical representation of the cube unit averages (3 replicates) for cross-reactivity experiments on Multiplex A. Each cluster of columns represents an assay of the multiplex device, and each coloured column represents cube units for the respective assay when 1 of the antigens is missing. “A All” stands for testing of top standard with all the antigens of the multiplex assays. For example, for A1AT, when the A1AT standard was missing, there was no signal as expected.

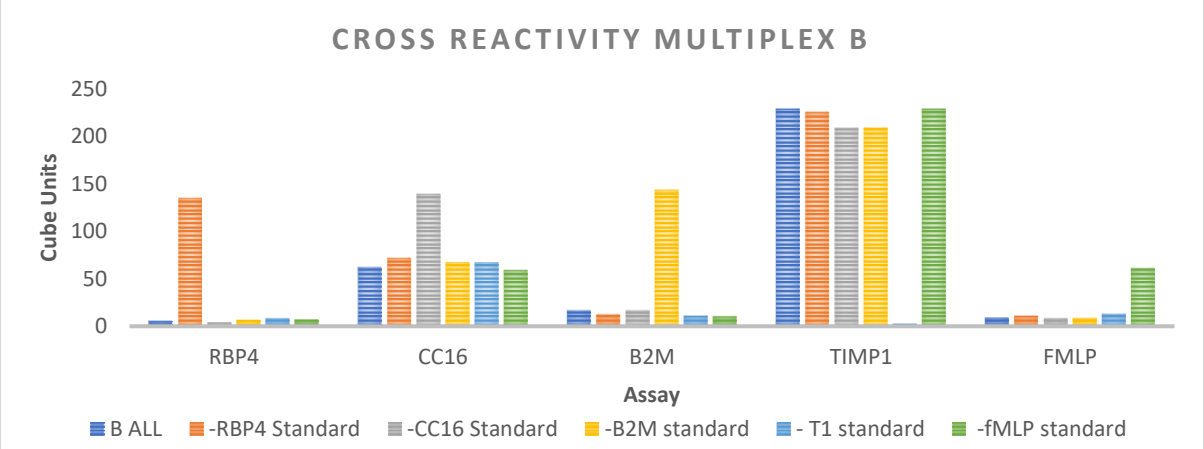


Figure 6.6. Graphical representation of the cube unit averages (3 replicates) for cross-reactivity experiments on Multiplex B. Each cluster of columns represents an assay of the multiplex device, and each coloured column represents cube units for the respective assay when 1 of the antigens is missing. “B All” stands for testing of top standard with all the antigens of the multiplex assays. For example, for RBP4, when the RBP4 standard was missing, there was a signal as expected.

6.3.4 Transport stability study

The objective of the transport study was to collect urines from healthy individuals (spiked) and test urine variability after the exposure to different conditions. The conditions tested were: ambient storage, transport storage and freeze-thaw at -80°C storage. Ten samples (5 for each multiplex) were stored in each test condition. Each set of samples for devices A & B was tested on the respective multiplex devices to yield initial timepoint reference values. Each aliquot stored at each condition was then tested at different timepoints (1, 4, 6, 8, 12, 15 days).

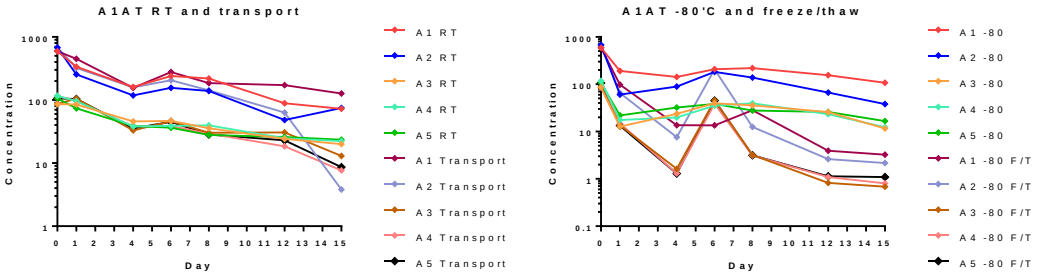
The first observation is that that the correlation between room temperature and transport results was very good across all assays. By way of example, the results will focus on RT, -80°C and freeze thawing.

- Regarding the sample variability of A1AT it was clearly shown that A1AT in fresh samples was not stable across the 15 days and was likely to continue to deteriorate further over time based on the downward trend. There was a >50% drop even at day 1 for the 2 high samples and less so for the low samples. When the sample was frozen there was an immediate decrease in levels of A1AT which was then maintained when frozen at -80°C storage. However, during multiple freeze thaw cycles the levels dropped further at each cycle.
- Levels of TIMP-2, similarly to A1AT, also decrease with time but not as dramatic at the start, there was possibly a 1-3-day lag time before it rapidly decreased. There was an effect of freezing the samples, though not as great a loss as A1AT but again, remained stable once frozen. There was minimal effect with multiple freeze thaw cycles.
- NGAL remained stable across the entire 15 days and also stable once frozen. There was some indication that there was deterioration with multiple freeze thaw cycles, the maximum allowance was 3 cycles.
- Fibrinogen appeared to be fairly stable both over time and with freezing. For the fresh samples, there was a slight decrease after 8 days. With freeze thawing, the results were quite erratic, there was one sample (A5) that demonstrated a significant decrease at the 3rd F/T cycle.
- CRP unexpectedly with the fresh sample deteriorated at day 4 of testing but then started to increase its level after day 12 but not back up to the levels obtained at day 0. No effect with freezing was observed and it remained stable once frozen and with up to 6 freeze thaw cycles.
- RBP4 had an unexpected trend where it increased for some samples with time. However, this was likely due to the position on the standard curve where it was not on the linear part

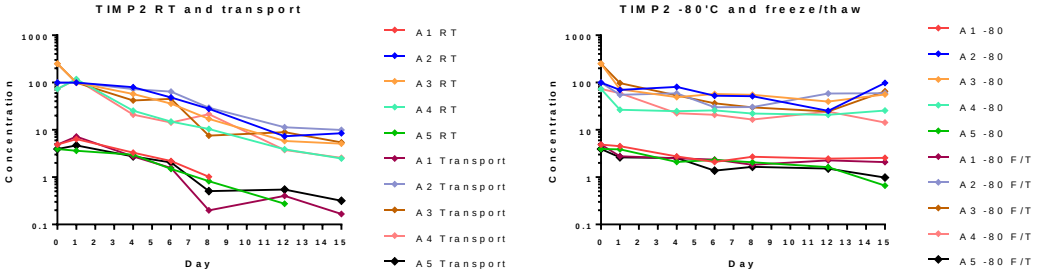
of the curve. Overall, stability was good over time and with freezing and multiple freeze thaw cycles.

- CC16 with the high samples was very stable under all conditions.
- B2M, Similar to CC16 was very stable under all conditions, however there was an upward trend with the fresh samples the reason for which was not understood.
- TIMP-1 deteriorated over the 15 days slightly though was stable at day 1 and up to day 8 with the high samples, but with the low samples there was a drop at day 4 onwards. Freezing the samples was acceptable and they remained stable. Freeze thawing samples appeared to cause some deterioration as shown by the variability with some samples.
- fMLP showed clear evidence that the analyte was not stable even at day 1 (not unexpected). There was some indication that freezing the samples improved the stability over time, especially when compared directly to the fresh samples, but there was a large variation that was not seen with any of the other assays. It was not clear if even when frozen for a longer period the analyte was stable. Freeze thawing the samples showed a gradual decrease in biomarker level at each cycle.

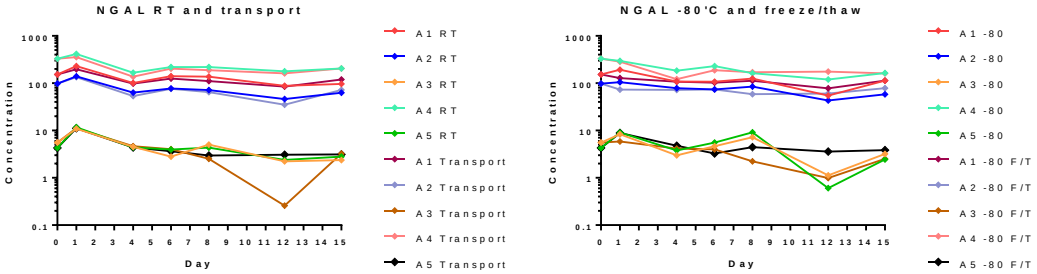
The overall summary was that for the majority of the assays, the sample needed to be tested on the day of collection and that results obtained from the fresh samples sent to Mologic that are days old should be regarded with caution. There was also evidence that frozen samples were not truly representative of fresh samples although likely to be proportionally related with the exception of fMLP and once the samples have been frozen they do remain stable. Freeze thaw samples not recommended, A1AT, NGAL, fibrinogen, TIMP-1, fMLP all showed deterioration with multiple freeze/thaw cycles.


A1AT RT and transport
Concentration
Day
A1 RT
A2 RT
A3 RT
A4 RT
A5 RT
A1 Transport
A2 Transport
A3 Transport
A4 Transport
A5 Transport

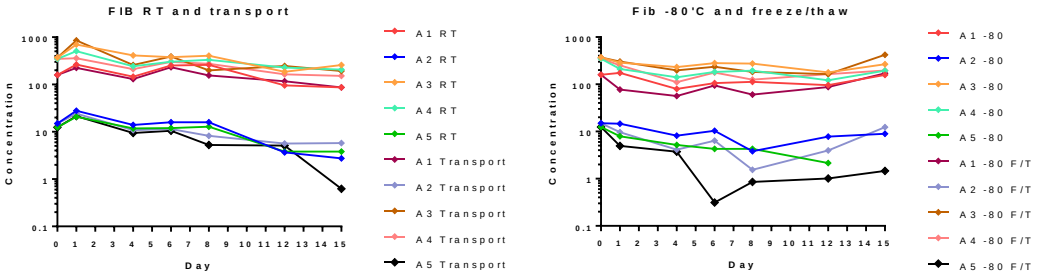
A1AT -80'C and freeze/thaw
Concentration
Day
A1 -80
A2 -80
A3 -80
A4 -80
A5 -80
A1 -80 F/T
A2 -80 F/T
A3 -80 F/T
A4 -80 F/T
A5 -80 F/T


TIMP2 RT and transport
Concentration
Day
A1 RT
A2 RT
A3 RT
A4 RT
A5 RT
A1 Transport
A2 Transport
A3 Transport
A4 Transport
A5 Transport

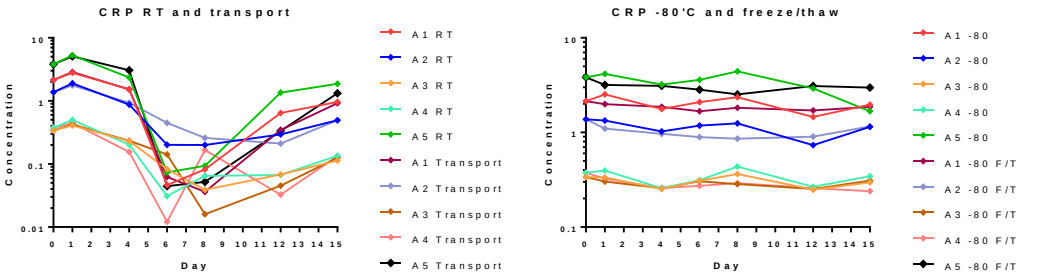
TIMP2 -80'C and freeze/thaw
Concentration
Day
A1 -80
A2 -80
A3 -80
A4 -80
A5 -80
A1 -80 F/T
A2 -80 F/T
A3 -80 F/T
A4 -80 F/T
A5 -80 F/T


NGAL RT and transport
Concentration
Day
A1 RT
A2 RT
A3 RT
A4 RT
A5 RT
A1 Transport
A2 Transport
A3 Transport
A4 Transport
A5 Transport

NGAL -80'C and freeze/thaw
Concentration
Day
A1 -80
A2 -80
A3 -80
A4 -80
A5 -80
A1 -80 F/T
A2 -80 F/T
A3 -80 F/T
A4 -80 F/T
A5 -80 F/T


FIB RT and transport
Concentration
Day
A1 RT
A2 RT
A3 RT
A4 RT
A5 RT
A1 Transport
A2 Transport
A3 Transport
A4 Transport
A5 Transport

Fib -80'C and freeze/thaw
Concentration
Day
A1 -80
A2 -80
A3 -80
A4 -80
A5 -80
A1 -80 F/T
A2 -80 F/T
A3 -80 F/T
A4 -80 F/T
A5 -80 F/T


CRP RT and transport
Concentration
Day
A1 RT
A2 RT
A3 RT
A4 RT
A5 RT
A1 Transport
A2 Transport
A3 Transport
A4 Transport
A5 Transport

CRP -80'C and freeze/thaw
Concentration
Day
A1 -80
A2 -80
A3 -80
A4 -80
A5 -80
A1 -80 F/T
A2 -80 F/T
A3 -80 F/T
A4 -80 F/T
A5 -80 F/T

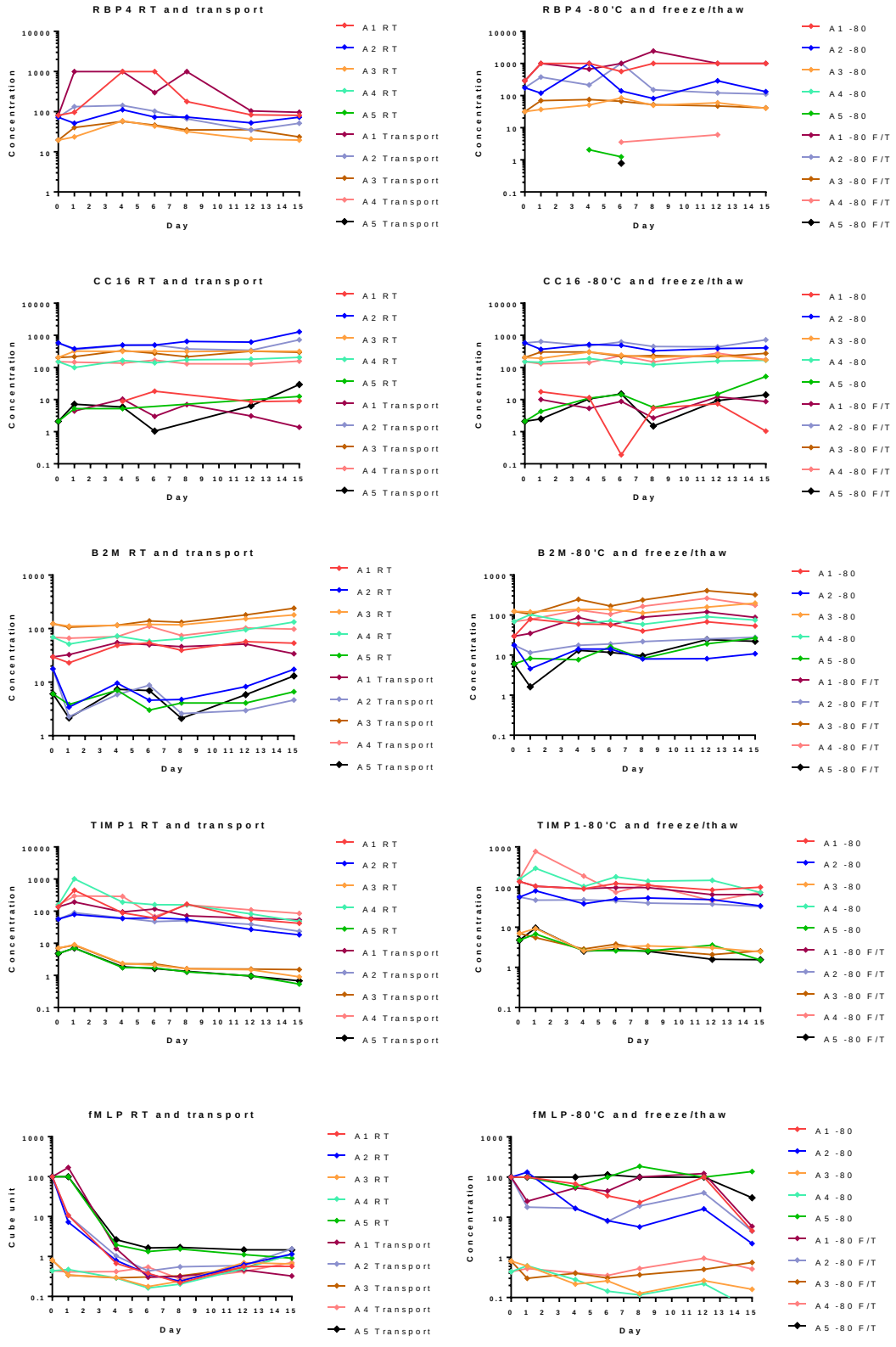


Figure 6.7. Graphical representation of the concentrations of each assay during the sample stability study. The concentrations of each biomarker under each condition across 15 days is shown. ” RT” indicates ambient storage, “Transport” indicates car transport storage, “- 80 °C” indicates storage at said temperature, ‘’F/T’’ indicates freeze-thawing one sample from 1 to 6 cycles from - 80 °C storage.

6.3.5 Fresh and frozen samples

Samples were tested fresh and freeze-thawed using both ELISA (figure 6.8) and multiplex LF (figure 6.9).

ELISA results were as follows:

A1AT, when measured fresh and freeze-thawed gave good correlation with an r^2 of 0.83 however, the slope of 0.5 indicated that the levels measured in the frozen samples when compared to the fresh samples was approximately 50% lower. This was confirmed with the Bland-Altman plots (Bias -77.39) where at higher concentrations, the points were outside the 95% confidence intervals (dotted lines located on the x axis).

TIMP-2 fresh vs. frozen samples, correlation was excellent (r^2 0.99) with a slope of 1 which indicated that both assays produced the same result, this was also confirmed by the Bland-Altman plot (Bias 202.5)

NGAL fresh vs. frozen samples, correlation was good (r^2 of 0.77) and a slope of 0.92 which indicated that both assays produced the same result, this was also confirmed by the Bland-Altman plot (Bias -0.39). There was one outlier that was not removed from the analysis.

Fibrinogen fresh vs. frozen samples, correlation was excellent (r^2 of 0.95) and slope of 1 which indicated that both assays produced the same result, this was also confirmed by the Bland-Altman plot (Bias -0.39) no trend was observed and the samples were aligned with y = '0' axis.

CRP fresh vs. frozen samples, correlation was excellent (r^2 of 0.92) and slope of 0.9 which indicated that both assays produced the same result, this was also confirmed by the bland-Altman plot (Bias -136.4), no trend was observed and samples were aligned with y = '0' axis.

RBP4 fresh vs. frozen samples, correlation was excellent (r^2 of 0.97) and a slope of 0.95 was obtained which indicated that both assays produced the same result, this was also confirmed by the bland-Altman plot (Bias 10859), no trend was observed and samples were aligned with y = '0' axis.

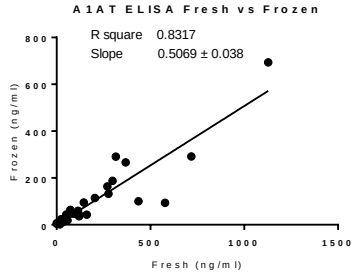
CC16 fresh vs. frozen samples, correlation was excellent (r^2 of 0.95) and slope was 1.1 which indicated that both assays produced the same result, this was also confirmed by the bland-Altman plot (Bias 6.15), no trend was observed and samples were aligned with y = '0' axis.

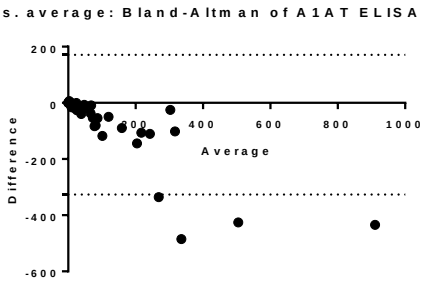
B2M fresh vs. frozen samples, correlation was less good (r^2 of 0.63) but a slope of 0.95 was obtained. However, the trend confirmed by the Bland-Altman plot (Bias 33.27), was within the ranges and samples were aligned with y = '0' axis.

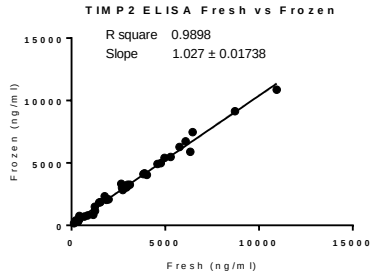
TIMP-1 fresh vs. frozen samples, correlation was excellent (r^2 of 0.99) and a slope of 1 was obtained which indicates that both assays produced the same results, this was also confirmed by the Bland-Altman plot (Bias 138.4), no trend was observed and samples were aligned with y = '0' axis.

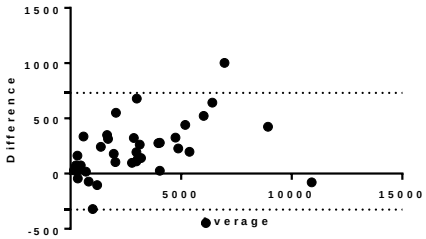
fMLP fresh vs. frozen samples, correlation was very poor (r^2 of 0.009) and slope was -0.1 which indicated that both assays were not the same. The Bland-Altman plot (Bias 0.26), however did not demonstrate this, no trend was observed, and samples were aligned with y = '0' axis.

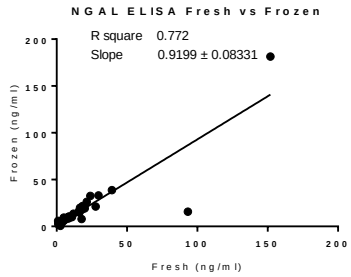
With the multiplex assay testing on fresh and freeze/thawed urine samples, the results were not as convincing as the ELISA results. Aside from A1AT, the slope results were comparable for the remaining nine assays indicating that the measurable levels within the samples for each biomarker were similar. The r^2 values were low for five of the assays: A1AT, RBP4, CC16, B2M, fMLP, but above 0.75 for the other 5 assays: TIMP-2, NGAL, fibrinogen, CRP, TIMP-1. The Bland-Altman plots did not add any value to the analysis so are not shown for the multiplex device data.

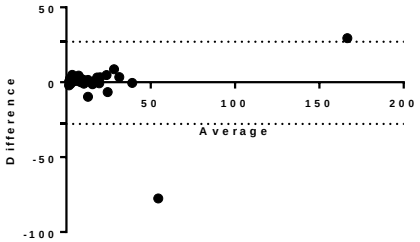

A1AT ELISA Fresh vs Frozen
R square 0.8317
Slope 0.5069 ± 0.038
Frozen (ng/ml)
Fresh (ng/ml)

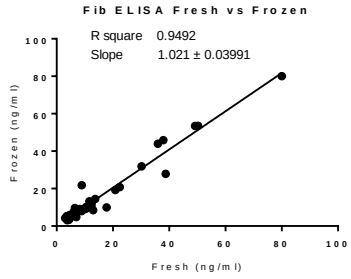

Difference vs. average: Bland-Altman of A1AT ELISA Fresh vs Frozen
Difference
Average

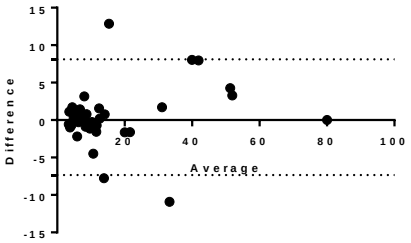

TIMP2 ELISA Fresh vs Frozen
R square 0.9898
Slope 1.027 ± 0.01738
Frozen (ng/ml)
Fresh (ng/ml)

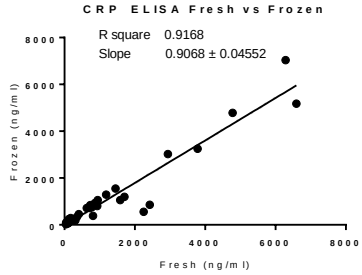

Difference vs. average: Bland-Altman of TIMP2 ELISA Fresh vs Frozen
Difference
Average

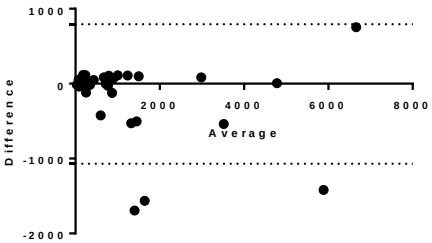

NGAL ELISA Fresh vs Frozen
R square 0.772
Slope 0.9199 ± 0.08331
Frozen (ng/ml)
Fresh (ng/ml)


Difference vs. average: Bland-Altman of NGAL ELISA Fresh vs Frozen
Difference
Average


Fib ELISA Fresh vs Frozen
R square 0.9492
Slope 1.021 ± 0.03991
Frozen (ng/ml)
Fresh (ng/ml)


Difference vs. average: Bland-Altman of Fib ELISA Fresh vs Frozen
Difference
Average


CRP ELISA Fresh vs Frozen
R square 0.9168
Slope 0.9068 ± 0.04552
Frozen (ng/ml)
Fresh (ng/ml)


Difference vs. average: Bland-Altman of CRP ELISA Fresh vs Frozen
Difference
Average

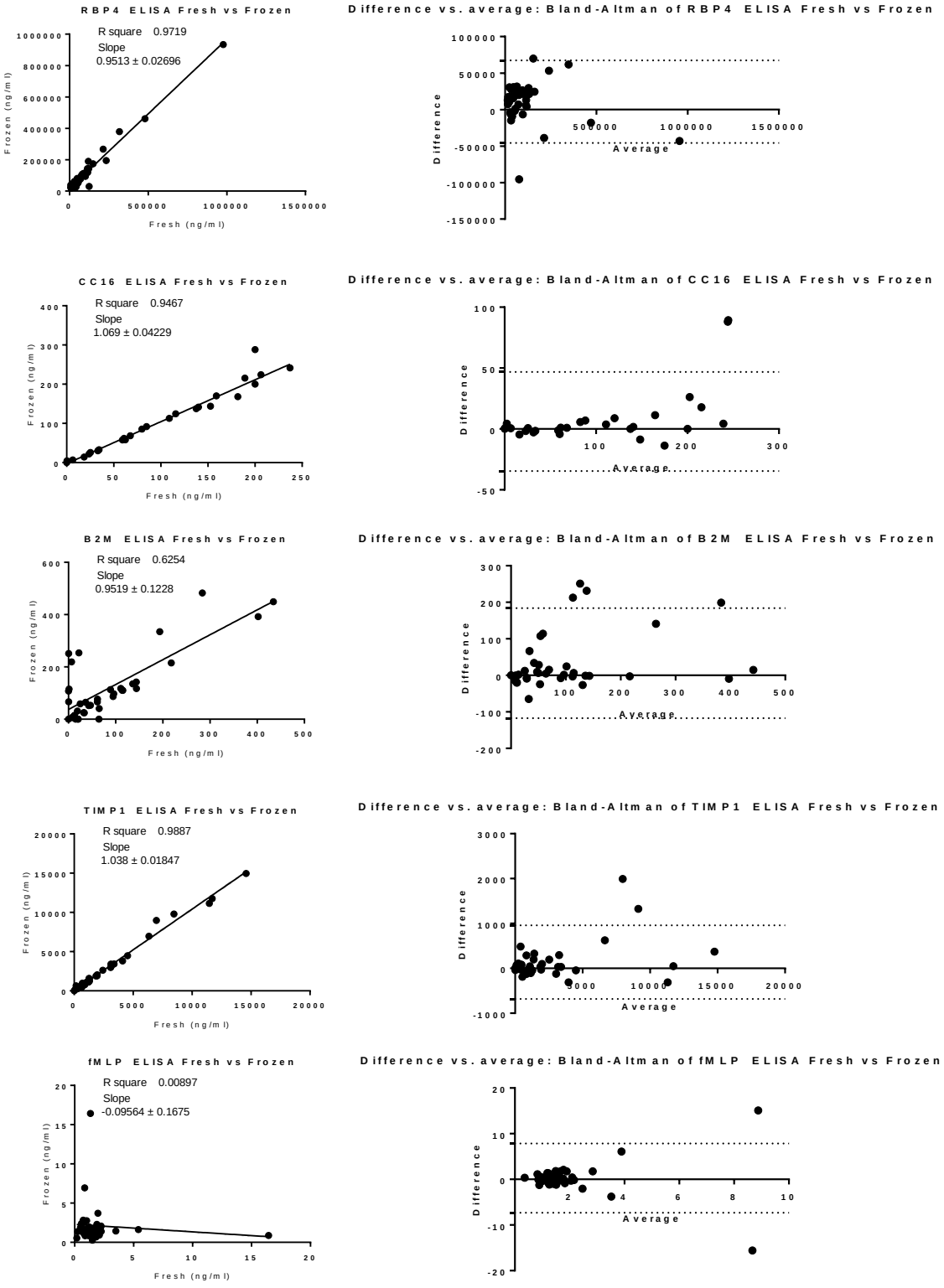


Figure 6.8. Graphical representation of the correlation of fresh and frozen samples for each asssay when tested by ELISA. For the correlation graph the r2 and slope values are shown. A value of 1 in each case reflects excellent correlation and agreement of estimated concentrations respectively. The Bland-Altman analysis demonstrates the equivalence of the assays when the bias is small and the results lie on the y = '0' line. The dotted lines show the +/- 95% confidence intervals.

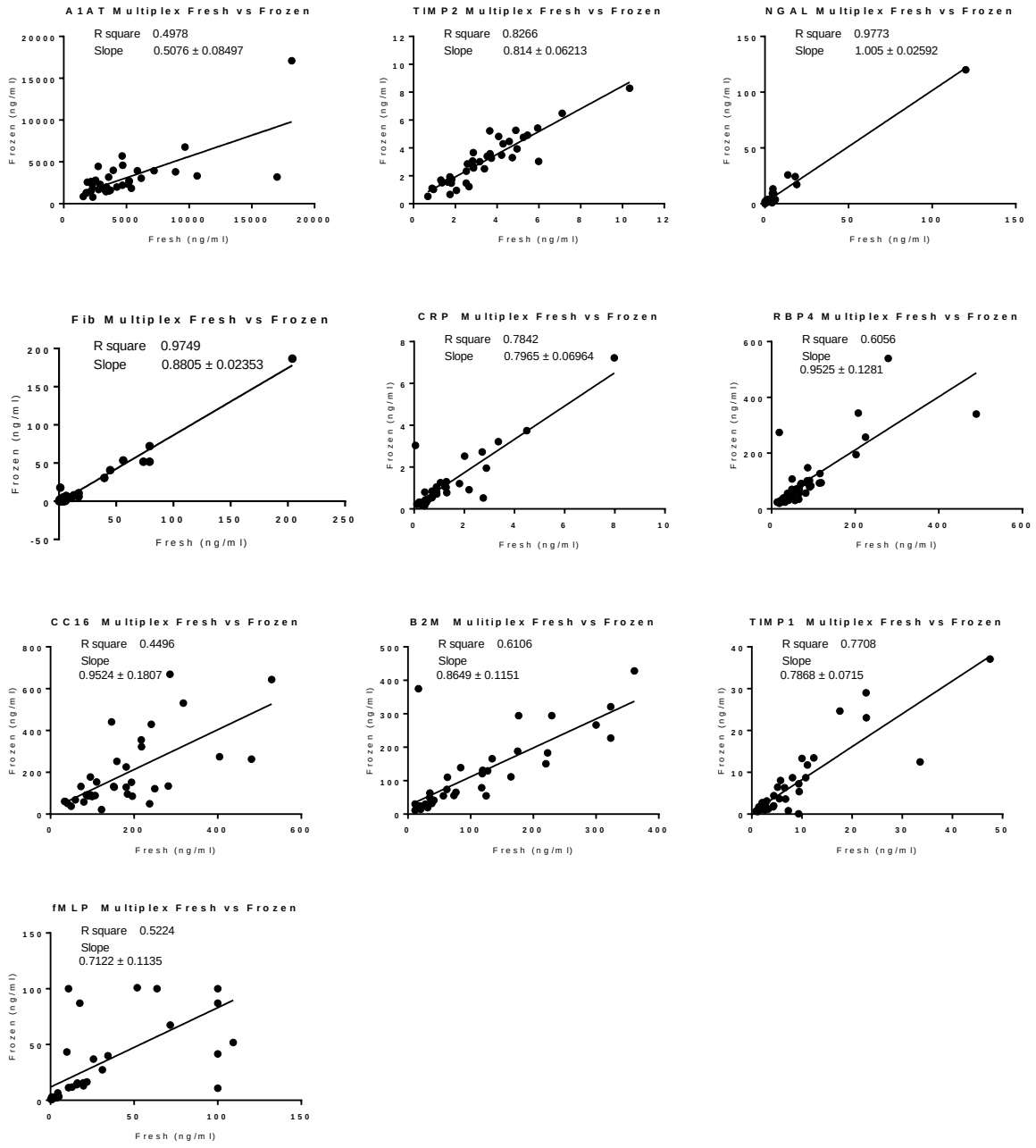


Figure 6.9. Graphical representation of the correlation of fresh and frozen samples for each asssay when tested by Multiplex Lateral Flow assays. For the correlation graph the r2, and slope values are shown. For both parameters a value of 1 in each case reflects excellent correlation and agreement of estimated concentrations respectively.

6.3.6 Stability of urine samples throughout the day

Six healthy urine samples were tested at intervals for 24 hours. The results are shown in the graphs illustrated in figure 6.10. A dotted line at 10 cube units represents the cut-off from what can be seen positively by the naked eye (>10 units). In the case of the A1AT assay, there was no obvious trend. A1AT in 2 of the 6 samples appeared to decrease throughout the day, one of the samples changed from a positive to negative result, this instability would give rise to an incorrect or false result for the patient. The other 4 samples appeared to remain stable. With regards to TIMP-2, in 3 of the 6 samples, the level of biomarker decreased within the 24-hour period. Fibrinogen did not appear to be stable, the samples all contained low levels of fibrinogen, but the difference for 4 of the 6 samples was a change from a positive to a negative result as shown by the error bars which would result in in a false positive. For CRP, all samples contained low/borderline levels of CRP, it cannot be determined by this testing whether the biomarker was stable. For RBP4 there was no obvious trend. As this was an inhibition assay, to show a deterioration, it would be expected for the cube units to increase, this was not the case for any of the samples. There was a trend with TIMP-1 that it decreased over 24 hours for the majority of the samples, only 1 sample changed from a clear positive to a borderline negative. NGAL, CC16, B2M and fMLP appeared stable throughout the time tested.

The conclusion was that the patient could test the urine at room temperature anytime up to 4 hours after producing the sample.

6.3.7 Line development

All the antigens showed the expected cube unit values for all 4 concentrations tested. In the case of sandwich assays such as A1AT, TIMP-2, NGAL, fibrinogen, CRP and TIMP-1, the values were initially low and increased with time, reaching a plateau at approximately 15 minutes. For the inhibition assays such as RBP4, CC16, B2M and fMLP, the cube units were initially low and increased with time reaching a plateau sooner at approximately 10 minutes (figure 6.11). As the sandwich assays continued to develop after 10 minutes it is important that the read time as specified is followed.

6.3.8 Batch to batch variation

All batches met the specifications set. Clearly some of the assays correlated perfectly as shown by the overlapping lines in figure 6.12. Those assays were A1AT, TIMP-2, NGAL, fibrinogen, RBP4, CC16 and TIMP-1. The other assays were still within the specifications set but were not exactly the same. In particular, in the case of fMLP, there was a larger variability when compared to the performance of the other assays.

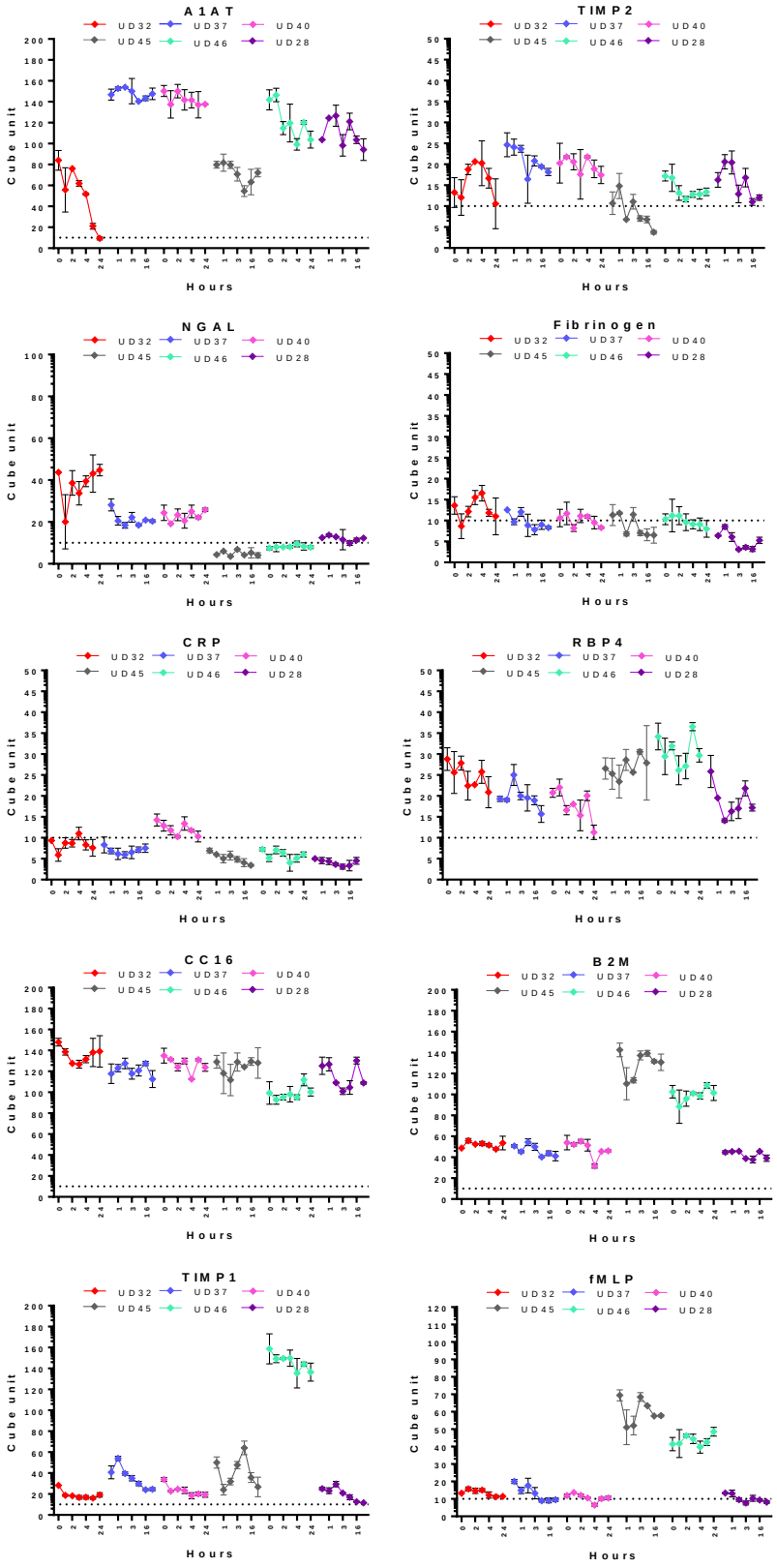


Figure 6.10. Graphical representation of the stability of fresh samples over a 24hr period for all 10 biomarkers. Six samples from healthy individuals were collected and stored at room temperature and tested periodically over 24 hours. Mean with range are shown. The dotted line represents the visual cut-off i.e. above 10 is a positive read.

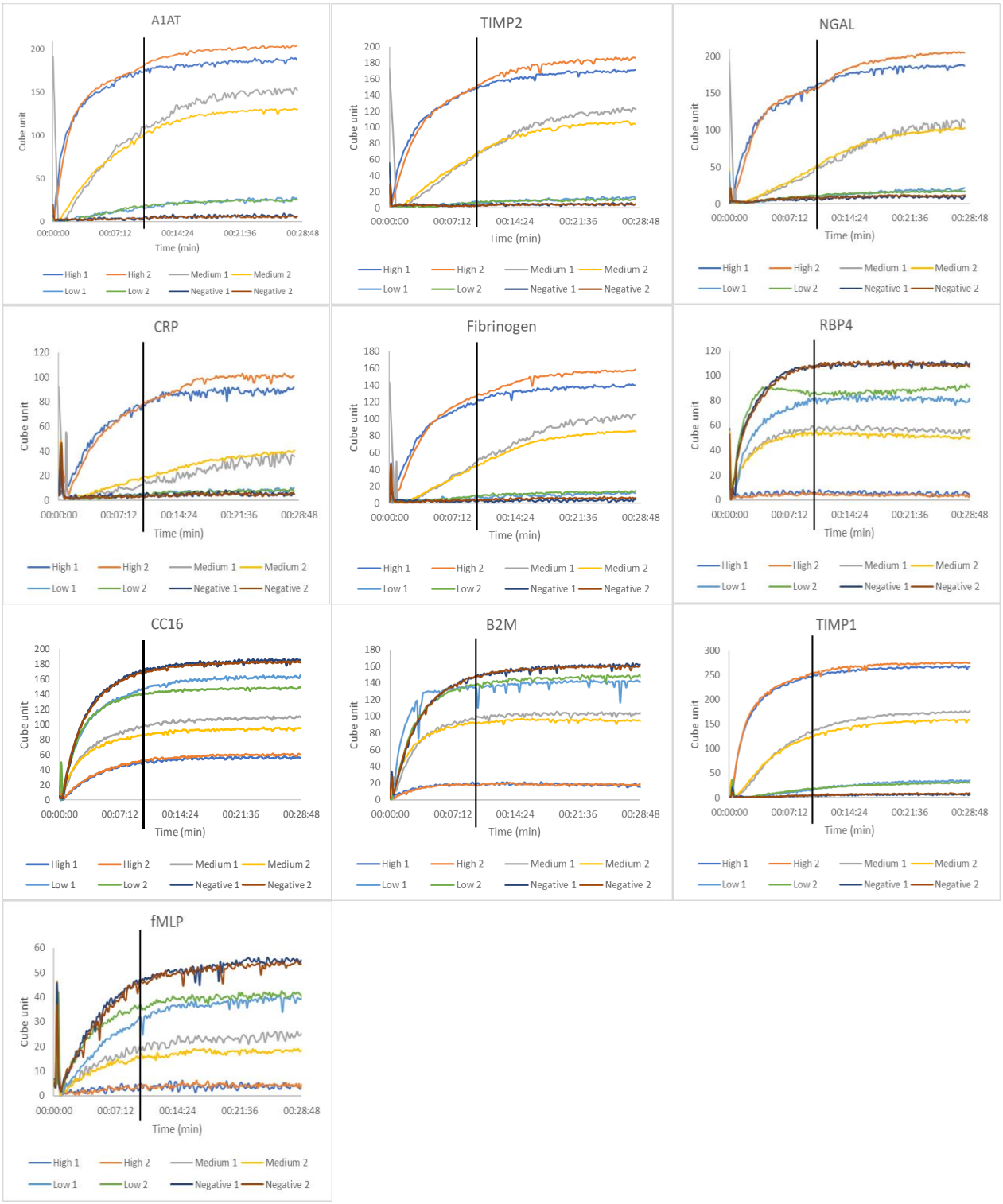


Figure 6.11. Graphical representation of the test line development for all assays. The lines show the time course of the cube reader output units over 28 minutes following addition of samples (high, medium low and negative). the black vertical line is the current read time of 10 minutes.

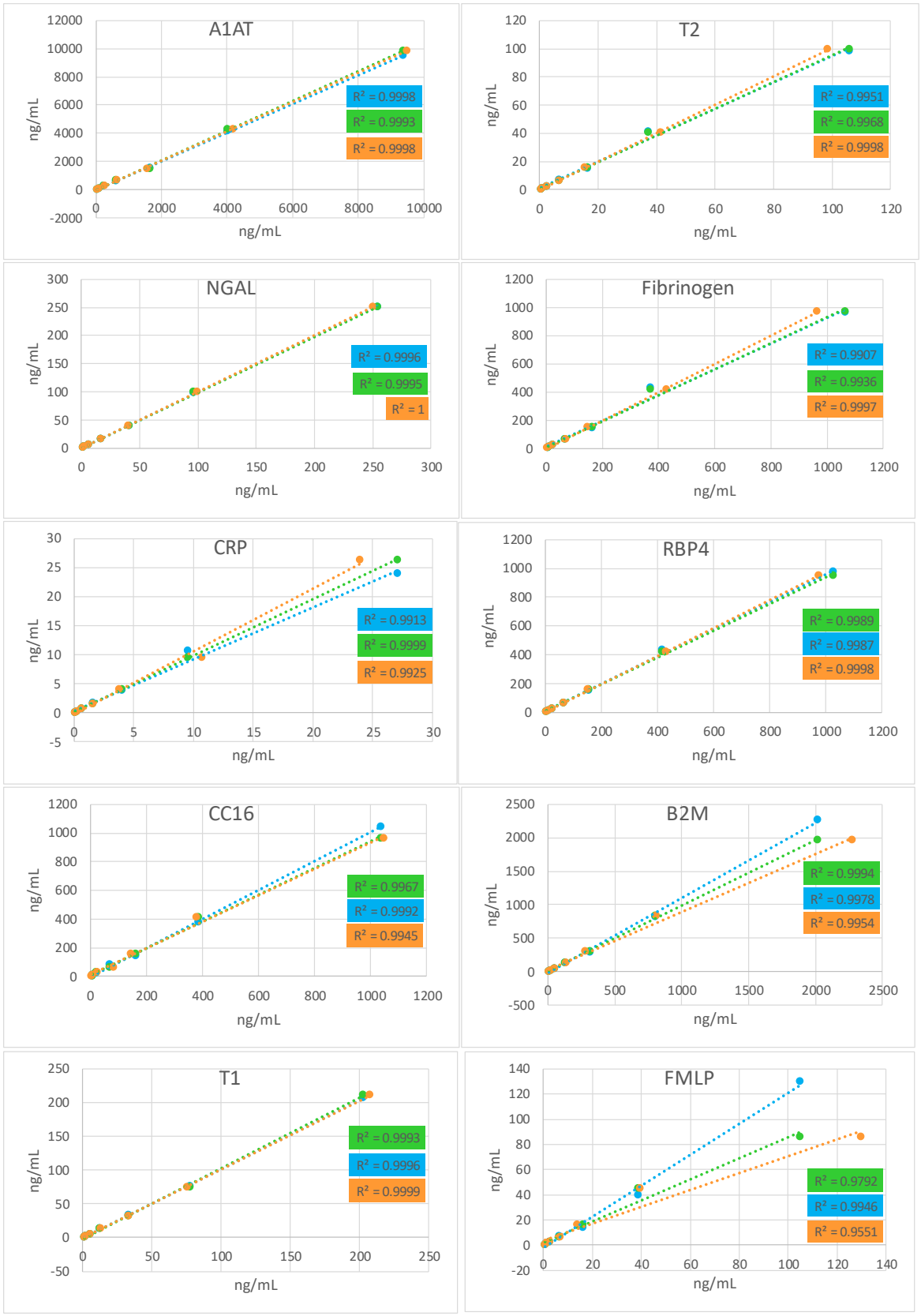


Figure 6.12. Graphical representation of Interbatch validation, comparison of three batches. Green represents the r^2 of batches 1 vs. 3, blue for batches 1 vs. 2 and orange for batches 2 vs. 3.

6.4 Discussion

Three methods of applying the sample to the test device were evaluated, the first 2 methods for dipping the device and the third method for the standard laboratory testing of applying a defined volume of sample to the device using a pipette. The hypothesis was that the tests were robust enough to allow use of any of these methods. It was predicted that that both dipping methods would be similar but either method would be more variable as the volumes of samples applied to the device would vary depending on the sample viscosity whereas applying a defined volume of sample to the device would be expected to result in lower CVs. In terms of signal, with all three methods similar results were obtained, though method 1 was had higher CVs out of the 2 dipping methods. Generally, if the CVs were high for certain samples, this was reflected for all methods.

Blood spiking into the sample at high levels, as indicated by the Multistix tests, demonstrated that apparent CC16, B2M and TIMP-1 results were affected. Accordingly, it is likely that the presence of significant haematuria would be an exclusion criterion for the use of the test in these cases.

There did not appear to be any cross reactivity with the analytes within each of the assays.

Samples were stored at room temperature, transported and evaluated at intervals over 15 days. The samples under both conditions were comparable indicating that time was the key variable. It was evident that certain biomarkers were not stable over time and that up to three days was the limit of acceptability, after 3 days, deterioration of certain biomarkers occurred that produced unacceptable change in some of the biomarkers tested with the multiplex assays. The biomarkers that appeared to be stable were NGAL, fibrinogen, RBP4, CC16, B2M, the biomarkers that appeared less stable were A1AT, TIMP-2, CRP, TIMP-1, fMLP. Of the unstable markers, A1AT, fMLP and TIMP-2 appear to be the most unstable and, for these markers in particular, the results from the returned samples from the planned observational study should be regarded with caution.

With regard to frozen samples stability, the samples that had unchanged biomarker levels (although variable) in fresh and frozen were NGAL, CRP, fibrinogen, RBP4, CC16, B2M, TIMP-1, fMLP. The samples that had decreased levels of biomarkers from fresh and frozen samples were A1AT, TIMP-2 but did remain stable after the initial drop. Based on the multiple freeze/thaw experiment, it is concluded that it is not recommended, A1AT, NGAL, fibrinogen, TIMP-1, fMLP all showed deterioration with multiple freeze/thaw cycles.

Further investigations into fresh and frozen urine samples with reference ELISAs in addition to the multiplex assays indicated that with the exception of A1AT and fMLP the slope values were close to ‘1’ for the remaining assays. This indicated that the actual concentration of biomarker in fresh and

freeze/thaw samples was unchanged. The biomarkers that correlated when run fresh and frozen with an r^2 >0.8 in ELISA were A1AT, TIMP-2, fibrinogen, CRP, RBP4, CC16 and TIMP-1. The biomarkers with reasonable correlation were NGAL and B2M and poor correlation was fMLP. In terms of stability of the biomarkers in the samples, the biomarkers with a slope close to 1 +/- 0.1 were TIMP-2, NGAL, fibrinogen, CRP, RBP4, CC16, B2M, TIMP-1. Biomarkers with changed levels were A1AT and fMLP. The results obtained from the Multiplex testing was not as convincing, only 5 of the 10 assays had an r^2 >0.75. The reduced lack of correlation for some of these assays in relation to the ELISA results suggests that there are potential matrix issues, influenced by the different storage conditions of the samples.

Stability of the urine samples over the day showed that that up to 4hrs gave acceptable results for all assays. From the line development study, it was clear that the sandwich assays continued to develop beyond 10 minutes whereas the inhibition assays were stabilised. However, using a 15 minute read time, the line development had completed for all assays. The requirement for a point of care assay is that the result should be obtained at 10 minutes, therefore it is concluded that it is important that the user follows the instructions rigidly for both testing immediately and reads the device immediately.

Batch to batch variation was minimal for all assays with the exception of fMLP. The QC specification for approving a batch is defined and the results indicate that the batches are reproducible with respect to these defined criteria.

The verification and validation experiments undertaken indicate that the multiplex assays perform appropriately, meet the requirements and are suitable for use in the designed clinical trial. Data not shown indicate that the assays are stable for up to 6 months at room temperature and at accelerated temperatures of 37°C and 44°C.

Chapter 7. Validation of biomarkers in longitudinal study

7.1 Introduction

The aim of this study was to assess the biomarkers in a large longitudinal observational study with the planned recruitment of 120 patients over 2 sites who would be testing daily for a period of 6 months. This would allow the examination of the biomarkers in fresh urine samples daily leading up an exacerbation through to recovery. This is the first study in the world where this has been possible as up to this point a) the frequency of urine samples collected has been at most monthly b) samples have been collected on the day when the patient has attended the clinic/centre and c) only frozen samples have been tested. It is hypothesised that certain biomarkers from the 10 selected biomarkers would prove to more useful in diagnosing an exacerbation early i.e. before symptoms present. Usually, samples were collected on the day of diagnosis and the actual time at which each biomarker appeared in relation to the exacerbation was unknown, the same applied to the recovery state and the return of the biomarkers back to 'baseline'. The outcome from this study would be to reduce the number of biomarkers from 10 to 5 so that all the assays could be incorporated into just one test device.

The point of care tests that have been developed as described in chapter 5 enabled the patient to test their own urine in the home in real time in just 10 minutes. Had the samples been sent daily to a laboratory for analysis using conventional laboratory assays, not only would this have involved significant time, cost and resource but some of the biomarkers that have proven to be unstable over time and during transport as demonstrated in chapter 6 would potentially have introduced variability and false results. Weekly urine samples from the patients during this study were additionally sent to the laboratory for further analysis and validation.

The patients were also asked to complete a 14 question "symptoms e-diary" and to send a sample back to the lab for verification testing once a week.

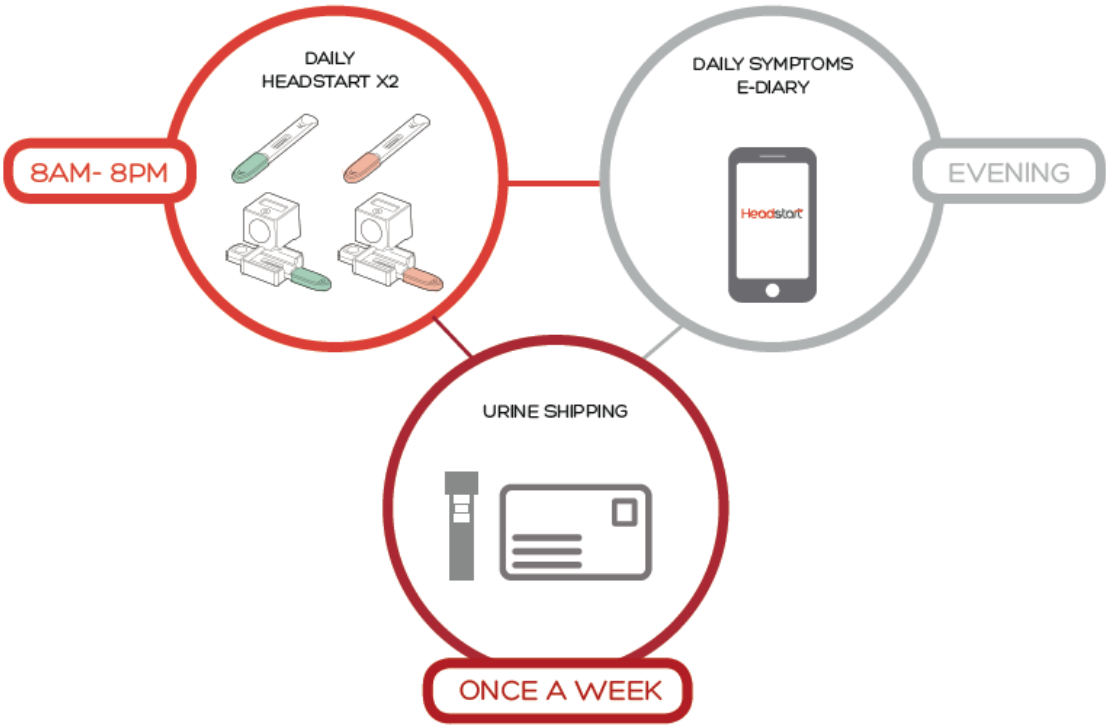


Figure 7.1. Schematic overview of the observational study. Each volunteer ran 2 tests per day for approximately 6 months, completed a symptom e-diary per day and sent 1 urine sample to the lab by post once a week.

7.2 Methods

7.2.1 Study design

Patients with COPD admitted to two hospitals (Glenfield Hospital, Leicester, and Prince Phillip Hospital, Llanelli) were approached from July 2017 to February 2018. Eligible patients were patients with a documented clinical diagnosis of COPD and two previous exacerbations in the previous year. Exclusion criteria included any clinically relevant lung disease other than COPD, diagnosed as being α1-anti-trypsin deficient (PiZ), a history of or current active pulmonary tuberculosis. The study was ethically approved (REC Ref: 08/H0406/189) – An open study to measure imaging biomarkers and inflammatory cells, mediators and biomarkers from blood, urine and airway samples from healthy volunteers, asthma patients and COPD patients in stable disease and during acute exacerbation. The Chief investigator was Professor Chris Brightling, Department of Respiratory Medicine, Glenfield Hospital, Leicester, UK and sponsored by the University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary, Leicester LE1 5WW. Subjects underwent symptom and clinical assessment and had blood, sputum (when possible) and a urine sample collected at recruitment, 3 months and at 6

months. If the subject had a suspected exacerbation they returned to the clinic where the same assessments were completed and samples were collected. In the case of a confirmed exacerbation, the subject was asked to return for a follow up visit at 6 weeks. As the cube reader had the limitation of storing only 1.5 months of data, the individual was asked to attend an additional visit at 1.5 and 4.5 months so that the data from the reader could be downloaded. The following were undertaken:

Scheduled visits (recruitment/month 3/month 6): Lung function tests were performed, and other parameters were recorded: Demographics (height, weight, BMI, gender, race), smoking history, COPD history, exacerbation history, medical history, drug history, physical examination, questionnaires (SGRQ, MRC, CAT), spirometry.

Exacerbation visit: An exacerbation was diagnosed by the clinician, this was based on 2 or more of the following symptoms: increased shortness of breath (SOB), increased chest tightness, increased cough, increased sputum volume and/or prevalence and/or change in colour of sputum.

Other parameters that were recorded: treatment, any previous exacerbations since last visit, medical history, drug history, vital signs, physical examination, spirometry, admission information if required.

Post exacerbation visit: parameters that were recorded: symptomatic information (SOB, chest tightness, cough, volume of sputum, sputum purulence), treatment details, any previous visit to GP, ED, secondary care visits, physical examination, vital signs, spirometry.

The patient received training at recruitment and received the starter pack and 3 month's supply of test devices, lot numbers were recorded for each component in the case report form (CRF).

Starter pack included:

- Cube reader (OpTricon, Berlin, Germany)
- Mobile phone (with App- e-diary designed and manufactured by Bond Digital Health solutions, Cardiff)
- Plastic jug for urine collection
- Shipping accessories for weekly urine sample (sample collection container, sealing tape, grip-seal bag, stamped addressed envelope, absorbent pad)
- 3 months supply of test devices (2 devices per pouch)

Instructions for volunteer provided as shown in figure 7.2.

Power calculation for study: the power calculation was calculated by an external contractor (JB Medical Ltd, Sudbury, UK). The primary study objective was to quantify the benefit of a range of biomarkers, either alone or in combination, as a predictor of acute exacerbation in COPD. This outcome mapped conveniently into a straightforward 2x2 contingency table: exacerbation/no exacerbation vs predictive biomarker changes/no predictive biomarker changes. This was then amenable to conventional chi-squared estimation to identify the presence or absence of an association, followed by calculation of the φ statistic, to assess the degree of the association.

In this circumstance, the fixed variable was the number of exacerbations. The predictor variable (biomarker result) could be altered to identify the characteristic or combination of characteristics that best predicted the occurrence of an exacerbation.

The power of the goodness of fit or chi-square independence test was given by:

$$1 - \beta = F_{df,\lambda}(x_{crit})$$

where F was the cumulative distribution function (cdf) for the noncentral chi-square distribution $\chi^2(df)$, x_{crit} was the $\chi^2(df)$ critical value for the given value of α, and $\lambda = w^2n$: the noncentrality parameter where w was the φ effect size. It was assumed w to be either 0.3, representing a moderate degree of association between the biomarker changes and exacerbation occurrence, or 0.5, representing a high degree of association. It was assumed an α of 0.05 and the power of the study was explored to detect a difference at either level, based on 120 recruited patients.

The number of expected exacerbations would depend on the clinical characteristics of the recruited population. A recently published UK retrospective study (160) used a GP database to identify the annual exacerbation rates in each of these groups, the results of which are shown in table 7.1. In order to maximise the number of evaluable exacerbations, it was recommended to focus recruitment on patients in GOLD categories. As shown, the expected number of exacerbations in these patients over 6 months would be 129. After making an assumption that 15% of patients would withdraw, drop out or fail to comply with the testing regimen, it was estimated that we should have 110 evaluable exacerbations at the end of the 6 month follow-up period.

Table 7.1. Expected number of exacerbations, based on GOLD categories

Category	Description	Expected exacerbations per year	Number of patients to be recruited	Number of exacerbations over 6 months
GOLD A	*FEV1>80%; 0-1 exacerbations; mMRC 0-1; CAT<10*	0.83	0	0
GOLD B	FEV1 50-79%; 0-1 exacerbations; mMRC≥2; CAT≥10	1.17	0	0
GOLD C	FEV1 30-49%; ≥2 exacerbations; mMRC 0-1; CAT<10	1.79	60	53.7
GOLD D	FEV1≤30%; 0-1 exacerbations; mMRC≥2; CAT≥10	2.51	60	75.3
TOTAL			**120**	**129**

Solving the above equation for x_{crit}, the power of the study was calculated to detect an association between the test and the outcome, based on a total number of exacerbations of 110. Based on this approach, the study would have an 81% power to capture a moderate association (w=0.3) between biomarker testing and exacerbation risk, at a critical p-value of 0.05. On the same basis, it had >99% power to detect a strong association (w=0.5).

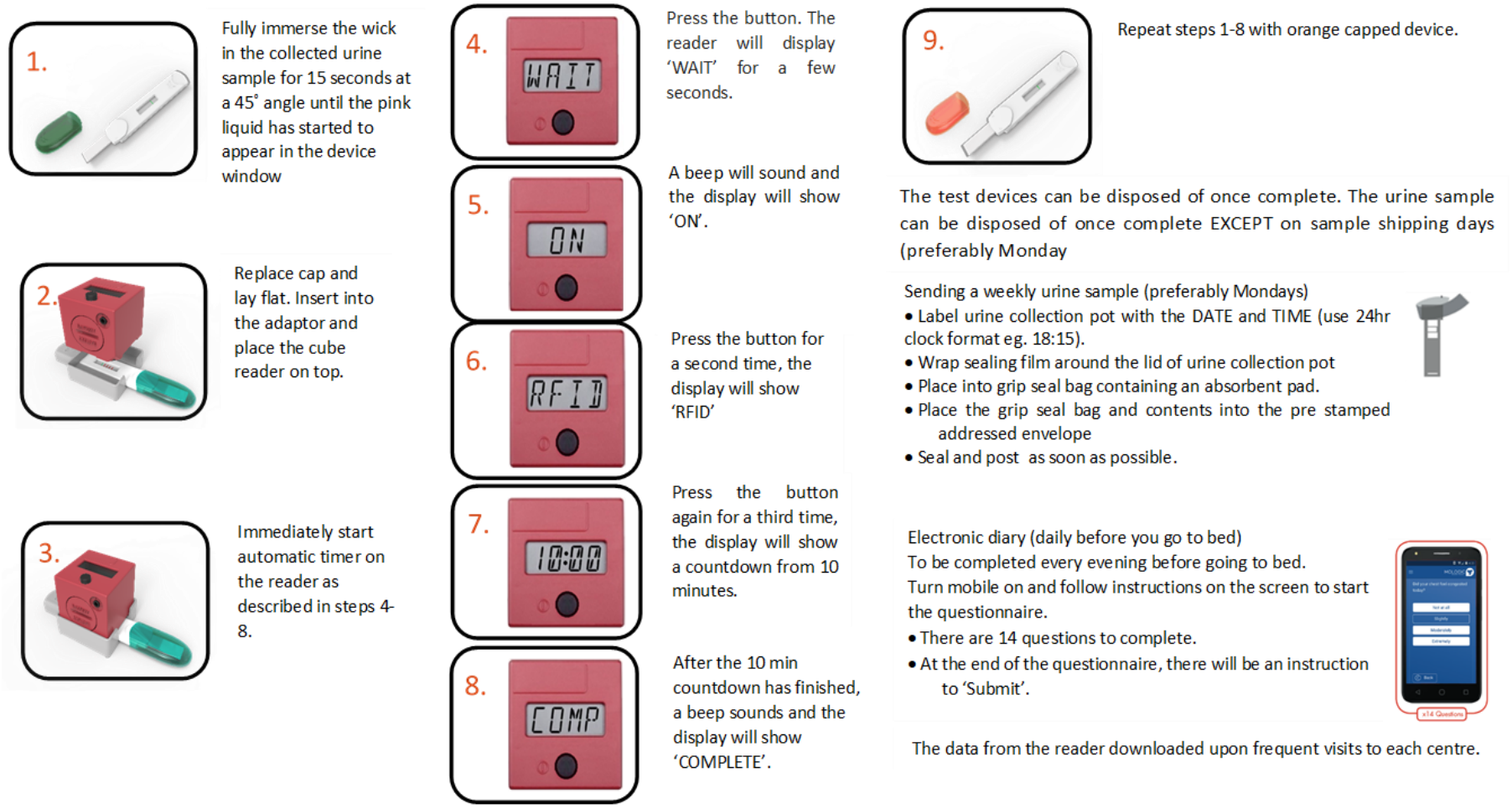


Figure 7.2. Schematic instructions for use provided to each patient. Urine sample collection- Collect a mid-stream sample of urine in the jug provided. Collect daily, same time of day between 8am—8pm. Two devices- green capped device first followed by the orange capped device, these represented the two tests that were developed, test a (A1AT, TIMP-2, NGAL, fibrinogen, CRP) and test b (RBP4, CC16, B2M, TIMP-1 and fMLP).

7.2.2 Individual patient biomarker profiles

An interim analysis consisting of 24 exacerbations was performed. For each individual biomarker, stable and exacerbation levels were compared with regards to concentration levels found in each state and if statistically different in each group.

All data were analysed using Graphpad PRISM Version 7. Data normality was explored and appropriate parametric or non-parametric tests chosen accordingly. Receiver-operator characteristic (ROC) analysis and paired Students t-test with significance levels $p<0.05$ were used to compare biomarker levels in different disease states with both transformed and non-transformed data.

7.2.3 Multiple biomarker analysis

The analysis performed was identical to what was done previously on the Leicester COPD data (Chapter 4, section 4.3.6.3). A baseline value was calculated for each individual patient which differed from the previous analysis whereby the baseline was calculated from 5 consecutive days which was not previously possible due to the infrequent collection of samples. A separate stable and exacerbation sample was selected, and the percentage difference was calculated. Using logistic regression analysis, the % difference values for both the stable and exacerbation samples were analysed for all 10 biomarkers and in addition, separate models were developed to determine the minimal number of biomarkers required to produce promising and acceptable results in terms of sensitivity, specificity, positive predictive values and negative predictive values.

All data were analysed using SPSS (version 21), Graphpad PRISM Version 7. Data normality was assessed, and appropriate parametric or non-parametric tests chosen accordingly. Receiver-operator characteristic (ROC) analysis and Wilcoxon's signed rank test with significance levels $p<0.05$ were used to compare biomarker levels in different disease states. Logistic regression was used to develop predictive models, combining biomarkers that determined the outcome of exacerbation. Internal validation was addressed by dividing the cases into 80% training set and 20% test set. This process was repeated 5 times using assignment to training and validation sets by random number generation in SPSS.

7.2.4 Patient profiles with algorithm

The logistic regression equation (s) generated from 7.2.3 was applied to all the data in the interim analysis. The probability risk scores were plotted for each patient using Graphpad prism V7. A rolling average of 3 days was calculated for the risk scores.

Added parameters were the EXACT-PRO total value which was collected during the study by the patient daily using the App. The EXACT® is a 14-item daily diary designed to provide a direct measure of patient-reported symptoms of COPD exacerbation by capturing unreported, symptom-defined events, and standardizing the evaluation of symptoms around medically treated events, including magnitude of change around events seen in the emergency room or clinic and before and after hospitalization. Advantages of a standardized, validated daily diary-based symptom assessment in exacerbation studies include uniform metrics, reduced recall bias, and the ability to fully characterize exacerbations of COPD, including the estimated 50% to 70% of events that are unreported.

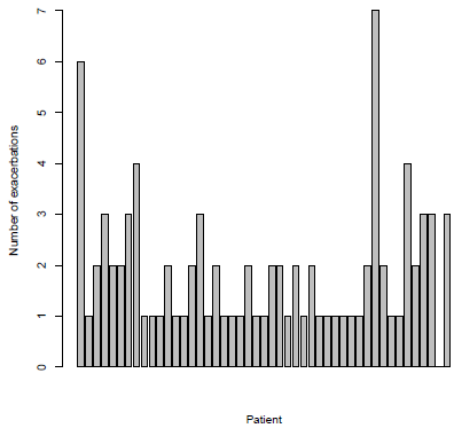
The EXACT® Total score is an interval-level scale ranging from 0 to 100, where higher scores indicate a more severe condition. The EXACT® Total score is used to assess exacerbations of COPD. The associated algorithm incorporates recalculating the threshold values periodically and post exacerbation.

7.3 Results

7.3.1 Outcomes from entire study

105 patients were recruited in the entire study, however, not all data were included due to withdrawals and missing data. Specifically, 9 patients (9%) withdraw from the study, 2 patients (2%) ran the test incorrectly and data from 7 patients (7%) was missing due to a Bluetooth issue with the readers. In total, there were 47 patients (45%) in the exacerbation set who took part in the study for a total of 8711 days and 40 patients (38%) in the non- exacerbation set who took part in the study for a total of 6514 days. For the exacerbation set, of those days, no exacerbation was recorded on 8622 days, a doctor confirmed exacerbation was recorded on 59 days and a patient confirmed exacerbation, with no doctor confirmation, was recorded on 30 days. This represents a total of 89 confirmed exacerbations. The exacerbations were spread across the patients who experienced them as in Figure 7.3a. As can be seen from the plot, most of the patients (n = 22) who experienced an exacerbation in the study experienced just a single exacerbation. However, some patients also experienced two exacerbations (n = 14), three exacerbations (n = 6), four exacerbations (n = 2), six exacerbations (n = 1) and seven exacerbations (n = 1). The doctor confirmed exacerbations (most reliable data as verified by a clinician) were spread across the patients who experienced them as in Figure 7.3b. These results were similar to the previous plot. The numbers of patients who experienced the different numbers of exacerbations in the study were: one exacerbation (n = 21), two exacerbations (n = 10), three exacerbations (n = 3), four exacerbations (n = 1) and five exacerbations (n = 1).

A



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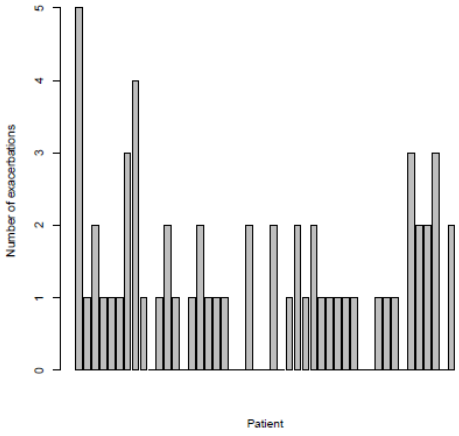


Figure 7.3. Distribution of exacerbations across all patients. a) The distribution of the doctor and patient confirmed exacerbations across all patients who experienced them. b) The distribution of the doctor confirmed exacerbations across all patient who experienced them.

7.3.2 Patient characteristics for all patients in the study

105 patients consented to the study. The majority of the patients were male (69%) and the biggest comorbidity was hypertension (13%). A summary of the demographics and characteristics is shown in table 7.2. According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017 strategy, the patient group fitted into the GOLD B criteria (FEV1 50-79%; 0-1 exacerbations; mMRC>2; CAT>10) with average FEV1 of 51.13, average mMRC and CAT score of 2.83 and 19.86 respectively. The majority of the patients did experience 0-1 exacerbation however, they were not followed over a 1 year period, some of the patients may have had further exacerbations in the following 6 month period. Although there was no obesity recorded, there was some indication by the BMI results (mean, SD 27 ± 5.5) that there would have been some patients with a BMI over 30 who would have been considered to be obese.

Table 7.2. Characteristics of the patients included in the entire observational study (n=105). Data are shown as mean (SD), mean (range) or number (%)

Male	**No (%)**	**72 (68.6%)**
Smoking, pack-years	Mean (SD)	46.33 (28.82)
Current smokers	Mean (%)	13 (12.38%)
Ex-smokers	No (%)	91 (86.67%)
BMI, kg/m2	Mean (SD)	27.11 (5.46)
CAT Score	Mean (SD)	19.86 (8.37)
mMRC Score	Mean (SD)	2.83 (1.19)
SGRQ-C Total Score	Mean (SD)	46.38 (21.63)
Comorbidities		
Heart failure	No (%)	4 (3.81%)
MI	No (%)	2 (1.90%)
Angina	No (%)	0 (0%)
HTN	No (%)	14 (13.33%)
Stroke	No (%)	1 (0.95%)
Lung cancer	No (%)	0
IBD	No (%)	0
Cirrhosis	No (%)	0
Bowel cancer	No (%)	0
Chronic Kidney disease	No (%)	0
Diabetes	No (%)	5 (4.76%)
Obesity	No (%)	0
Prostate cancer	No (%)	1 (0.95%)
Bladder cancer	No (%)	0
Anxiety and depression	No (%)	2 (1.90%)
no changes since last visit	No (%)	0
None	No (%)	2 (1.90%)
Other	No (%)	20 (19.05%)
Physiology and Imaging		
heart_rate_bpm	Mean (SD)	77.34 (13.67)
saturation	Mean (SD)	95.81 (1.87)
respiratory rate	Mean (SD)	17.07 (2.51)
fev1_predicted1	Mean (SD)	2.62 (0.53)
fvc_predicted	Mean (SD)	3.36 (0.74)
fev1_measured	Mean (SD)	1.36 (0.59)
pre_bd_fev1_predicted	Mean (SD)	51.96 (19.81)
pre_bd_fvc	Mean (SD)	2.72 (0.71)
pre_bd_fvc_predicted	Mean (SD)	80.37 (21.24)
pre_bd_fev1_fvc	Mean (SD)	50.63 (14.93)
post_bd_fev1	Mean (SD)	1.45 (0.65)
post_bd_fev1_measured	Mean (SD)	55.43 (23.13)
post_bd_fvc_measured	Mean (SD)	2.83 (0.83)
post_bd_fvc	Mean (SD)	82.69 (25.03)
post_bd_fev1_fvc	Mean (SD)	51.13 (16.62)
GP visits in 12 months prior to recruitment (n = 80)	Mean (range)	0.9875 (0-8)
Hospital visits in 12 months prior to recruitment (n = 80)	Mean (range)	0.375 (0-8)

7.3.3 Verification of results from patients

The 4-parameter logistic regression (4PL) was used to convert the cube raw values to concentration values. As the name implies, it has 4 parameters that need to be estimated in order to "fit the curve". The equation for the model was:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

Where x = the independent variable and y = the dependent variable.

The four estimated parameters consist of the following:

a = the minimum value that can be obtained (i.e. what happens at 0 dose)

d = the maximum value that can be obtained (i.e. what happens at infinite dose)

c = the point of inflection (i.e. the point on the S shaped curve halfway between a and d)

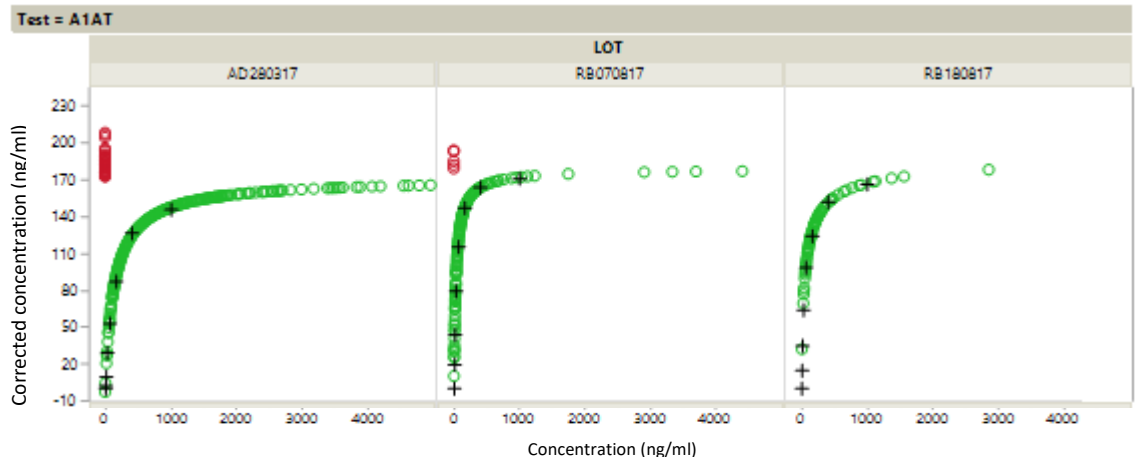
b = Hill's slope of the curve (i.e. this is related to the steepness of the curve at point c).

The 4PL curve fit included a background correction step, the mean of the blank sample (buffer only) was subtracted from the raw data measurements. The blank-corrected values were then used in the fitting. The standard data points (concentration vs. measurement) were plotted on semi-log axes and a 4PL fit was applied to the data points. The concentrations of the samples were determined from the fit. The conversion of cube values to concentration values was performed using Excel.

To determine if the patient data were within the standard curve the concentrations and raw values were plotted on the X and Y axes on the same graph as the standard curve (figure 7.4). As each batch of devices represented different production lots, each lot had a separate standard curve. For this analysis three lots were taken AD280317, RB070817 and RB180817 and all data available per lot were plotted i.e. multiple patient data. As can be seen in figure 7.4 there is significant variability between different lots with batch AD280317 assays with a smaller dynamic range and lower top standard compared to the other 2 batches. This was the first lot assessed and based on the data coming from the patients, improvements were made to the standard curves for subsequent batches.

The large dynamic range required for each assay was challenged in this study with fresh sample testing from a variety of patients. A1AT was the one assay which did not encompass the majority of patients. The levels of A1AT in fresh samples were higher than those levels found in frozen samples therefore the assay was "too sensitive" so some samples had raw signals that were higher than the dynamic range of the assay with the risk of hooking (decreasing in signal with extremely high A1AT

levels). In the case of the TIMP-2, fibrinogen, CRP and TIMP-1 assays, the higher levels were all within the standard curve but there were a few samples below the standard curve. This was not deemed to be of concern as it is expected from previous studies to date that for some patients where their baseline levels are on the lower part of the curve, the increase in signal in the event of an exacerbation would be measurable and be within the standard curve range. The assay range for NGAL was improved after the first batch which led to a better range and samples tested with the remaining batches were within range. All the competition assays experienced a lack of sensitivity, with a high proportion of samples with raw signals higher than the standard curve (classified as negative). The only competition assay that caused a level of concern was fMLP where by the assay range became narrower after batch AD280417 resulting in fewer samples with raw signals that fell within the dynamic range.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RB070817
RB180817
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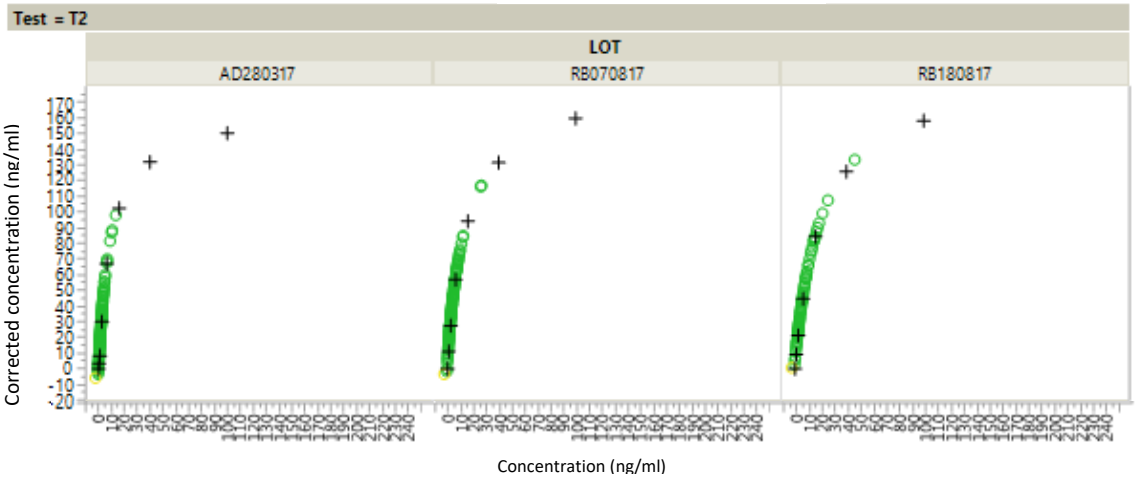
Concentration (ng/ml)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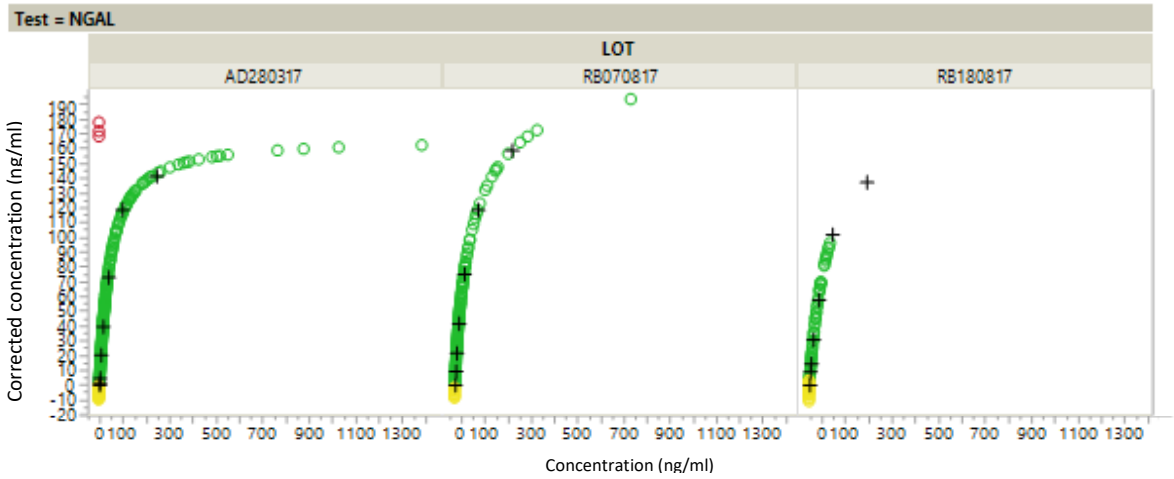
LOT
AD280317
RB070817
RB180817
Corrected concentration (ng/ml)
Concentration (ng/ml)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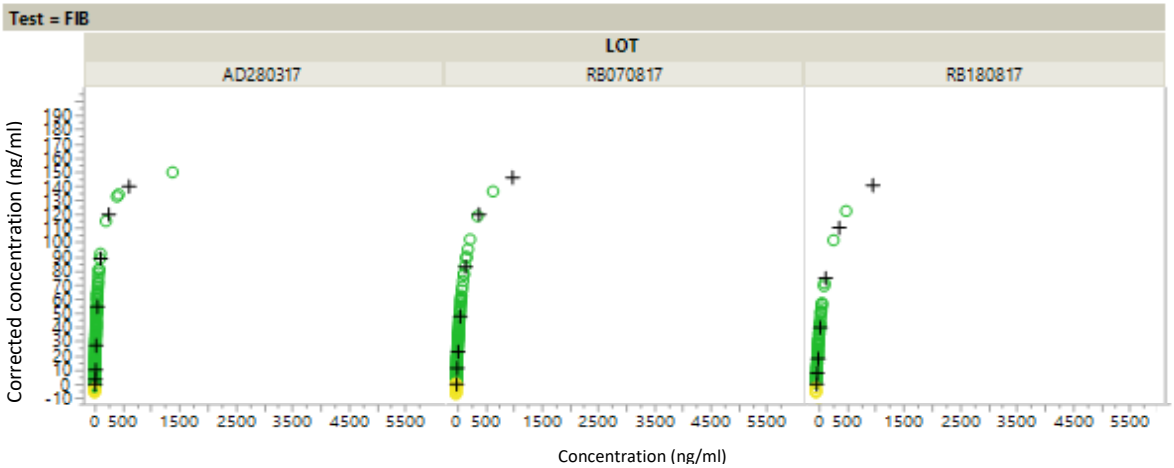
Concentration (ng/ml)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LOT
AD280317
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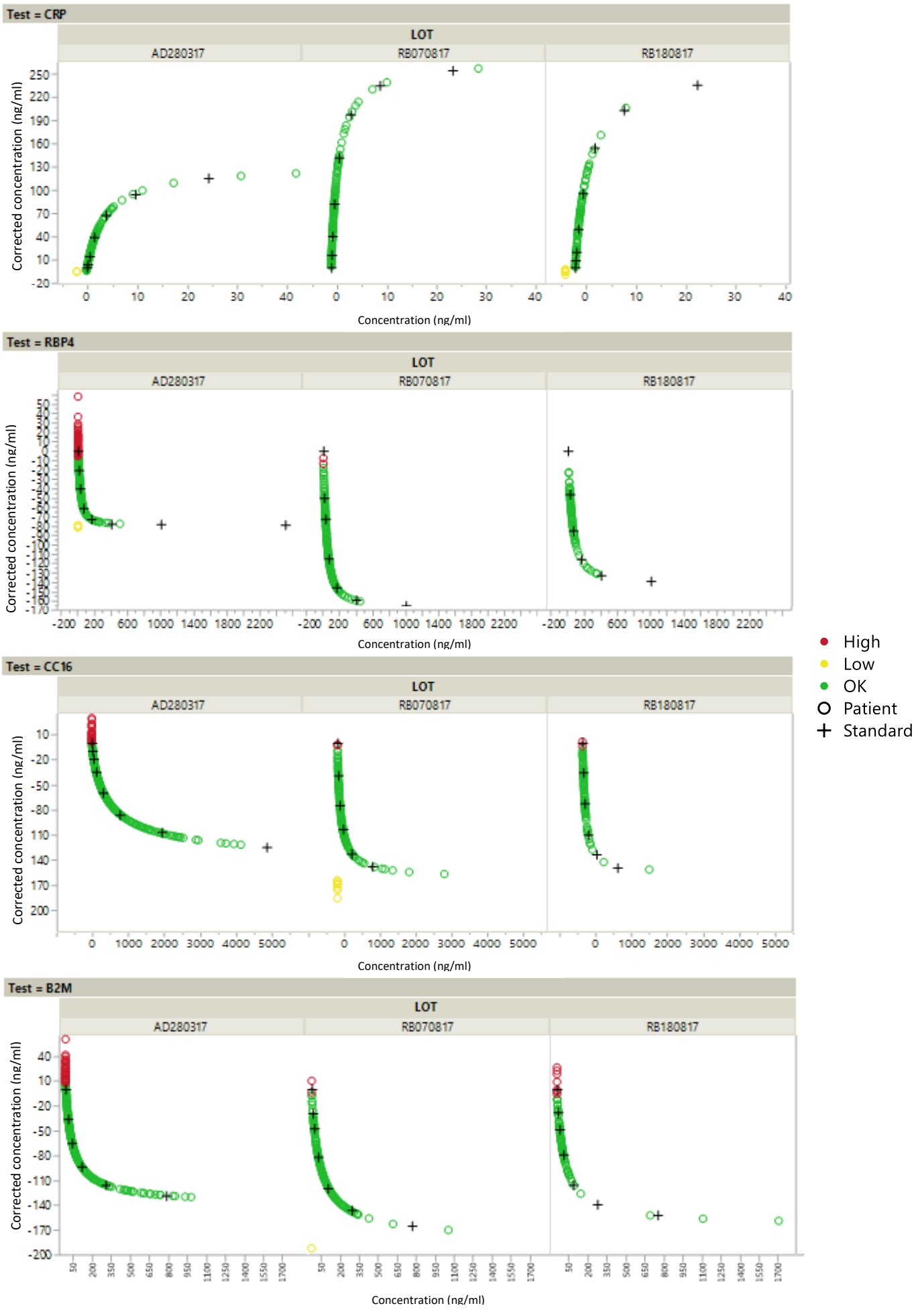
RB180817
Corrected concentration (ng/ml)
Concentration (ng/ml)


High
Low
OK
Patient
Standard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LOT
AD280317
RB070817
RB180817
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Concentration (ng/ml)
Test = RBP4
LOT
AD280317
RB070817
RB180817
Corrected concentration (ng/ml)
Concentration (ng/ml)
Test = CC16
LOT
AD280317
RB070817
RB180817
Corrected concentration (ng/ml)
Concentration (ng/ml)
Test = B2M
LOT
AD280317
RB070817
RB180817
Corrected concentration (ng/ml)
Concentration (ng/ml)
High
Low
OK
Patient
Standard

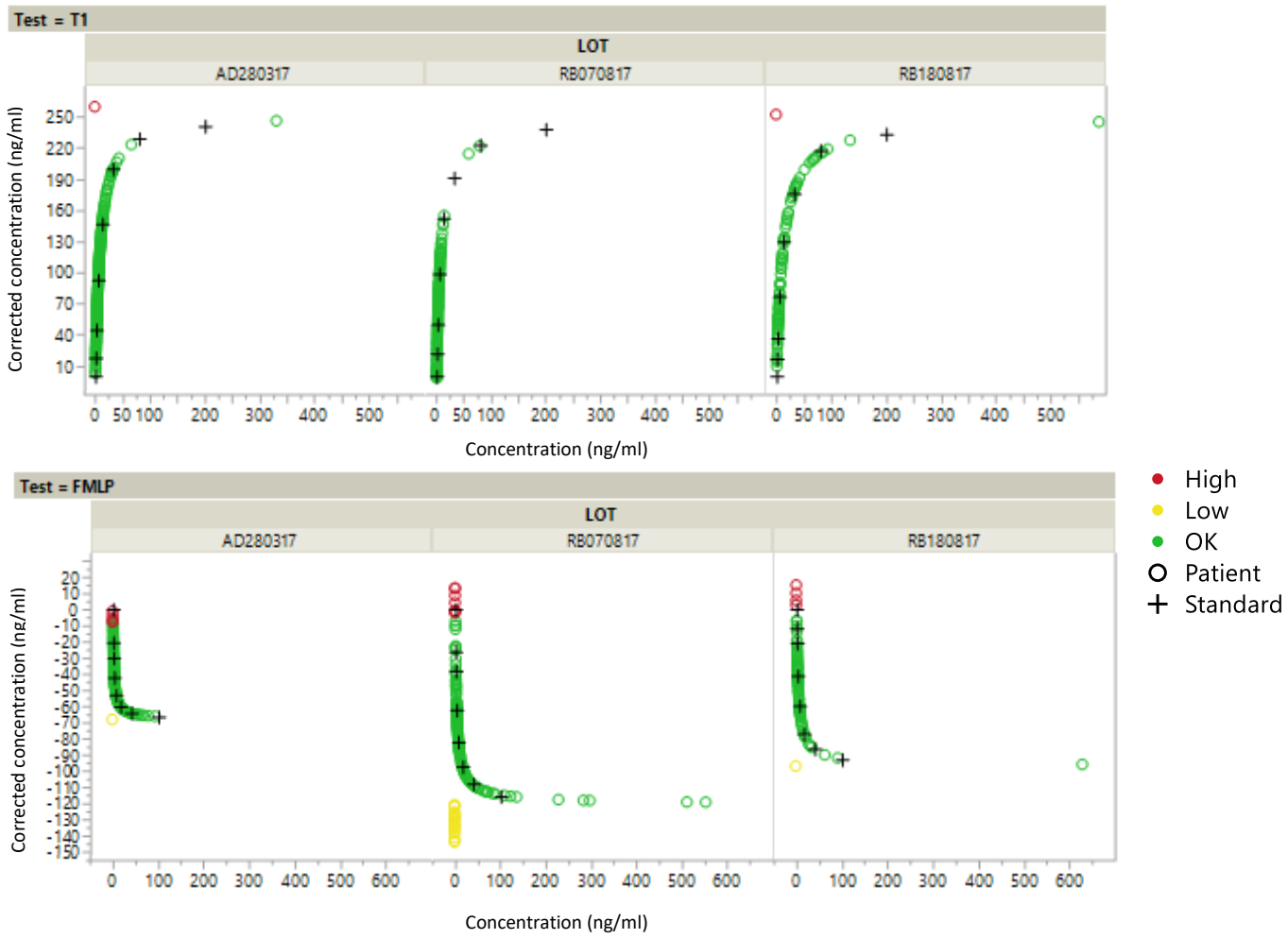


Figure 7.4. Concentration plotted against raw cube value and representation on the standard curve for three batches of devices. Each row represents each individual assay and three different batches of the multiplex devices. The measured biomarker concentrations received from the patients home testing was plotted against the calculated concentrations from each standard curve. If the measured samples were within the dynamic range of the assay, then all would be green. Red = High (over the standard curve, yellow = low (below the standard curve, Green= within the standard curved. O = patient data, + = Standard curve values. Graphs were created by Dr Clare Lendrem (Newcastle university)

7.3.4 Outcomes from the interim analysis

In the interim analysis, the data for all the patients who experienced an exacerbation were considered during the study. This consisted of a subset of 22 patients described below for a total of 2985 days.

From the interim analysis, in 22 patients, 33 exacerbation events occurred. Most of the patients (n = 17) who experienced an exacerbation in the sub study experienced just a single exacerbation. However, some patients also experienced two exacerbations (n = 3) and three exacerbations (n = 2). As exacerbation results were not available for all (received on the actual day), 24 exacerbations were taken forward for analysis (taken across 22 patients).

7.3.4.1 individual patient biomarker profiles

Stable and exacerbation samples were selected as shown in table 7.3. The stable sample was selected based on being at least 30 days prior to or after an exacerbation event. There was just one sample that did not conform to this rule which was taken just 5 days prior to the exacerbation (Head092), this was due to the limited number of available stable samples for this patient.

The biomarker levels in both of these groups are shown in table 7.4. Even with the small sample size it was possible to see significance (p value <0.05) for some of the biomarkers, namely A1AT, fibrinogen, RBP4, CC16 and fMLP when looking at raw or transformed data. The criteria for the AUC was > 0.6 or <0.4. Of the 10 biomarkers, A1AT, RBP4 and CC16 met these specifications.

Table 7.3. selected data for analysis and indication of days pre- or post-exacerbation. The number of days between the stable and exacerbation sample are shown.

Patient ID	Day of stable sample	Day of exacerbation	Difference (days)
Head001	35	91	56
Head001	78	164	43
Head002	96	119	23
Head004	51	78	27
Head006	140	179	39
Head009	15	36	21
Head011	85	122	37
Head013	46	179	133
Head019	11	25	14
Head019	158	187	29
Head028	15	37	22
Head030	3	16	13
Head031	12	42	30
Head035	36	57	21
Head037	2	15	13
Head050	40	53	13
Head055	24	49	25
Head106	2	12	10
Head118	3	15	12
Head092	4	9	5
Head099	41	61	20
Head102	41	57	16
Head074	20	31	11
Head076	11	27	16

Table 7.4. Biomarker levels at stable and exacerbation with statistical tests

Biomarker	Unit	Stable	Exacerbation	Statistical tests		
		Median (IQR)	Median (IQR)	Paired t test	Paired t test (transformed data)	AUC
A1AT	ng/ml	313.70 (92.06-962.50)	108.80 (44.51-562.00)	0.2440	0.0478	0.36
TIMP-2	ng/ml	1.41 (1.23-2.76)	2.28 (0.92-3.33)	0.0843	0.5987	0.54
NGAL	ng/ml	8.69 (2.98-24.83)	4.91 (1.84-18.36)	0.4974	0.5513	0.43
Fibrinogen	ng/ml	15.97 (5.18-46.42)	7.90 (2.91-21.04)	0.0404	0.0553	0.36
CRP	ng/ml	0.44 (0.20-0.96)	0.51 (0.12-2.04)	0.2051	0.4919	0.55
RBP4	ng/ml	44.35 (17.19-97.98)	68.96 (31.73-162.40)	0.0184	0.0093	0.62
CC16	ng/ml	99.82 (27.46-207.20)	164.10 (61.52-543.20)	0.2248	0.0480	0.60
B2M	ng/ml	49.17 (24.96-132.10)	58.10 (38.57-165.20)	0.0530	0.1063	0.59
TIMP-1	ng/ml	3.26 (1.20-8.04)	2.78 (1.05-5.71)	0.3880	0.1219	0.42
fMLP	ng/ml	5.00 (2.91-16.95)	6.43 (3.33-18.89)	0.0320	0.1031	0.56

7.3.4.2 Multiplex biomarker analysis

When assessing the final 10 biomarkers on the Leicester BEAT-COPD study, an AUC of 0.84 (95% confidence interval 0.76 to 0.92) was achieved (chapter 4). At an optimal cut-off of 0.4065, the sensitivity and specificity were 80% and 76.36 respectively with a PPV of 77.19% and NPV of 79.25%. The minimal number of biomarkers that could be used without compromising the results was 5. The selected 5 biomarkers using logistic regression modelling was CC16, CRP, B2M, A1AT and RBP4 which gave an AUC of 0.8304 (95% confidence interval 0.7479 to 0.9129). At an optimal cut-off of 0.4049, the sensitivity and specificity were 81.82% and 80% respectively with a PPV of 80.36% and NPV of 81.48%.

The same analysis was applied to the results from the observational study. The baseline was calculated from n=5 days either prior to or after the exacerbation depending on the availability of data. The selected baseline values for each patient with reference to the stable and exacerbation samples are shown in table 7.5. For all exacerbation events, the stable sample n=1 was selected for the analysis at least 10 days prior to the event. For 79% of the exacerbation events, the baseline samples n=5 were selected prior to the exacerbation n=19 and but for 5 patients the BL had to be calculated post event (Head030, Head037, Head106, Head118 and Head092).

The percentage change for the stable and exacerbation samples was calculated from the average baseline value as explained in the methods section of this chapter.

For all 10 biomarkers, an AUC of 0.86 was obtained (95% confidence interval 0.75-0.96. At an optimal cut-off of 0.577, the sensitivity and specificity were 75% and 91.67% respectively with a PPV of 90% and NPV of 78.57%.

A second model with the minimal number of biomarkers was developed, with just 5 biomarkers, namely, TIMP-2, fibrinogen, CRP, CC16 and B2M. In this case an AUC of 0.82 was obtained (95% confidence interval 0.7-0.94). At an optimal cut-off of 0.5159, the sensitivity and specificity were 75% and 83.33% respectively with a PPV of 81.82 and NPV of 76.92.

Table 7.5. selected data for analysis and indication of days pre- or post-exacerbation

Patient ID	Baseline samples	Day of stable sample	Day of exacerbation	BL Difference from exacerbation (days)	Stable Difference from exacerbation (days)
Head001	19-23	35	91	68	56
Head001	19-23	78	164	141	43
Head002	81-85	96	119	34	23
Head004	26-30	51	78	48	27
Head006	124-128	140	179	51	39
Head009	2-6	15	36	30	21
Head011	62-66	85	122	56	37
Head013	30-34	46	179	145	133
Head019	1-5	11	25	20	14
Head019	1-5	158	187	182	29
Head028	1-5	15	37	32	22
Head030	74-78	3	16	-62	13
Head031	1-5	12	42	37	30
Head035	21-25	36	57	32	21
Head037	75-79	2	15	-64	13
Head050	23-27	40	53	26	13
Head055	12-16	24	49	33	25
Head106	46-50	2	12	-38	10
Head118	33-37	3	15	-22	12
Head092	60-64	4	9	-55	5
Head099	23-26	41	61	35	20
Head102	21-25	41	57	32	16
Head074	3-7	20	31	24	11
Head076	1-5	11	27	22	16

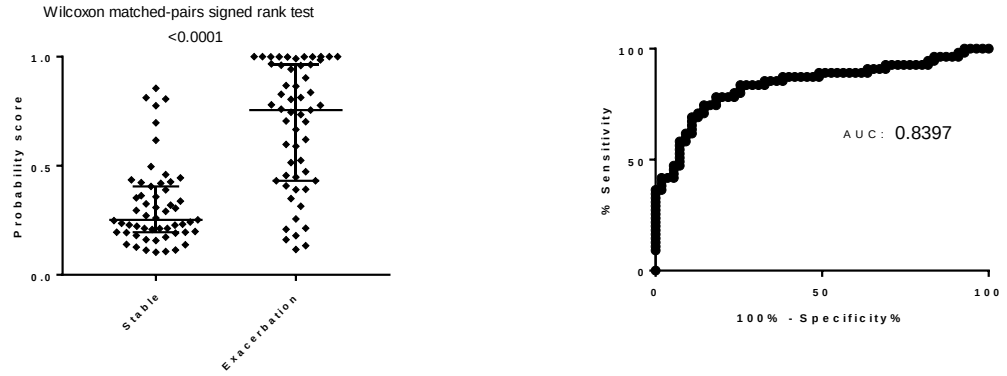


Figure 7.5. Combined Male and females n=55 with final 10 selected biomarkers (BEAT-COPD study). (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values)

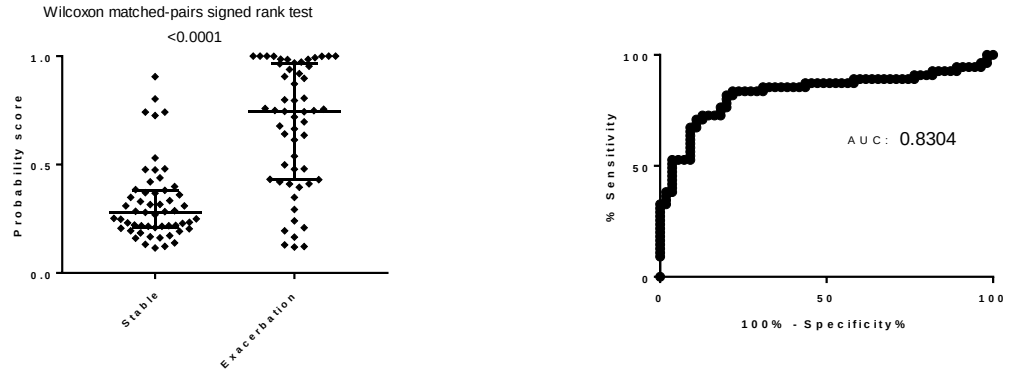


Figure 7.6. Combined Male and females n=55 with 5 selected biomarkers (BEAT-COPD study). (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values)

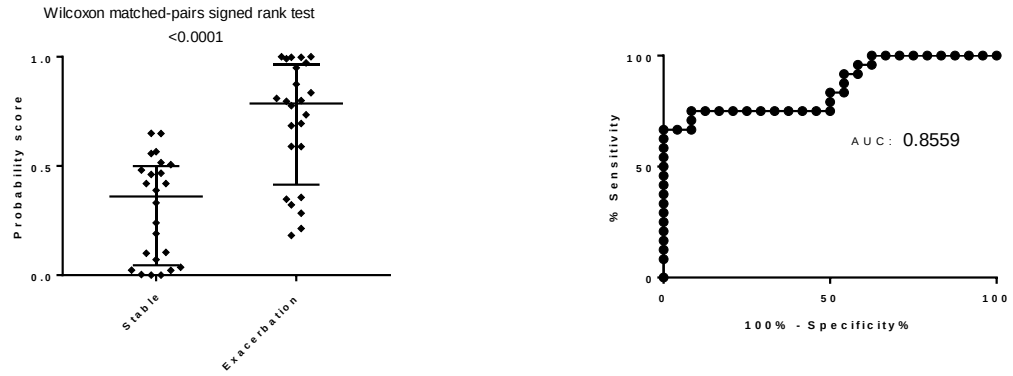


Figure 7.7. Combined Male and females n=24 with all 10 selected biomarkers (new study). (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values)

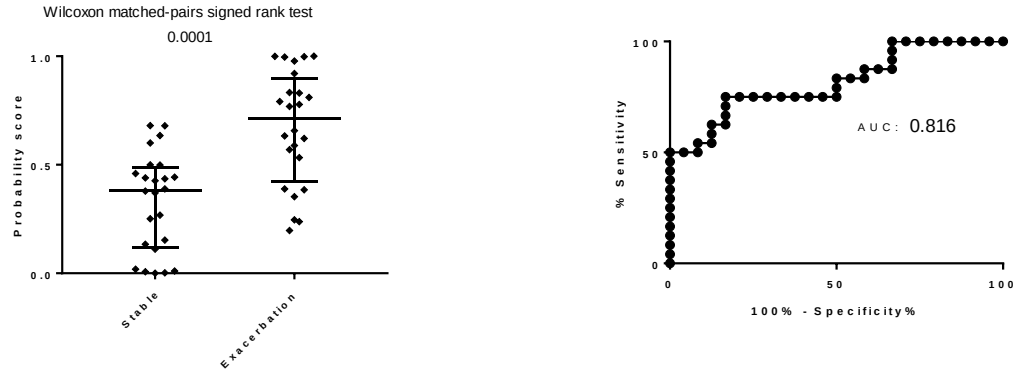


Figure 7.8. Combined Male and females n=24 with 5 selected biomarkers (new study). (a) Scatter plot of the predictive probabilities from models generated for combined male and female model (b) ROC curve (and AUC values).

7.3.4.3 Patient biomarker profiles with Algorithm (5 biomarkers)

The logistic regression equation generated for the second algorithm (5 biomarkers) was applied to all the daily results from all 22 patients. The risk scores generated were plotted for 6 patients that experienced 1-2 exacerbations. A rolling average was calculated - an average of the last 3 values in each data set to allow for missing data and to smooth out short fluctuations and highlight longer-term trends. The EXACT-PRO total score which was calculated from a set of 14 questions was also plotted. EXACT-PRO is used as a research tool for prediction of exacerbations and to determine the severity of the exacerbation. It has its own algorithm that uses the total score generated, which is re-calibrated monthly and in the case of an event a new baseline is calculated. There are set criteria for predicting an exacerbation looking a difference from the baseline. Only the raw total scores are shown with the risk score.

Interpretation of the 6 different profiles (figure 7.9):

Head011: The patient had 1 exacerbation at day 122, this was predicted by the risk score where it appeared to be raised at day 104, 18 days prior to the reported exacerbation. The EXACT-PRO total score was also raised but only 2 days prior to the exacerbation. The exacerbation was not resolved at day 168 as shown by the risk score, on average, the recovery time post exacerbation was 6 weeks as indicated by clinical experts.

Head004: This patient had 2 reported exacerbations at day 78 and day 143. For the first exacerbation, the risk score was not predictive, it was raised prior to the exacerbation on day 49 onwards (29 days prior to reporting the event). The risk scores were significantly raised, for a long period of time (day 89-124) before returning back to baseline for approximately a week. It is predicted that this was a severe exacerbation which was not reported early enough and hence un - resolved resulting in a re-exacerbation on day 143 (the risk scores started to become elevated on consecutive days after day 133 (10 days prior to the second exacerbation. As reflected by the EXACT-PRO total score, the first exacerbation was also not predicted at the time of exacerbation or after but there were peaks on day 43, day 52 which did correlate with the biomarker risk score and was raised at the second exacerbation just 2 days prior.

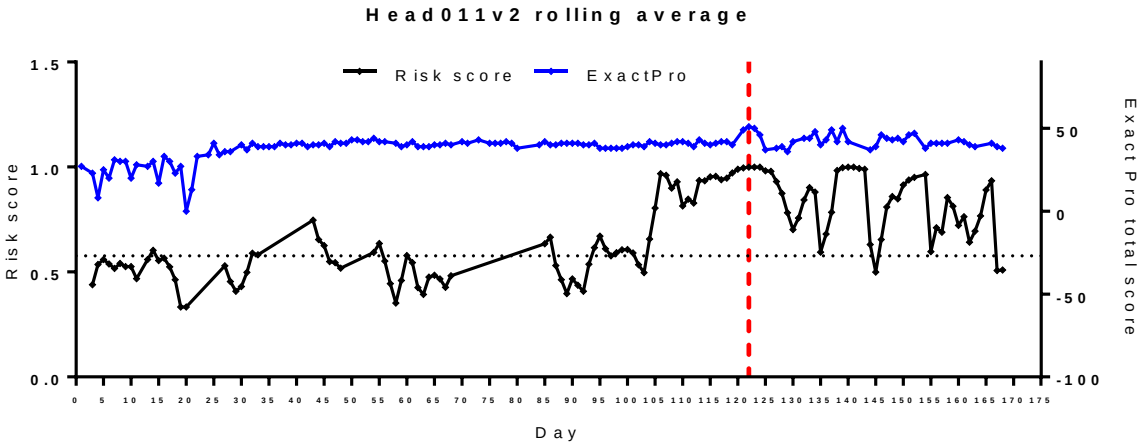
Head019: this patient had 2 reported exacerbations at day 25 and day 187. Both these exacerbations were predicted by the risk scores. However, it is not so obvious as the profile is variable. The trend is such that that the scores did return back to baseline levels after day 33 until day 184 but there were times during this period where they were above the cut-off set. For exacerbation 1, the number of days prediction for the test was 1 day and for EXACT-PRO, approximately 3 days. For the second exacerbation, the number of days prediction for the biomarkers was approximately 2 days and for

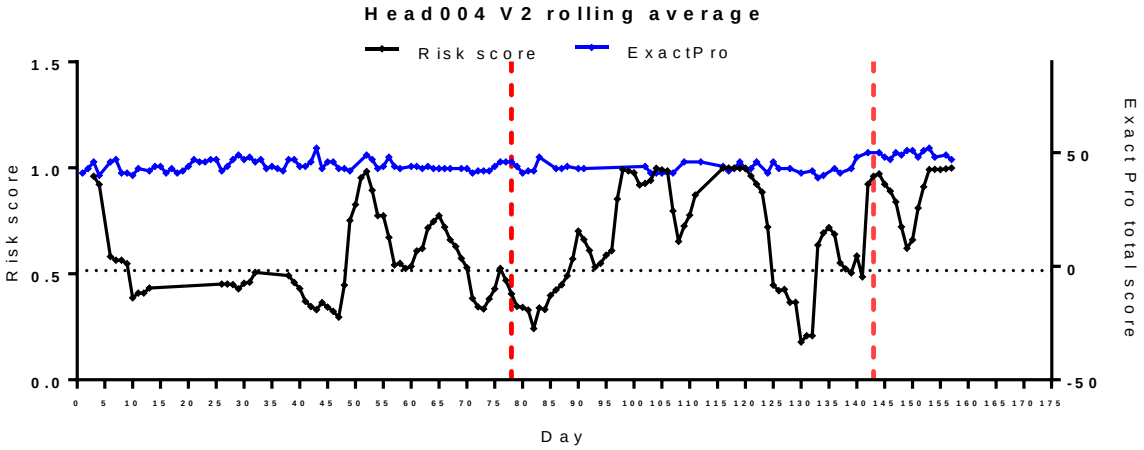
EXACT-PRO, although there were missing data, it appears to be 5 days. Due to the correlation of the biomarkers and symptoms, it does confirm that these 2 events were 'real' exacerbations.

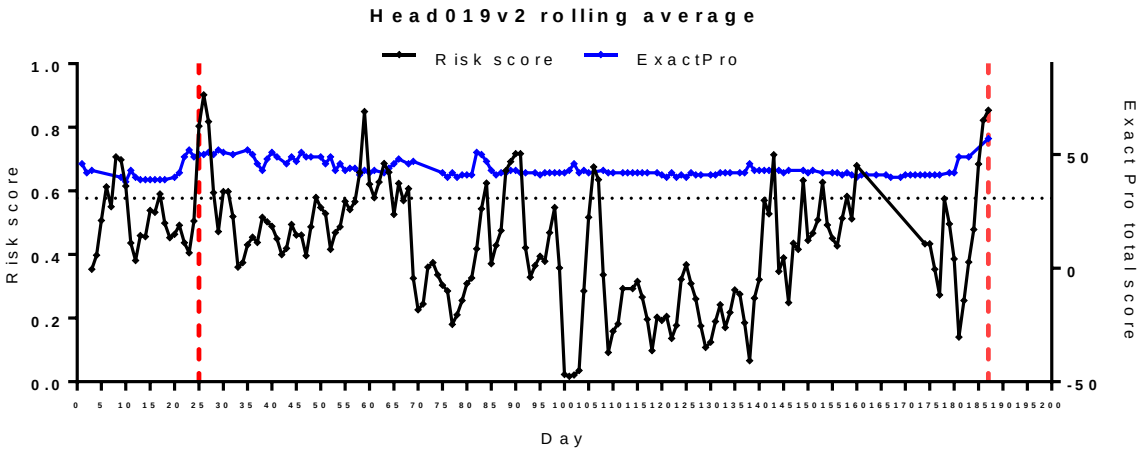
Head030: The patient had 1 exacerbation at day 16, this was predicted by the risk score on the actual day and continued to be raised until day 19 (3 days post exacerbation). EXACT-PRO also confirmed that this was an exacerbation and the number of predictive days prior to the exacerbation event on day 16 was 1 day. Overall the total EXACT-PRO score was higher than most which is indicative that this patient's condition was more severe.

Head035: the patient had 1 exacerbation on day 57. The risk score was able to identify this exacerbation and this was on day 51 (6 days prior to the exacerbation event). The risk score remained high up to the last day (day 126), either indicating that the exacerbation was not resolved or the algorithm needs to be recalibrated after each exacerbation. EXACT-PRO total score was also raised prior to the exacerbation but not as early (day 54) and then remained at this level on average.

Head106: the patient had 1 exacerbation on day 12. This patient profile was selected as the data was not used for the analysis and therefore used as a test data set. The risk score was raised prior to the exacerbation day at day 5 (7 days prior to the exacerbation) and seemed to be returning back to the baseline levels at day 50. There was no indication that the EXACT-PRO score was able to predict the exacerbation but did have a high value on day 12 and then peaked again on day 36 which is a similar time as the second risk score peak.


Head011v2 rolling average
Risk score
ExactPro
Risk score
ExactPro total score
Day


Head004 V2 rolling average
Risk score
ExactPro
Risk score
ExactPro total score
Day


Head019v2 rolling average
Risk score
ExactPro
Risk score
ExactPro total score
Day

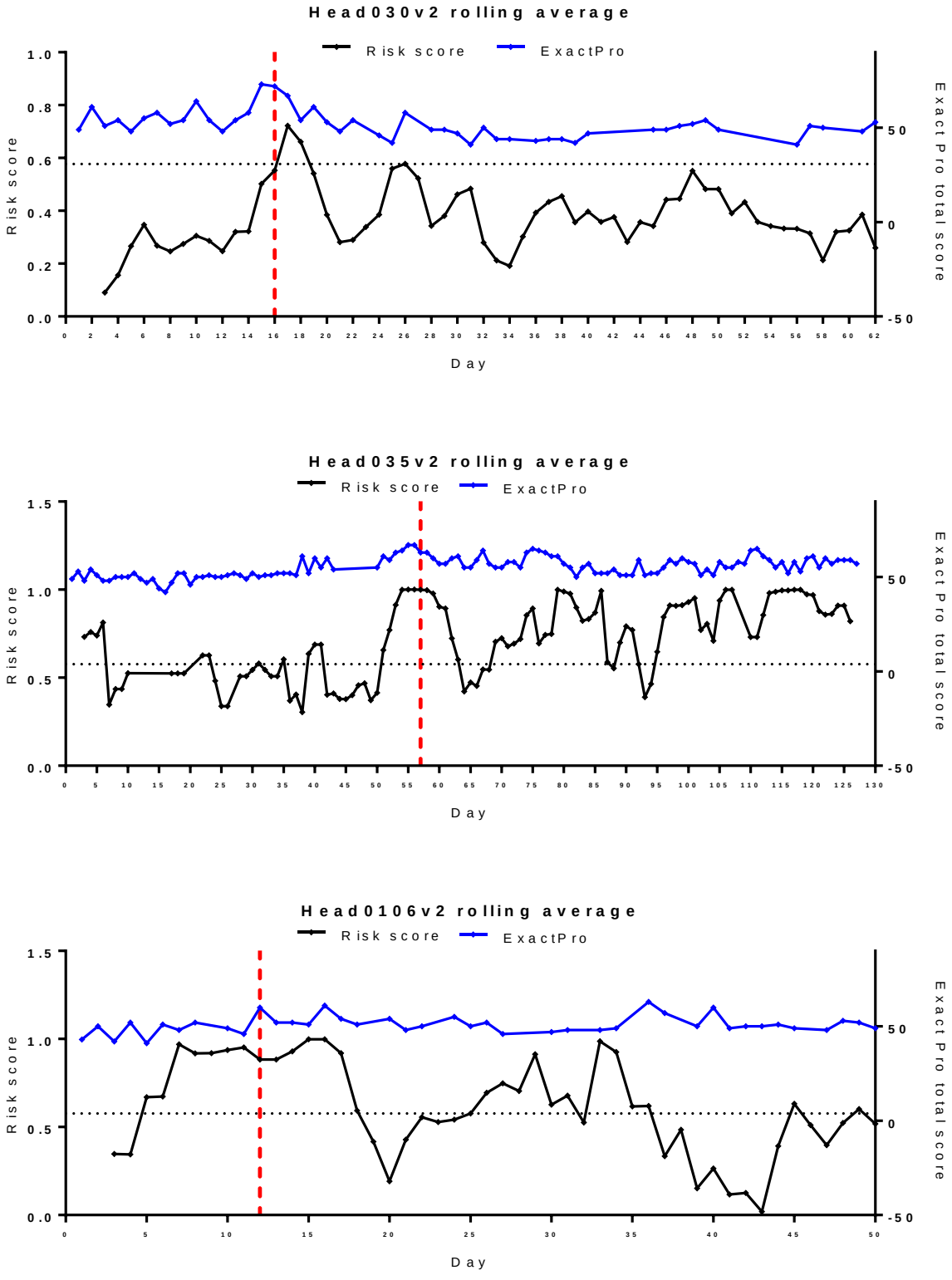


Figure 7.9. Patient profiles with probability risk scores plotted against day with EXACT-PRO total score. The risk score is derived from the applied algorithm to the biomarker measurements. The EXACT-PRO total score is derived from the symptom questionnaire completed by the patient. Red dotted line is day of exacerbation. Grey dotted line is a universal cut-off level based on previous analysis.

7.4 Discussion

The chosen biomarkers were the same whether selected on the basis of freeze/thawed samples or fresh sample analysis. Although the 10 biomarkers were not selected from just one study but from a total of 3 (Birmingham, AERIS and Leicester BEAT-COPD), the same methodology used for analysis on the BEAT-COPD study when applied to the current study produced comparable performance results. Evaluation of the 10 markers in the BEAT-COPD study (frozen samples) provided an AUC of 0.83 whereas, in fresh samples from the current study a comparable AUC of 0.86 was obtained. This is an excellent outcome, as it confirms that the selected biomarkers not only show promise in different cohorts but also in fresh samples since, to date, all analysis has been conducted on frozen and older samples. Additionally, the measurement of the biomarkers was different, namely, the original measurements were performed by trained operators using more accurate and sophisticated ELISAs whereas the new measurements were all from lateral flow technology, a 10 minute read time with the added variability of being performed by the patients themselves, all of them elderly with moderate to severe COPD.

Analysis was undertaken that refined the 10 biomarkers to 5 or less based on 2 studies (Leicester BEAT-COPD and the recent observational study). On the basis of the Leicester BEAT-COPD analysis, the most promising panel was CC16, CRP, B2M, A1AT and RBP4, whereas for the observational study, the selected biomarkers were CC16, CRP, B2M, fibrinogen, and TIMP-2. Thus, the three common biomarkers are CC16, CRP and B2M. For the observational study, A1AT was excluded from the refined biomarker selection due to the poor quality of the data where it was found that urine levels of A1AT in fresh samples were much higher than those in frozen samples and the assay range was not sufficient to encompass these levels. Therefore, this could be an explanation as to why the same 5 biomarkers were not selected for both studies (BEAT-COPD and current study). It does confirm that the three common biomarkers add the most weight to the panel with some added value by including 2 more biomarkers.

In the current study, it was shown that that the algorithm generated from the selected baseline/stable and exacerbation sample data when applied to the remainder of the unseen data yielded meaningful patient profiles relating to recovery, pre-exacerbation (prediction) and the EXACT-PRO pro total scores. Included in the ‘’unseen’’ set were additional exacerbation events for example: patient 106 was not included in the training set and neither was the second exacerbation event for patient Head004, the resulting risk scores did reflect the state of the patient with known information. At a glance, Head004 is interesting as this could have been a case where, had the patient reported the exacerbation early and started treatment early, the severity of the event could

have been reduced. However, this is only speculation and the effectiveness of the test would need to be assessed in a randomised controlled trial.

If the results from the final analysis continue to look promising there is a significant benefit to introducing a simple point of care test to enable the patients to monitor their condition in the home. Based on a survey feedback from the patients in the study indicated that the test was simple to use, was not burdensome and the majority would be happy to test on a frequent basis - daily or every three days.

Limitations of the study, the point of care assay, quality of the data received and the statistical analysis.

The planned recruitment of 120 patients into the study was not achieved due to a lack of patients available to participate at each of the sites within the time frame of the study as well as delays with providing the kits to the sites in a timely manner. The number of patients that were recruited was 105 of which 9 withdrew from the study. In addition, there was an unexpected issue with the Bluetooth cube readers where data failed to upload to the cloud and was not saved resulting in a loss of data (n=7). The key reason for not reaching the target number of exacerbations of 110 was due to the population group. The COPD patients recruited fell more into the lower risk category as shown by the demographic data rather than the high risk/more severe population resulting in a total of 89 exacerbations of which 30 exacerbations were self-diagnosed by the patient. The failure of the patients to visit the centre for a diagnosis by the clinician demonstrates the need for a home diagnostic test as often the patients live in remote locations and find it difficult to travel especially when they feel unwell. The lower numbers of exacerbations as well as the severity of the patients would have to be considered when analysing the final set of data.

The data that was received back from the patient was checked and '' cleaned up'' by this, only valid results were included in the data analysis. The control line was an indication on whether the test devices were run correctly, and those results that were below the cut off were excluded. There were 2 patients who were not able to run the test correctly despite training, the entire results from these patients were excluded. There was an assumption that the volunteer was complying with the instructions provided, for example, to read the test after exactly 10 minutes and that the midstream urine collected was used immediately. The time of testing was recorded by the reader therefore, it was clear that in all cases device B was run immediately after device A and that they were run daily as requested. The timing was not consistent in that whilst there were a few individuals that tested at a set time each day, some individuals tested at random times even throughout the night.

The A1AT and fMLP results were used with caution as the assay ranges for both of these were not optimal for the urine samples. For the A1AT assay there was a possible hook effect– high levels of A1AT would potentially be erroneously lower for some patients. For fMLP, the narrow range of the assay did not allow small changes in the biomarker levels to be measured. For the final selection of the biomarkers these two assays were excluded from the analysis and the result was that they were not needed in order to achieve the desired assay performance and the other 8 biomarkers were sufficient.

The statistical analysis performed is not the only method that could be used to generate a final monitoring algorithm. In order to choose the best five biomarkers from the ten candidates in the trial there are a number of statistical approaches that could be evaluated. For example, a time series model, called a Dynamic Linear Model (DLM), could be fitted to the biomarkers from each patient, which would then model the progression of each biomarker for each patient over time. This model could then be used to make predictions of the biomarker levels for each patient one day, two days, etc. into the future. Another method of analysis could be using a Bayesian approach or neural networks, which is a type of machine learning.

Chapter 8. General discussion

8.1 Introduction

The purpose of the work described in this thesis was to explore if and how key biomarkers of lung tissue degradation (caused by neutrophil-driven inflammation) partition into urine, where they can be quantified and used as a new diagnostic tool.

Specific objectives: -

- Develop and characterise assays for degradation products of collagen, elastin and other molecules
- Develop assays for neutrophil enzymes (e.g. MMPs, Elastase), and protease inhibitors, to study the different ratios involved in disease progression

List of questions:

- Which biomarkers released by inflamed lungs find their way into urine?
- Does molecular size influence the extent to which individual biomarkers in the urine reflect the state of lung inflammation?
- Can reliable immunoassays be made for key biomarkers (in particular, small molecules) of interest to this study?
- Is it possible to utilise the kidney as a "sentinel" of inflammatory activity elsewhere in the body?
- Are there any patterns in the urinary biomarker profile to indicate which organ/tissue is the source?

Thirty-Six biomarkers were selected for analysis based on known biological pathways involved in COPD. Assays in the form of ELISA, lateral flow or fluorescent substrate were acquired, those that were not commercially available were developed which involved immunogen design, bio-conjugation techniques, antibody generation/characterisation and assay development. Two of the most complex assays are described in Chapter 2, desmosine being a small molecule, where antibody generation was challenging, the resulting assay was validated against the gold standard method LC-MS/MS and MMP activity where current lateral flow assays were not sensitive enough to measure low levels of active MMP-9 (and other MMPs) in urine. The assays were used to quantify analytes in urine from normal donors, patients with inflammatory lung disease in a stable state and during exacerbation. With a subset of samples, parallel assays on blood as well as urine were conducted to determine if this could provide an insight as to whether certain biomarkers were transmitted through the kidneys or synthesised by the kidney. Of the thirty-six biomarkers there were just two

that were not measurable in urine, these were TNFα and MBP. There was no relation to molecular weight observed. Biomarkers found in urine that were higher than what has been reported.

8.2 Main findings

In discussing these complex relationships, simple, integrated diagrams are used to clarify the key patterns that can be derived from the results.

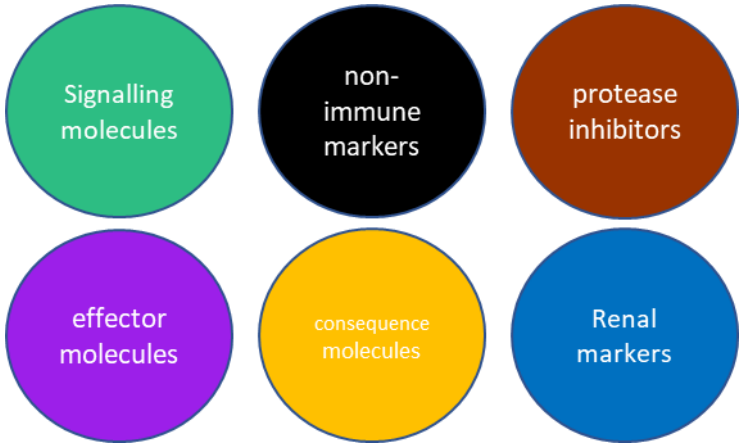


Figure 8.1. KEY: Colour code for biomarker groupings

8.2.1 Urinary biomarkers in health, disease and severity

The first observation is that levels of biomarkers in urine from both stable COPD and CF are significantly different compared to samples taken from healthy individuals. This has already been proven in other sample matrices such as sputum, blood, EBC and BAL fluid, but to the best of our knowledge, has not previously been demonstrated in urine with the exception of desmosine where levels have been found to be increased in stable disease and exacerbations. Out of the 17 biomarkers tested, when comparing to the healthy urine samples, there were 3 markers associated with CF (creatinine, NGAL and MMP-8) which were elevated and for COPD there were 3 biomarkers (Fibrinogen, IL-6 and IL-1β) which were also elevated however, they were also elevated in samples obtained from people with suspected UTI indicating that they were not specific to COPD. There were 9 biomarkers that overlapped both CF and COPD, 4 of which were also increased in UTI. There were three biomarkers NGAL, HNE, RBP4 from the panel of 17 whereby the levels found in urine were not significantly different to that found in healthy urines but were elevated in UTI, this is summarised in Figure 8.1. Of note is that from the 17 biomarkers evaluated, it was the proteases/ effector molecules that were associated with UTI. This was not unexpected as the infection is local and such molecules might be anticipated to be present in the urine. Limitations are that the control group

were not age matched to either the COPD or CF group and in addition, the sample size of the control group was small (n=40) compared to all the other cohorts.

The second observation was that there were increased levels of proteases in urine samples collected from females compared to males, similarly there were higher levels of protease inhibitors in males compared to females (figure 8.2). The elevation of NGAL in females relative to males has been demonstrated previously in studies evaluating NGAL as an acute kidney infection (AKI) marker though the cause of the difference is unknown (161). The general observation of increased proteases in females and increased protease inhibitors in males is a novel finding not previously reported. With the understanding that women are more susceptible to the development of COPD (162), it does suggest that the elevated unregulated proteases found in the urine could be related to this greater susceptibility.

The third observation is that there were clusters of biomarkers that correlated with each other and the correlations were stronger when stratified by gender. Cluster 1 consisted of 7 biomarkers, the majority of which were effector molecules (MMP-8, MPO, Calprotectin, NGAL, HNE, MMP-9 and IL-8), it is shown that the chemokine - IL-8 correlated with all biomarkers in the cluster with the exception of calprotectin in females but not in males. This could be due to the fact that there were higher levels of all these biomarkers in females such that there were measurable included compared to the males where there was lower (perhaps absent) levels. This result was consistent in not only COPD but also CF (one of the cohorts in particular). Cluster 2 consisted of again 7 biomarkers, a mixture of different groups, protease inhibitors, signalling molecules, other molecules and consequence molecules (TIMP-2, Cystatin C, fMLP, B2M, RBP4, Creatinine and desmosine). In males there were more biomarkers that correlate, albeit not strongly (Spearman's r values of >0.7), with certain biomarker pairings i.e. fMLP and creatinine, fMLP and TIMP-2, TIMP-2 and Desmosine, RBP4 and cystatin C, RBP4 and B2M, RBP4 and creatinine. Interestingly, RBP4 did not appear to be gender specific, yet it correlated with a greater number of biomarkers in males relative to females. Cluster 1 biomarkers; HNE and MPO consistently correlated in stable/recovery and exacerbation states, cluster 2 biomarkers; TIMP-2 consistently correlated with creatinine. In COPD exacerbations (AERIS cohort), 4 biomarkers (MMP-8, MMP-9, HNE and MPO) from cluster 1 remained with a high correlation (Spearman's r >0.8) whereas the correlation with NGAL was lost in AECOPD, in this instance, the levels of NGAL were significantly higher in AECOPD (Wilcoxon matched-pairs signed rank test) but unchanged for the other 4 biomarkers. In cluster 2, there were 3 biomarkers that correlated strongly (Spearman's r >0.8) with each other in both stable and AECOPD (B2M, Cystatin C and creatinine), however, the correlation with TIMP-2 was lost in AECOPD, in this instance the levels of TIMP-2 were unchanged as were the other three biomarkers therefore the reasoning is not clear.

The same strong correlations that exist with urine biomarkers were not found in blood and in addition the correlations overall were weaker in blood with all biomarkers (figure 8.5). Interestingly in a study evaluating neutrophil mobilisation during stable and exacerbation states (92), a secondary finding was that MPO and HNE were strongly correlated in blood (ρ=0.72, P,0.001, Spearman rank correlation) in stable state and modestly in exacerbation state (ρ=0.4, P,0.05, Spearman rank correlation). In the analysis performed as shown in figure 8.5, HNE was not tested therefore the correlation with MPO could not be calculated for the comparator analysis, however based on previous correlations between MPO and HNE, the findings from the Andelid study were not as strong as the correlations found in urine (AERIS study r=0.87). There are limited studies reporting correlations between different biomarkers in other sample matrices but from the analysis performed here, it is concluded that in urine, the expected correlations with biomarkers with shared cellular origin are strong and this is not replicated in blood. An explanation for this is that biomarkers in urine can reflect physiological or pathophysiological changes better than in blood where mechanisms are in place in order to maintain homeostasis.

The fourth observation was that there were biomarkers in the urine that could differentiate between severity of disease. For instance, in 2 COPD cohorts, a single biomarker (IL-1β) was significantly increased in the frequent exacerbator compared to the infrequent exacerbator. IL-1β, an innate immune cytokine involved in the initiation and persistence of inflammation, has been shown to be increased in frequent exacerbators compared to infrequent exacerbators by measurement of gene and protein expression in sputum with a p value of 0.018 and 0.065 respectively (163). This association was weaker that that shown in urine described in this thesis, indicating that urinary IL1β might be a better indicator of disease severity than in other biological fluids such as serum.

In addition, a combination of markers was able to predict the conversion of an infrequent exacerbator to a frequent exacerbator 1 year in advance, a better predictor than previous history of exacerbations which is currently used in practice. This would need to be evaluated in a different cohort and with a larger number of subjects in order to confirm this result.

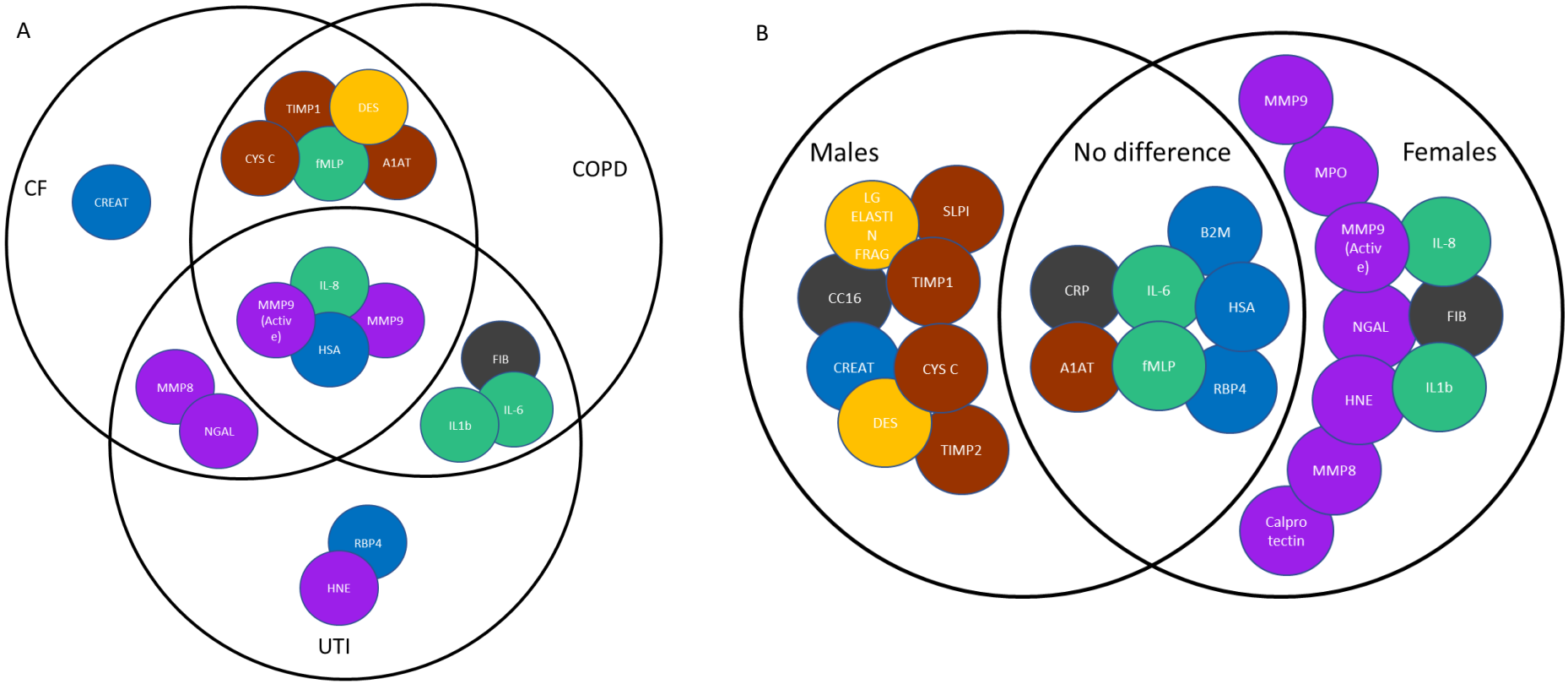


Figure 8.2. Biomarkers that were significantly different in health and disease and gender specific biomarkers. A) biomarkers that were shown to be significantly different (non-parametric unpaired t test $p < 0.05$) in different disease state, stable CF, stable COPD and UTI compared to control group. Overlapping biomarkers across all three groups were IL-6, Active MMP, MMP-9 total and HSA, biomarkers specific to lung disease CF and COPD shown. B) biomarkers that were different between males and females derived from results obtained from 1 x stable COPD cohort and 2x stable CF cohort. The colours of each biomarker represent different classes based on previous literature. Purple indicates effector molecules, brown for protease inhibitors, black for non-immune biomarkers, yellow for consequence molecules and blue for assumed renal biomarkers

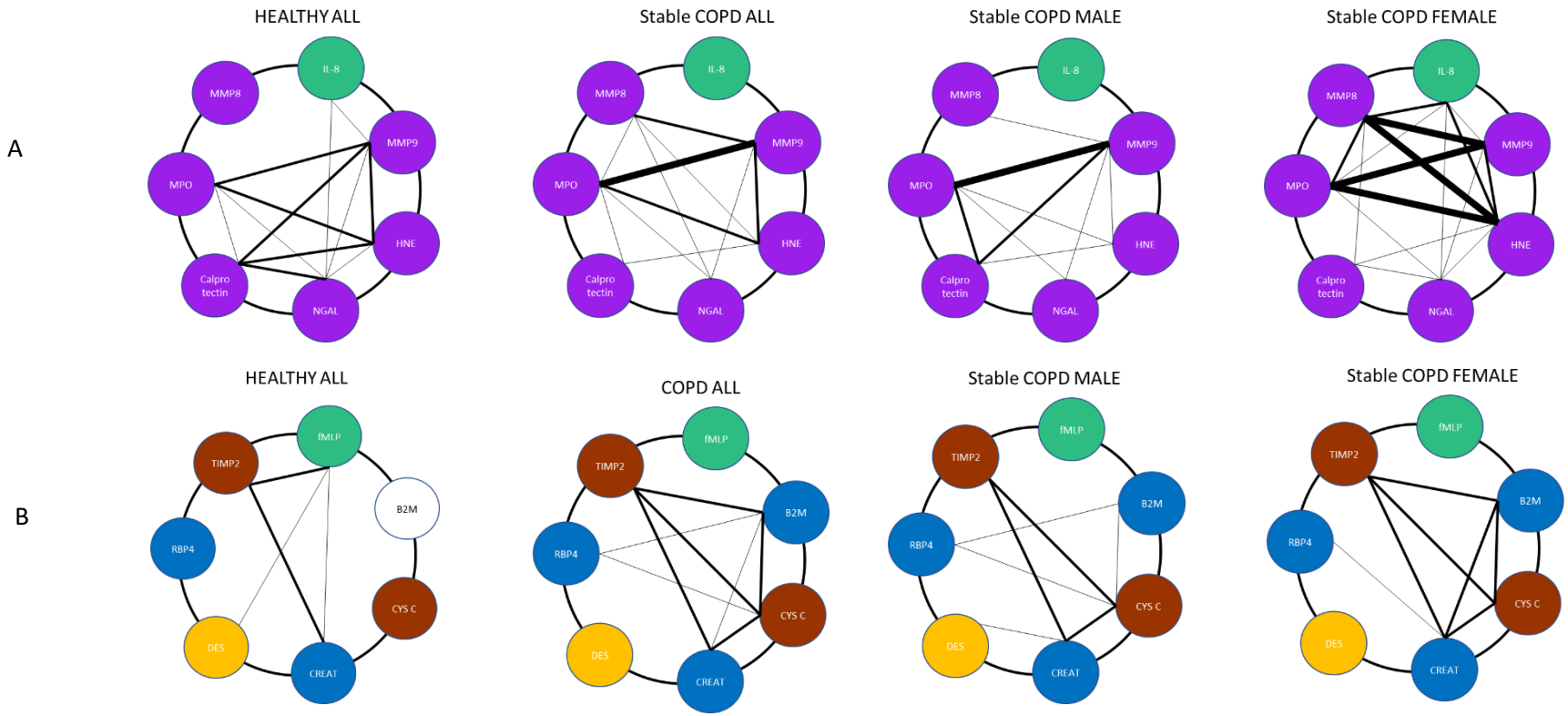
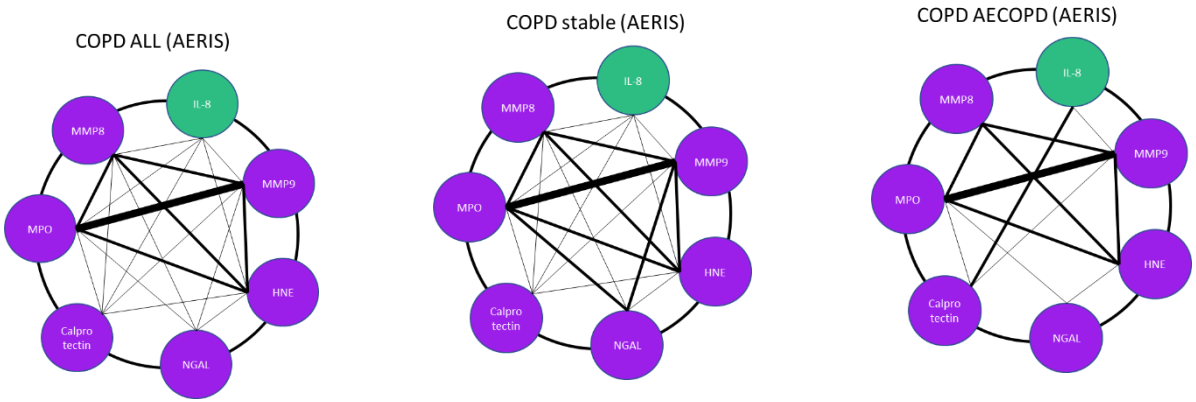


Figure 8.3. Correlations between urinary biomarkers in stable state. A) cluster 1, healthy, stable COPD, stable COPD males, stable COPD females and B) cluster 2, healthy, stable COPD, stable COPD males, stable COPD females. Cluster 1 consists of mostly effector molecules (purple) + chemokine IL-8 (green) and cluster 2 consists of a mixture of protease inhibitors (brown), renal markers (blue), elastin degradation molecule (yellow) and signalling molecule fMLP (green). Strength of correlation was measured by non-parametric Spearman's r with 3 different gradients of line thickness; thin line between 0.7-0.8; median thickness line 0.8-0.9; and thick line 0.9-1. For cluster 1, in females, there was an excellent correlation between 0.9-1 for neutrophil degranulation proteins MMP-9, MPO, HNE and MMP-8 with correlations with fMLP. In males, strong correlations >0.8 for MPO, MMP-9 and calprotectin. For cluster 2, in females, there were strong correlations between 0.8-0.9 for protease inhibitors cystatin C, TIMP-2 and renal markers, creatinine and B2M. In males, strong correlations 0.8-0.9 with cystatin C, TIMP-2 and creatinine and good correlation with desmosine (0.7-0.8) and creatinine.

A



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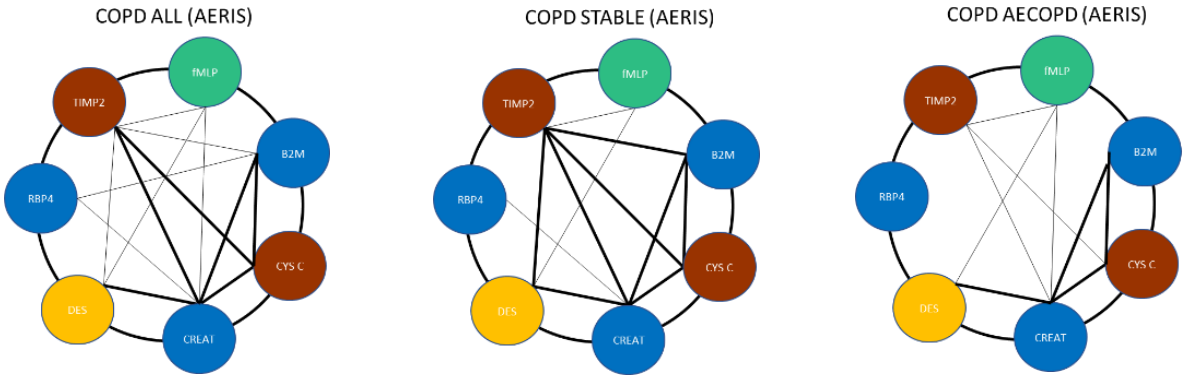

Figure 8.4. Correlations between urinary biomarkers in stable and exacerbation. A) cluster 1, COPD, stable COPD, AECOPD and B) cluster 2 COPD, stable COPD, AECOPD. Cluster 1 consists of all effector molecules (purple) + chemokine IL-8 (green) and cluster 2 consists of a mixture of protease inhibitors (brown), renal markers (blue), elastin degradation molecule (yellow) and signalling molecule fMLP (green). Strength of correlation was measured by non-parametric spearman's r with 3 different gradients of line thickness; thin line between 0.7-0.8; median thickness line 0.8-0.9; and thick line 0.9-1. For cluster 1, Four biomarkers correlated in all 3 groups (>0.8), these were MMP-9, MPO, HNE and MMP-8, there were more biomarkers that correlated in stable state compared to exacerbation this also applied to cluster 2 biomarkers. this indicated that in AECOPD there were dysregulation of biomarkers that resulted in a lack of correlation i.e. changes in the biological pathways.

8.2.2 Changes in levels of certain urinary biomarkers are indicative of exacerbation in retrospective samples

Biomarkers found in urine reflected pathological changes occurring in the lungs both in stable disease and in exacerbation. It was found that certain biomarker levels were lower in the stable state and elevated in the exacerbation state, these findings were in line with results obtained from other biological fluids such as sputum, BAL fluid and blood. Samples from three different COPD cohorts were analysed (all retrospective samples), and were collected prior to the exacerbation, or after or both. Statistical analysis, simply comparing levels of biomarkers measured in stable/recovery and exacerbation states, showed significant differences in levels ($p<0.05$) for certain biomarkers (figure 8.4). Elevated biomarkers included effector molecules, protease inhibitors, non-immune markers; CRP, fibrinogen, CC16 and renal markers. A1AT was significantly increased in AECOPD for all cohorts indicating that this was a robust marker of exacerbation. Biomarker that failed to meet significance between stable and AECOPD in all three cohorts were signalling molecules and consequence molecules which could be due to the timing of when the exacerbation sample was collected i.e. too late for signalling molecules and too early for the consequence molecules. In urine samples, the biomarkers are cleared unlike in blood where they may remain for prolonged periods of time (persistent systemic inflammation).

The relative differences in levels of the other biomarkers and differentiation between stable and exacerbations states with the three cohorts could be for the following reasons. Firstly, not all biomarkers were evaluated in all three cohorts i.e. CRP, RNASE3, CHI3L1, CC16 were not tested in the Birmingham cohort, therefore it is unknown if levels of these markers were significantly different between the two disease states Secondly, although the 3 cohorts were similar (demographics shown in Chapter 4), there could be some underlying factors that cause differences in baseline levels i.e. the AERIS cohort patients were more severe than Leicester for example, with 60% GOLD3/4 compared to 52%. Thirdly, as previously stated, the time of sample collection may have an impact. For the Birmingham cohort the samples were collected at exacerbation and a follow up recovery sample at 6 weeks compared to the other two studies whereby a stable sample was collected followed by an exacerbation sample. It could be that the levels of biomarkers at recovery are different from a true stable sample. For the AERIS study; pre-exacerbation samples were collected between 3-66 days before the exacerbation therefore, not all are ''true stable'' samples whereas for the Leicester study, the stable samples were collected in good time prior to the exacerbation in most cases so would be classed as ''true stables'' These were all factors that needed to be considered when selecting biomarkers for the final device.

The demonstration of significance using population threshold averages was encouraging as from previous studies it was established that the it was the change from baseline levels of the urinary biomarkers to exacerbation that was fundamental when looking at this type of data. It was shown in chapter 3 that there were differences due to gender, age and severity that would influence the baseline values. As a result, a different method of analysis was required in order to establish the true utility of urinary biomarkers levels. This was achieved by calculating the % change from stable to AECOPD or AECOPD to recovery for each individual and a change of greater than 10% was deemed to be reflective of a positive change from the 'baseline'. Limitations were that only a single time point was available and in practice a baseline would be calculated from more frequent data points taken at a stable state. The Leicester cohort was more representative of what would be done in practice as samples were collected at multiple stable time points and an average of these provided a more representative baseline. In addition, a single biomarker was not likely to be effective and a combination of biomarkers would be required, the analysis of which was undertaken manually for the Birmingham and AERIS data and by logistic regression analysis for the BEAT-COPD data. Through the multiple analysis methods, the most promising 10 biomarkers were selected for further analysis.

A further limitation was that these samples were frozen, and verification and validation studies undertaken in Chapter 6 demonstrated that there were differences in the levels of some of the biomarkers when comparing fresh and frozen samples although the correlations were comparable.

The measurement of biomarker levels was undertaken using more sophisticated and accurate assays such as ELISAs which would not be suitable for point of care testing by individuals in the home. The question is whether a simple lateral flow assay will be reproducible, robust and quantitative to be able to replicate these results.

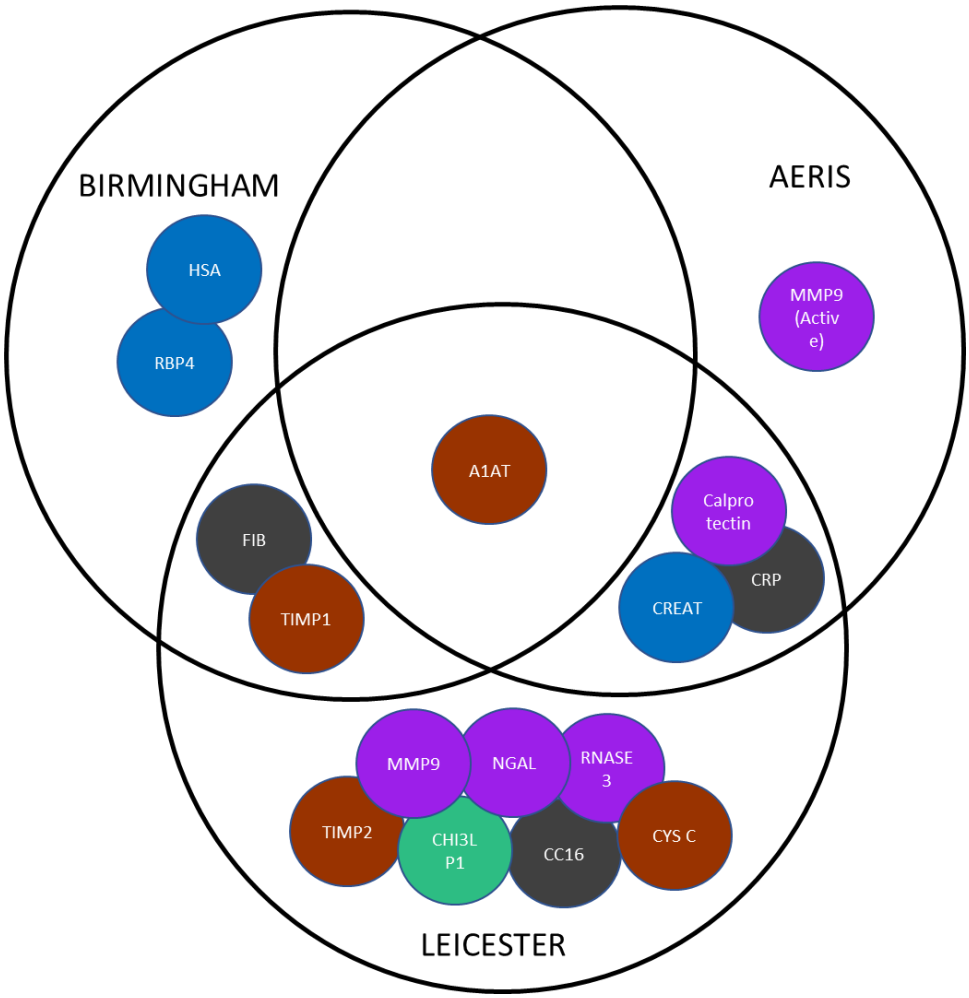

Figure 8.5. Urine biomarkers associated with AECOPD. Biomarkers that were increased in exacerbation compared to a stable or recovered state with significance levels $p < 0.05$. Birmingham cohort consisted of samples collected from patients at exacerbation and at 6 weeks. The AERIS cohort comprised samples collected at stable (or pre-exacerbation, sometimes only days prior) and at exacerbation. The Leicester cohort consisted of samples collected at stable state and at exacerbation.

8.2.3 Changes in levels of selected urinary biomarkers are indicative of exacerbation in prospective samples

Daily samples were collected and tested by people with COPD for 10 biomarkers over a period of approximately 6 months. An interim analysis of this study indicated that there were 5 biomarkers that, when combined, could be useful in predicting and diagnosing an exacerbation. This was the first true longitudinal study where daily inputs other than symptoms or lung function tests (spirometry) could be collected. Other longitudinal studies involved collection of samples less frequently during stable disease i.e. monthly, thence more frequently during the exacerbation period for the first 2 weeks and subsequently at 4-6 weeks when recovered. The details of some of these studies are presented in the introduction, table 1.1. The 10 biomarkers that were measured with the point of care tests were A1AT, TIMP-2, NGAL, Fibrinogen, CRP, RBP4, CC16, B2M, TIMP-1 and fMLP. In our study, in addition to the daily urine testing undertaken by the patient in the home, scheduled visits were arranged at day 0, and every 1.5 months till the end of the study and unscheduled visits; exacerbation and follow up after 2 weeks. At all these visits blood samples and urine samples were collected. The correlations of the biomarkers in blood relative to urine were very different, the clusters of urine biomarkers already reported in Chapter 3 were not replicated in blood. Notably, the effector molecule correlations (cluster 1) were not observed and in stable state there were 2 very interesting pairings in blood – A1AT and B2M and CRP and TIMP-1 and in exacerbations, Cystatin C and CHI3LP1. In urine, a new pairing was observed, namely IL1β and periostin, which could be attributed to the fact that these were fresh samples in this particular study, although it would need to be validated, the biomarkers may have been degraded in stored or freeze/thawed samples.

The model developed used 5 biomarkers, TIMP-2, Fibrinogen, CRP, CC16 and B2M. The performance obtained using logistic regression analysis gave an AUC of 0.82 (95% confidence interval 0.6957-0.9363). This was based on results from the prospective, longitudinal study where data were obtained from patients running daily tests.

The results that were obtained from the study described here were screened for validity, namely, results that were classed as being incorrect were removed where the control line result did not fall within a specified range. Usually, the invalid tests were due to the incorrect positioning of the reader or device in the holder. The patients were monitored for the first 2 weeks and additional training was provided if required, however, there still remained results that were incorrect post-training highlighting the need to make the system more user-friendly. Samples were also sent to the laboratory once a week where the tests were repeated. It was not possible to confirm if the results

from the same samples were equivalent due to the time difference from when the sample was collected and received (1day + later), some biomarkers were shown to be unstable over time and under transport conditions. We were therefore reliant on the testing results received from the patients.

It was observed that due to the different baseline values for each individual the biomarker results for some of the assays did not always fit within the dynamic range. The A1AT assay for example, was too sensitive and there were several measurements that were above the standard curve. This was anticipated as results from a previous cystic fibrosis home study had highlighted a difference between fresh and frozen samples. However, the extent of this was not known until the present study. The assay was modified and introduced during the study, however, it did complicate the analysis as data obtained from the new assay would need to be analysed separately thus reducing the sample size and not possible for this interim analysis with limited data.

The statistical analysis used to select the 5 biomarkers took just one baseline (average of results taken from 5 consecutive days), one stable result and one exacerbation result. Subsequently, the % change of the stable and exacerbation result from the baseline was calculated for each individual biomarker and it was these variables that were inputs for the logistic regression analysis to determine how the probability of an exacerbation occurring depended on each variable. This determined a set of weights that could be applied to the terms to produce an index or risk score. Whilst this provided an early insight there are other approaches that could be used to improve accuracy a) a continuous re-calculation of the baseline b) use of variables based on slope as well as extent of change calculated from previous days on a continuous basis c) use of other more sophisticated methods other than logistic regression such as 'artificial' neural networks (ANN). ANN captures associations or discovers regularities within a set of patterns and can cope with noise, complexity and non-linearity found in biological data. It is often used in cases where the relationships are difficult to describe adequately with conventional approaches. This type of machine learning on such data is likely to be the most appropriate due to the complexity of the data and the changes that occur over time as the disease becomes more severe and exacerbations become more frequent.

The date of exacerbation was defined as the day on which the patient contacted the research centre and a diagnosis was made. In certain instances, the patient may have waited several days before making the initial contact, therefore we were reliant on the patient to provide correct history of previous events and dates for when the symptoms first presented. This is a practical problem and underpins the reason such a test would be valuable to the patients. The patients recruited to the

trial completed the daily EXACT-PRO symptoms e- diary which has been proven to be successful in several trials, concluding that the EXACT tool was reliable, could determine frequency, severity and duration of AECOPD (164) (165). However, a poor performance in AECOPD detection has also been reported (166), which highlights the complexities of defining an exacerbation due to the heterogenous nature of the exacerbations. This notwithstanding, the electronic symptom recording is far superior to paper-based recording with compliance of 94% and 73% respectively (167) and is one of the most accurate methods developed to date for recording patient symptoms during clinical trials. The reason for the poorer compliance with the patient diaries is retrospective backfilling of diary entries and sometimes forward-filled diary cards for upcoming, future assessment points.

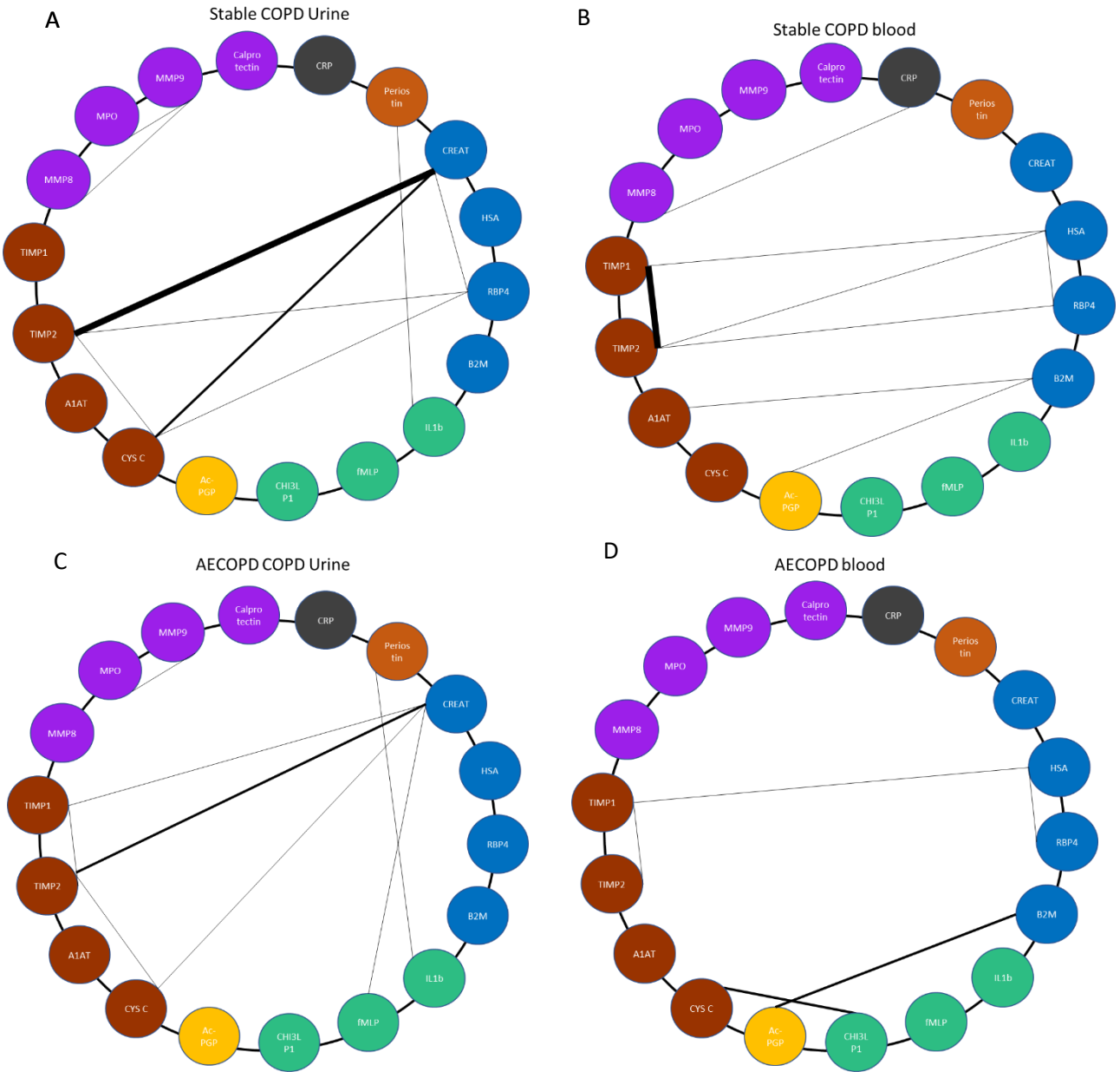

Figure 8.6. Biomarker correlations associated with AECOPD as shown in blood and urine. A) paired urine and B) blood samples collected from patients in stable state C) paired urine and D) blood samples collected from patients at exacerbation. Stable and exacerbation samples were matched from the same patient. Only the biomarkers where correlation was obtained are shown. At a glance, both TIMP-1 and TIMP-2 correlated in blood but not in urine, B2M correlated with A1AT in blood but not urine (weak correlation) and B2M correlated with Ac-PGP in blood and not urine and finally, CHI3LP1 correlated with Cystatin C at exacerbation but not in stable state.

8.2.4 Urinary biomarker profiles in individual patients confirm already documented biological pathways

From knowledge of the biomarker biological pathway it was expected that the order in which the 10 selected biomarkers presented themselves would start with the signalling molecules i.e. fMLP followed by IL-6 induced CRP/FIB/B2M, expected thereafter would be the effector molecules (NGAL) and lastly, the protease inhibitors (TIMP-1/TIMP-2/A1AT). It is not apparent where RBP4 and CC16 fit into the biological pathway as they are not recognised biomarkers of COPD exacerbations and their functionality in this regard is accordingly unclear.

From inspection of some of the daily biomarker recordings and time series, there were 3 observations:

- Biomarker levels varied from one patient to another and would therefore require standardisation
- The delay between a change in biomarker level and the diagnosis of an exacerbation was variable
- There may be interactions between individual biomarkers that will influence the overall predictive power of the method

An appropriate statistical method/analysis would be able to establish from this complex data which biomarkers individually are relevant and that would feed into the algorithm. Most time-series analysis techniques involve some form of filtering out the noise in order to make the pattern more apparent. Two examples of individual patient profiles taken from the observational study are shown in figure 8.6

In one patient profile (Head51) for AECOPD 1, indicated by the dotted red lines, RBP4 and NGAL are raised 5 days prior to the event which occurred on day 55 and CRP 4 days prior with a second burst 5 days after the event (day 60), however for AECOPD 2, it was RBP at 4 days prior, NGAL 3 days prior and CRP 2 days prior and the second burst was in this case RBP4 and NGAL with a very slight increase in CRP levels. This could be because the 2nd exacerbation event is within 6 weeks of the 1st event and the kidneys are “leakier”. In some cases (data not shown) RBP4 is released post-exacerbation therefore this profile is not reproduced in all patients. In the case of another patient (Head72), there was 1 exacerbation event at day 57, B2M, RBP4, CC16 levels become raised at day 47 (10 days prior to the event), TIMP-1 at day 51 (7 days prior to the event). The order of ‘peaks’ was CC16 at day 52, followed by TIMP-1 at day 56, B2M at day 58 and RBP4 at day 63, there were 2nd peaks at day 65, 68,

63 and day 70 and the 4 biomarkers returned back to baseline levels, in particular TIMP-1 at day 74. The other biomarkers were present but at lower levels.

The differences could possibly be explained as a result of treatment, some treatments could affect the levels of biomarkers, in this instance, inhibition of biomarker during treatment might be expected followed by an increase following cessation of treatment. This could also explain the second peak for some of the biomarkers shown Head072 (figure 8.6). Macrolide antibiotics, include erythromycin, azithromycin, clarithromycin and roxithromycin, exhibit antibacterial and anti-inflammatory actions (168) and have been implicated in altering the production of a wide variety of molecules and parameters that influence the inflammatory response (cytokines, oxidant production, chemotaxis and degranulation of neutrophils). Doxycycline, a potent inhibitor of MMP enzymes is also known to reduce CRP levels in plasma (169).

Although all the patients were given steroids (some were also prescribed antibiotics), the exacerbations from the observational study have not yet been stratified into those with bacterial or viral infections i.e. determination of neutrophil or eosinophil derived exacerbations. Further stratification may provide further insights and understanding on the differences between the biomarker profiles for individual patients.

While there may be different responses relating to individual physiological states or idiosyncratic biochemical anomalies, each example constitutes a real biological phenomenon. If such an example makes biological sense there is reason to take the occurrence seriously, in that it can provide an understanding of possible biologic profiles that may be encountered in at least some of the subjects enrolled in future clinical trials, or in subsequent routine use.

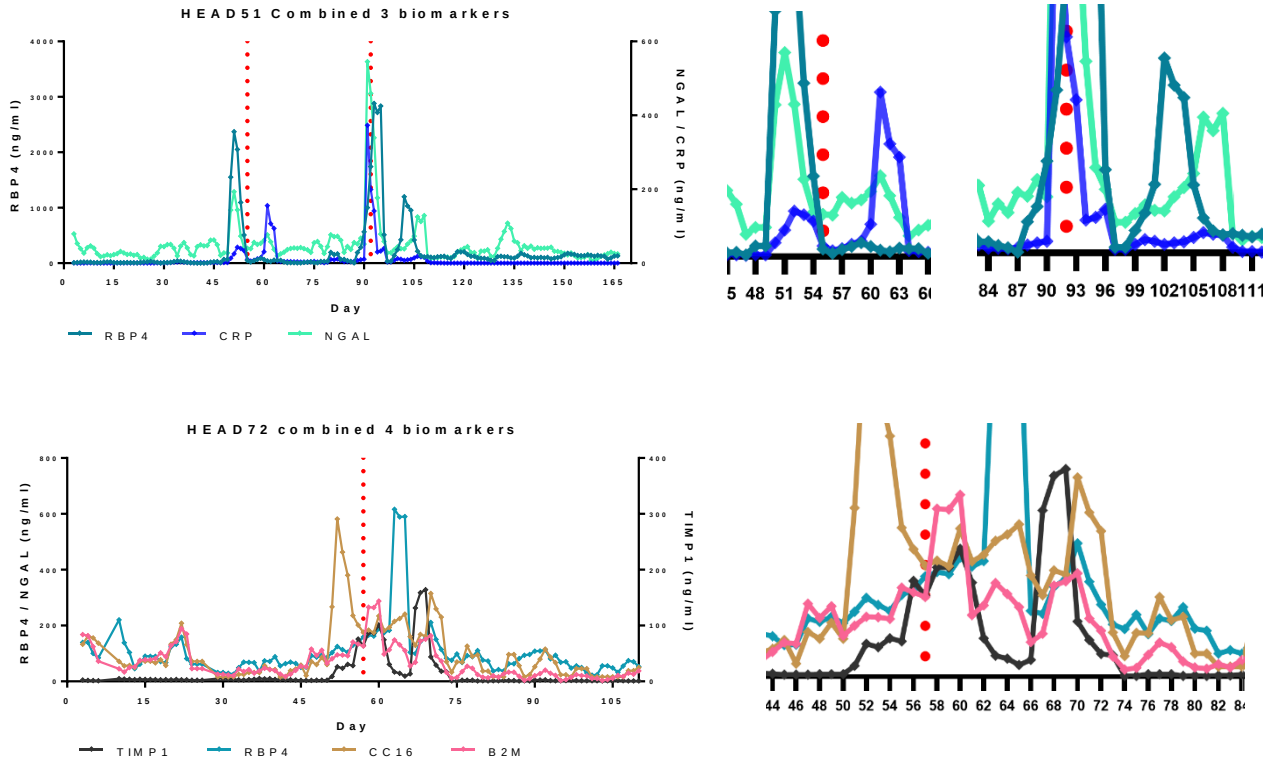

Figure 8.7. Biomarker profiles established through clinical study. Two profiles are shown on the left with close up graphs for the exacerbations shown on the right. Patient “Head51” experienced 2 exacerbation events shown by the dotted red line and Patient “Head72” experienced 1 exacerbation event shown by the dotted red line. For Head51, 3 biomarker profiles are shown that demonstrated an increase in level from stable to exacerbation. For Head72, 3 different biomarker profiles are shown that increased leading up to the exacerbation. RBP4 in this instance increased after the event but before the events for Head51.

8.3 Biological relevance of selected biomarkers contributing to prediction of exacerbations

Based on documented roles and origins, a diagram has been constructed to show the interactions between the biomarkers evaluated in this thesis (figure 8.7). B2M, RBP4 and CC16 have not been rigorously evaluated in any sample matrix with regard to their association with COPD exacerbations. B2M has been shown to be elevated in chronic inflammation, which is consistent with the fact that the surface of lymphocytes and monocytes (intimately associated with inflammatory processes) are particularly rich in B2M. Free B2M circulates in the blood as a result of shedding from cell surfaces or intracellular release regulated by cytokines(170). Once released, B2M is cleared from the blood by glomerular filtration, a physiological feature that has been used for estimation of the glomerular filtration rate (171). Although much is known about the source, fate and function of B2M, further work is required to identify the relationship of these three biomarkers with the processes underlying COPD exacerbation. Very little is known about the other 7 biomarkers in ''urine'' (from the final selection of 10 biomarkers), especially with regard to their origin and whether they are lung derived or produced locally in the kidneys. Whilst these details are not yet known, it is clear that they are useful in prediction of exacerbations.

It has been demonstrated through the research described in this thesis that the presence of particular products in urine reflect physiological or pathophysiological changes that occur in the lungs. The findings support the original hypothesis, which can now be refined with the addition of the new evidence (including 2 other promising biomarkers that did not end up in the final 10 due to the restriction in numbers that could be taken forward).

To summarise, the key elements of the hypothesis are supported by evidence from these studies, or the evidence must be evaluated in the light of known complicating factors adopted into the hypothesis, as follows:

- As neutrophil leukocytes, in particular, infiltrate the lungs and become activated, large amounts of proteases and other molecular biomarkers (indicated below) are produced and these spill-over into the blood. Significant elevations in levels of neutrophil-associated biomarkers (above the levels found during stable disease, or on recovery) at exacerbation are clearly consistent with this hypothesis. The biomarkers involved are: -
    - signalling molecule fMLP
    - cytokine-induced fibrinogen, CRP and B2M
    - tissue-derived protease inhibitors; TIMP-1, TIMP-2, A1AT, Cystatin C
    - lung-derived marker: CC16
    - Elastin degradation product: Desmosine

- Any active protease in the blood will quickly encounter the kidneys and, as the kidneys have a copious vascular supply and high blood perfusion rates, they cannot risk any protease-mediated tissue damage, so, they produce their own inhibitor supplies (TIMP-1, TIMP-2). There is a lack of direct evidence to support this, but blood concentrations and paired urine concentrations do NOT correlate, which indicates that the biomarkers are derived from elsewhere locally, pointing to the kidneys as the source of the inhibitors. There is already evidence that the kidney has the capacity to express substantial amounts of TIMP-1 and TIMP-2 (172) and SLPI (173).
- During episodes of acute inflammation, the kidneys are affected by the presence of active inflammatory mediators in the blood, causing inflammation-related changes in the molecular permeability of the glomeruli and, consequently, measurable changes in urinary concentration of certain biomarker molecules. Evidence to support this is to be found in the significantly higher levels of certain biomarkers at exacerbation than the concentrations in stable or recovered states (such as the changes reported for RBP4 and B2M) and the finding of large molecular weight molecules in the urine such as A1AT, CRP and fibrinogen during episodes of acute lung inflammation.
- Consequently, kidneys can be utilised as sentinel organs, releasing molecular messages that warn of impending exacerbation, although the messages need to be de-convoluted in order to be understood.
- Small but measurable amounts of inflammation biomarkers traverse the kidney to become detectable in the urine it is to be expected that differential filtration and metabolic effects can effectively scramble the overall biomarker message.

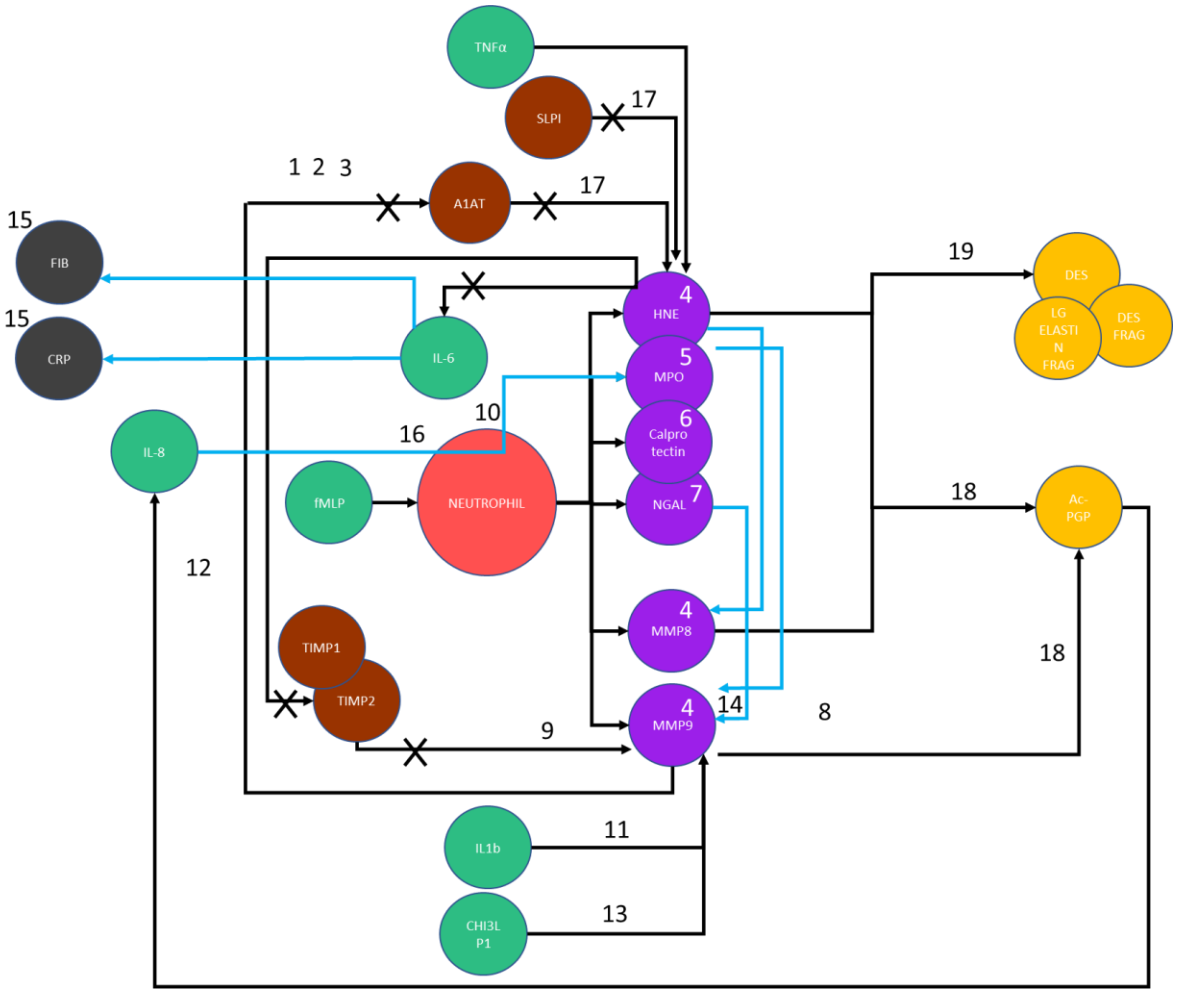

Figure 8.8. Interactions between the biomarkers involved in COPD exacerbations 1) inactivation of A1AT caused by A1AT deficiency 2) inactivation/oxidation of A1AT caused by ROS 3) inactivation caused by active MMP-9 4) HNE derived degradation of collagen and/or elastin 5) MPO resulting in destroying of bacteria 6) calprotectin involvement of inflammatory cell recruitment 7) NGAL reduction of available iron required for bacterial growth 8) HNE activation of MMP-9 9) inactivation of TIMPs resulting in increased MMP-9 10) IL-8 induced released of MPO from neutrophils 11) IL1β induced release of MMP-9 12) Ac-PGP activation of CXCR2 resulting in increased IL-8 13) induced release of MMP-9 14) NGAL/MMP-9 complex inhibits MMP-9 inactivation resulting prolonging MMP-9 damage 15) systemic inflammation associated with poor clinical outcomes (exacerbations and mortality) 16) IL-8 stimulated migration of neutrophils to site of injury 17) decreased inhibition function resulting in increased HNE 18) release of Ac-PGP from degradation of collagen 19) release of desmosine and fragments from degradation of elastin.

8.4 Future impacts of the research

COPD is characterised by daily symptoms of breathlessness, cough and wheeze with persistent impairment in lung function tests. At times there is worsening of the symptoms leading to exacerbations. COPD patients already monitor their health at home – not by means of biomarkers of disease status but by patient perceivable signs and symptoms. When their symptoms worsen, they contact emergency services or their GP. Exacerbation symptoms are often unclear and patients' ability to recognize them is variable. Some patients seek help promptly, whilst others delay, increasing the likelihood of hospital treatment.

Exacerbations are caused by several different triggers, including major events such as viral and bacterial infections as well as a series of smaller disturbances culminating in destabilisation of the disease. In most COPD exacerbations there is evidence of airway inflammation which could be either a cause of the exacerbation or a consequence of a new infection. Most measurements of inflammation have concentrated on sampling blood or sputum at the time of the exacerbation, compared with values from samples taken several weeks apart during periods of disease stability. These approaches have not led to an adopted test, due to lack of clinical sensitivity and specificity and challenges in obtaining the samples at times when management could be usefully altered. The few telemonitoring strategies to predict onset of COPD exacerbations have not been successful, possibly due to the absence of direct and objective measures of inflammation, moreover, the largest trial to date demonstrated that telemonitoring had no significant clinical benefits but posed a substantial impact on workload for healthcare providers (174). The challenge is to develop reliable near-patient tests of inflammation which can be measured frequently in different settings, including the patient's home that can be introduced into the existing patient care pathways. To be successful, such tests need to be simple for the user, yet sophisticated enough to deconvolute the heterogeneous inflammatory responses that precede the clinical presentation of an exacerbation.

Through the use of urine as the sample, the proposed point-of-care test is minimally invasive, easy to use, rapid in time-to-result and with simple-to-understand results. With these characteristics it can easily be integrated into the patients' routine at home. The simplicity-in-use will encourage the patient to maintain a high frequency of use to improve accuracy and extent of warning. Sputum is far from an ideal sample, due to the invasive method of collection and the complexities of processing the sample which makes it less suitable for point-of-care testing. Blood may be more convenient but too invasive for frequent testing. Profiling of inflammatory mediators in urine samples provides a simple and robust measure of respiratory inflammation in COPD patients and can be done repeatedly within a patient's own home or in the clinic.

The potential benefits of improved advanced warning of an exacerbation are:

- An early warning or reassurance, and additional information to patients to manage their condition more efficiently. Experts suggest 20-40% patients struggle to recognise exacerbations.
- Help for healthcare professionals in planning and prioritising primary care/community service interventions in a more clinically- and cost-effective way.
- Prompts for patients to seek help earlier for exacerbations, especially for those who would otherwise seek help too late
- Reduction in unnecessary referrals to secondary care and hospital admissions
- Reduction in misuse of rescue packs, ambulance usage, and emergency presentations to A&E

Should this test be able to provide an early indication that an exacerbation is imminent, clinicians would have an opportunity to treat more patients in the community, reducing visits to hospital and emergency admissions. For the patient, access to this technology would result in a better quality of life by empowering the patient to take control in managing their own condition.

Before the test can be adopted (i.e. used in practice by patients) more diagnostic evidence is required, especially with regard to the performance of the test (sensitivity and specificity, negative and positive predictive value), usability data and realistic insights in health economics, care pathways and patient attitudes and concerns. The development of health economic models would provide robust evidence on clinical utility and cost-effectiveness of the test, data for which should be gained from a randomised clinical study where the benefits of early diagnosis would be demonstrated. Early models have been developed by the MedTech and In Vitro Diagnostics Co-operative (MIC) in Newcastle. Without these trials it would not be possible for such a test to be adopted, as sufficient evidence is essential to convincingly demonstrate benefits and to prove that the introduction of the test does not cause harm to the patient (e.g. anxiety or the intrusion of increased contact with the healthcare professionals or, the worst-case scenario, a missed exacerbation diagnosis leading to death.

8.5 Limitations and strengths

Prior to the observational study, the only clinical samples made available for this research were kindly donated via various clinical partners from sample banks populated by samples collected in clinical studies not designed with longitudinal collection or with appropriate frequency over sufficiently long timescales. It was, therefore, not possible to access samples gained from studies designed to test the core hypothesis that urinary biomarker profiles can predict or confirm exacerbation. This introduced bias to the some of the early analyses. For example, the UTI cohort consisted of female volunteers only, the healthy cohort patients were not age matched and, for the COPD and CF cohorts, any potential effects of treatments for COPD were not assessed as a potential confounding factors. These need to be taken into consideration upon reviewing the results, as some of the groups may not always be comparable.

A power calculation was conducted for the number of patients recruited into the observational study but sample size estimation was not taken into account for the other studies (this, of course, was limited by the availability of samples). The number of samples in the cohorts was deemed acceptable, based on previous calculations with >100 individual samples in the COPD ECLIPSE cohort, CF imperial study, CF QUB study, UTI Cardiff study. The COPD matched stable/exacerbation/recovery samples were from >50 patients in each of the COPD Birmingham, Leicester and Aeris cohorts. In these cases, the sub analyses did create smaller groups that were not always large enough to enable normally acceptable levels of significance to be reached.

Other methods of analysis for multiple comparisons would be more appropriate than what was used in chapter 3. A correction for multiple testing would have been more suitable and representative of the data.

For biomarker selections samples were tested from multiple cohorts (n=3). Final biomarker selection was based on results from all studies, to compensate for COPD heterogeneity. Although it was not possible to perform the same analyses for all individual studies, common biomarkers were found to be promising and robust.

8.6 Conclusion

Prior to the research described in this thesis, biomarkers involved in known biological pathways had been identified and quantified through studies undertaken by analysis of lung biopsies, sputum, BAL fluid and blood. However, this thesis describes the first investigation of a large panel of biomarkers detected in urine samples from subjects in various stages of COPD. This has provided new insights into the relevance and origin of the biomarkers. Prototype point-of-care tests were developed that

could be used routinely by patients in their own homes to monitor their inflammation status and predict pulmonary exacerbations. This was evaluated in a prospective observational study, results of which were used to develop a simple algorithm that showed the potential for differentiating between stable state and exacerbation events. Technology that enables patients with COPD to measure biomarker levels on a daily basis in the home would make it possible to harness the otherwise hidden time-course of the selected biomarker levels as the basis for diagnosis and prediction of exacerbation. The research described here is part and parcel of a major research programme carried out within the Mologic R&D group and constitutes investigations designed and directed by the author, and conclusions derived from the author's analysis of the data collected by the biomarker immunoassays. The findings constitute a key scientific foundation for a new approach to personalised medicine for COPD sufferers.

8.5 Future work

The findings and conclusions need to be comprehensively validated in line with medical device regulations and clinical best-practice before consideration for adoption. Other questions to be answered are:

- Is there evidence for renal response to circulating inflammatory agents, resulting in renal production of biomarkers?
- What are the factors that may influence trans-renal passage, or the renal production of biomarkers in response to circulating inflammatory mediators?
- What is the influence of co-morbidities and medications on biomarker baseline levels as well as any masking effect?

Further work needs to be done on COPD biomarkers where the origin and functionality is unknown. In order to determine the origin of the biomarkers that end-up in the urine and their association with the kidneys in particular, it will be necessary to use various complementary techniques such as immunoblotting, qRT-PCR, immunocytochemistry and *in situ* hybridisation. These techniques could be used to localise and quantify the selected biomarkers in biopsy samples. Immunoblotting and qRT-PCR using samples derived from kidney cell lines *in vitro* (podocyte, glomerular endothelial, tubular) would permit accurate quantification of relative expression at RNA and protein levels. Immunocytochemistry would enable localisation of proteins in clinical samples although not adequate for quantitation. *In situ* hybridisation would permit localisation of the mRNA in tissue and show whether the proteins in question are being synthesised at that site.

Development of a sophisticated algorithm that could learn the patients profile in real time and improve the prediction on a personalised level. This type of machine learning could also incorporate other factors such as comorbidities, treatment, gender, severity of disease and symptoms and make sense of noisy data.

Further investigation of desmosine and active MMPs, in particular MMP-9 and MMP12 would be worthwhile. Although these markers were not selected for further analysis due to the lack of evidence derived from the retrospective sample testing to support the usefulness of these markers in exacerbation, it could have been that these markers presented earlier than the day of diagnosis which could have been approximately 3 days post symptoms. The assays have been developed and are available to be incorporated in a study with more frequent testing leading up the exacerbation.

Other applications could be for other respiratory diseases such as CF. CF exacerbations are mainly caused by bacterial infections whereby COPD exacerbations are of bacterial and viral origin. Studies to date have been promising however, not as extensive investigated compared to COPD.

Further clinical trials are required to prove that that the test has the required diagnostic accuracy, demonstrate patient benefit and cost savings and help identify the best ways to integrate these products into existing NHS care pathways.

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Appendix

I. Development and validation of novel assays

I.i Desmosine

Recent advances in detection techniques have been focused on sophisticated laboratory methods, especially liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (125, 175) and high-performance capillary electrophoresis with laser-induced fluorescence (176). These techniques have allowed quantification of desmosine at a concentration as low as 0.1ng/ml in urine (177, 178) and enabled great progress in understanding desmosine as a means for identifying exacerbation and monitoring therapeutic intervention in COPD (97, 98). Previously reported immunoassays for desmosine and isodesmosine appear to have been based on antibodies with low affinity and low specificity. Although there are a few reports of enzyme immunoassay (EIA) and radioimmunoassay (RIA) methods (179, 180), they have not been widely adopted or validated against the reference standard LC-MS/MS methods.

The aim was to develop an EIA and a lateral flow assay (LF) assay for accurate quantification and high throughput testing of desmosine in urine samples in the laboratory, at the point of care (PoC) or in the home. Both the EIA and LF assays were configured in the competitive assay format, with an ovalbumin-desmosine conjugate presented on the solid-phase (as the capture reagent) and a tracer antibody attached to either an enzyme label (alkaline phosphatase) or particulate label (40nm gold particles). The particular technical challenges in developing these assays include a) poor immunogenicity of desmosine, even when conjugated as a hapten to an immunogenic carrier molecule (181), b) the close structural similarity of related collagen cross-linkers (isodesmosine, pyridinoline (PYD) and deoxypyridinoline (DPD)) which are also present in urine (182) c) the presence of desmosine as mixtures of free DES and DES containing peptides in test samples (extreme molecular heterogeneity) (182, 183) and d) the need for agreement between the immunoassay results and a reference isotope dilution LC-MS/MS assay (97, 182).

These challenges could not be overcome by manipulating the assay format or assay type but by seeking antibodies with optimum performance at the level of molecular recognition. The solution to the problem was, therefore, to generate and refine high-performing antibodies in terms of affinity and specificity.

I.i.i Materials and Methods

Antibody development and characterisation. Desmosine was conjugated to Keyhole limpet hemocyanin (KLH) as a carrier protein with glutaraldehyde as the cross-linking agent following standard, well-known procedures. The KLH-desmosine conjugate (2mg) was emulsified with 2ml

Freunds complete adjuvant and 2ml saline and injected subcutaneously into two sheep. The sheep were then boosted once a month for 4 months with 0.5mg of the KLH-desmosine conjugate in incomplete Freund's adjuvant and bled 2 weeks after each injection, according to normal, approved procedures. At week 32 sheep CF1316 was put on hold for 5 months, with no further booster injections until week 52 to allow the B-cell response to mature. At week 52, it was re-boosted with a new batch of KLH-desmosine (sonicated) in incomplete Freund's adjuvant. The cycle of immunisations was repeated using 0.1mg per injection.

Measurement of antibody titre by ELISA. The materials and reagents are described in an earlier section 2.2.4.4 The titre of the anti-desmosine antibody in the serum was measured by serial dilution of the serum in sample diluent (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20 and 1% (w/v) BSA) and evaluated using the following protocol. Diluted serum samples (100µl) were added to duplicate wells and incubated for 1 hour at room temperature with gentle agitation. Donkey anti-sheep IgG alkaline phosphate conjugate (Sigma, Cat No, A5187) was diluted 1 in 30,000 in the sample diluent and added to each microtitre well (100µl), incubated for 1hour with gentle agitation. After the final plate wash, the colorimetric detection step was initiated by the addition of 100µl of pNPP solution to each well. Once colour had been allowed to develop, the absorbance was measured at 405nm using an Omega plate reader. The sheep anti-desmosine CF1316 antibodies were sampled at various stages of the immunisation process to detect desmosine was tested at a dilution of 1 in 3200 over the 29 months.

Competitive EIA for urinary desmosine. As described in section 2.2.4.4.

Desmosine LF competitive immunoassay. Desmosine-ovalbumin conjugate was immobilised onto Sartorius CN140 nitrocellulose membrane at 1mg/ml using an Isoflow flatbed dispenser (Imagene Technology), dried in a tunnel dryer (Hedinair, UK) at 60°C and stored with desiccant prior to use. Affinity purified sheep anti-desmosine (CF1316) antibody was conjugated to 40nm gold colloid in a suspension buffer of 20mM borate pH9.3 to a final concentration of 15µg/ml. Following a 10min incubation, any unbound colloid was blocked with a final concentration of 2mg/ml BSA in PBS. The sheep anti-desmosine gold conjugate was sprayed onto Millipore GO41 glass fibre pads in a deposition buffer containing 3% (w/v) BSA, 5% (w/v) sucrose and 1% (v/v) Tween 20 using an Isoflow flatbed dispenser. The sprayed conjugate pads were dried in a tunnel dryer at 60°C and stored with desiccant prior to use. Both prepared membranes and conjugate pads were laminated and assembled into LF devices (VWR Cat No SLINM00810) according to an in-house protocol. Samples were diluted 1 in 5 in sample diluent (PBS, 0.1% (v/v) Tween20, 1% (w/v) BSA) and 80µl was applied

to the device. Following a 10min incubation the signals generated were quantified using a LF device reader (LFDR101; Forsite Diagnostics, York, UK).

Assay specificity. The specificity of both EIA and LF assays were evaluated by measuring the degree of cross-reactivity of 3 known cross reactive compounds, isodesmosine, PYD and DPD. Test samples were made with each compound covering a wide range of concentrations (0.05 – 500,000 ng/ml), including desmosine as the definitive analyte. The cross reactivity was determined by calculating the concentration required to generate a signal equivalent to 50% of the maximum desmosine signal when the back-ground signal was subtracted. For each compound, the 50% inhibitory concentration was then expressed as a percentage of the concentration of desmosine required to give a 50% signal reduction.

The Octet QK (ForteBio, CA, USA) system was used to measure the intermolecular binding between the antibody and the target compounds. The Octet instrument used streptavidin biosensors to immobilise the biotin-desmosine (Pierce EZ-link Sulfo-NHS-biotinylation kit Cat No 21425) to the tip of the biosensors enabling further interactions with the anti-desmosine antibody for binding measurements. Ligand/protein loading concentrations were optimized prior to this experiment to obtain affinity and kinetic measurements. Samples were dispensed into 96-well micro-titre plates (Greiner bio-one GmbH, Cat No 655209) at a volume of 200µl per well. The operating temperature was maintained at 30°C. Streptavidin-coated biosensor tips (Fortebio) were pre-wetted with kinetic buffer (Fortebio) for 20 minutes in order to establish a baseline. After a 60 second kinetic buffer wash step, biotinylated desmosine, PYD and DPD (10µg/ml) were contacted with the streptavidin sensors for 30 seconds. Subsequently the sensors were added to the wells containing anti-desmosine CF1316 at decreasing concentrations for kinetic measurements (association step -1300 seconds and dissociation step-1500 seconds).

Validation of assays with an isotope dilution LC-MS/MS reference method. Urine samples were analysed for desmosine by 3 methods – liquid chromatography tandem mass spectrometry (LC-MS/MS) chosen as the reference standard, and the 2 new methods (EIA and LF). Total urinary desmosine and isodesmosine were measured using a validated isotope dilution LC-MS/MS assay (125) with modifications described previously (178). The lower limit of quantification is 0.1ng/ml. Hydrolysed samples were analysed on the LC-MS/MS and EIA. Non-hydrolysed samples were analysed on both the EIA and LF assay. Creatinine measurements were obtained using a creatinine parameter assay kit from R&D systems (Cat KGE005).

Clinical validation of assays. Patients with acute exacerbations of COPD (AECOPD) admitted to 2 hospitals (Birmingham Heartlands Hospital and University Hospital Birmingham) were recruited

between September 2012 and January 2014. Patients were eligible for the study if they had (i) major symptom deterioration (reduced sputum volume, altered sputum colour, development of dyspnoea) for 2 or more consecutive days at home and (ii) documented clinical diagnosis of COPD. Patients were excluded if they had a documented history of cancer of the bronchus, interstitial lung disease, active pulmonary tuberculosis, pneumonia or any other severe disease likely to confound results. The study was approved by the local ethics committee and informed written consent was obtained within 24 hours of admission. Subjects underwent symptom and clinical assessment, completed the COPD assessment test, they were imaged by computed axial tomography (CAT), and urine samples were collected. Diary cards for symptoms were also given out at recruitment and post broncho-dilator spirometry was performed at day 56.

Refinement of antibodies to improve assay specificity. Epoxy-activated Sepharose 6B was obtained from GE Healthcare (cat No 17-7087-01). Samples of PYD and DPD were purchased from TLC Pharmachem (Cat No 1543-050A2 and 1543-048A2 respectively). The coupling buffer was (0.2M NaH2CO3 pH 9.0), the blocking buffer was (1.0M Ethanolamine pH 8.0), wash buffer A was (0.1M sodium acetate, 0.5M NaCl pH 4.0) and wash buffer B was (0.1M Tris 0.5M NaCl pH 8.0) The desmosine column was prepared previously using the same method as described below.

Preparation of PYD and DPD epoxy sepharose columns: The method of coupling followed standard protocols as recommended by the manufacturer. PYD or DPD (3mg in each case) were dissolved in coupling buffer and solutions were added to the epoxy sepharose gels prepared as instructed and mixed for 18 hours at 37°C. The coupling fluid was decanted off and the absorbance at 280nm was measured to gain an indication of the extent of coupling. The absorbance for both filtrates had reduced by about 50% suggesting that about 1.5mg of each had bound to the epoxy sepharose. After a wash and blocking step, the gels were allowed to stand overnight at room temperature and subsequently washed with buffer A, buffer B and finally, PBS wash before packing into columns. Each column was stored in PBS buffer with sodium azide.

Affinity purifications: For the initial experiments a simple antibody extraction/purification step was used, based on a desmosine affinity column to extract anti-desmosine antibodies from the whole anti-serum. This fraction is referred to as 'pre-purification' reagent hereafter. To further enhance specificity in subsequent experiments a multi-step refinement scheme (figure A) was used to produce the 'post-purification' reagent, starting with 1.2 µm pre-filtered whole anti-serum (20ml). This was passed through the PYD column as the first step. The fall- through was passed through the DPD column and, finally, the fall-through from that column was passed through the desmosine column to produce the fully refined antibody (post-purification reagent). This procedure was carried

out with an AKTA purification system. The antibodies were eluted from each column with 0.1M glycine pH 2.7, which was immediately neutralized with 60µl of 1M tris pH 9 per ml of sample before being dialyzed against PBS overnight. After dialysis, sodium azide was added to a final concentration of 0.05% (w/v) before concentration with a Vivaspin 6 (Sartorius AG). The refined antibody concentrations were determined in terms of absorbance at 280nm, assuming an extinction coefficient of 1.4 = 1mg/ml.

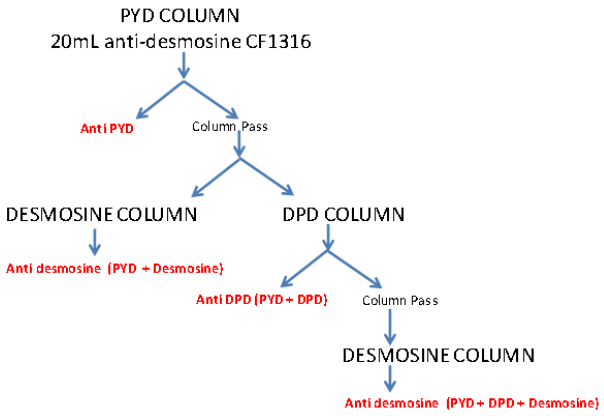


Figure A. Purification scheme for sheep anti desmosine CF1316, by sequential immunoadsorption on a set of antigen affinity columns

Evaluation of refined antibody in assays: EIA and LF assays were developed with the refined antibody and the specificity of the assays was established by testing these with desmosine, PYD and DPD as described above.

Statistical methods. All data were analysed using GraphPad Prism 5 (Graphpad, software, La Jolla, CA, USA). Data normality was identified and appropriate parametric or non-parametric tests chosen accordingly. Two tailed tests were used throughout. Mann-Whitney or students t-test were used to compare levels of urinary biomarker excretion, normalized to urinary creatinine. Significance was assumed if $p<0.05$.

I.i.ii Results

Antibody development and characterisation. The antibody titres from each test-bleed determined by EIA after a 1:3200 dilution are shown in figure B. Although some sheep produced higher affinity antibodies, the anti-serum from sheep CF1316 was selected on the basis of its performance in the immunoassays. High titres (signifying stronger B-lymphocyte responses and/or affinity maturation) started after week 52, following a beneficial holding period and repeated immunization. Increases in titre continued up to week 116.

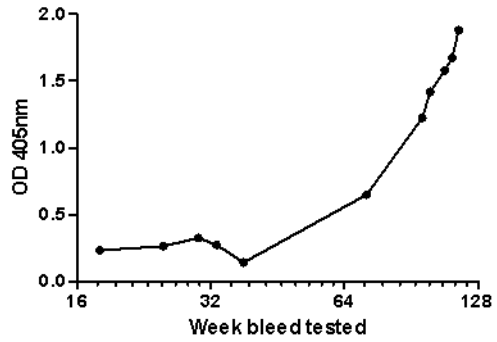


Figure B. The progression of antibody binding efficiency from Sheep anti desmosine CF1316 collected over 78 weeks of immunisations, with a 20-week rest after the 32-week booster.

Analytical validation. Both EIA and LF assays underwent rigorous testing to determine the lowest limit of detection (LLOD), spike recovery and linearity with urine samples and intra and inter-assay repeatability as described in the FDA Bioanalytical Method Validation Guidance for Industry. The typical calibration curves of both assays are depicted in figure C.

a) b)

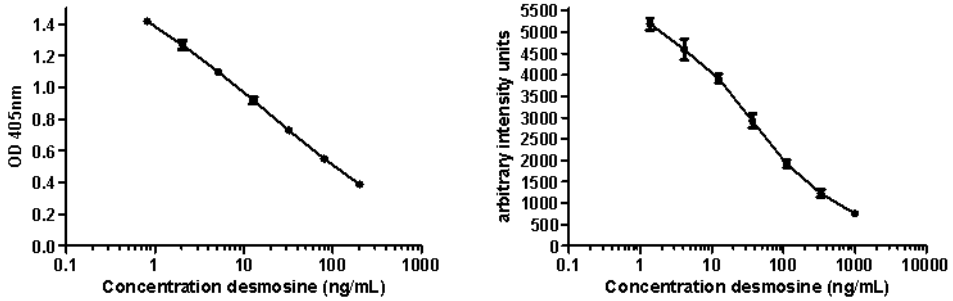


Figure C. Example desmosine calibration curves for (a) EIA; each data point represents the mean of replicate measurements (n=12) of each calibrator giving a curve fit (r^2) of 0.9995, (b) LF assay; each data point represents the mean of replicate measurements (n=12) with each standard with an r^2 of 0.9997.

LLOD: The lower limit of detection for the EIA was 0.82ng/ml with an upper limit of 200ng/ml. For the LF assay, the range was from 1.37ng/ml-1000ng/ml which fully covers the clinical range.

Spike recovery: Six urine samples spiked with 250ng/ml desmosine were diluted 1 in 5 in sample diluent. These were then run in the EIA to give percentage recoveries ranging from 79% to 120%, with an average recovery of 100.7%. For the LF assay, 3 urine samples spiked with 80ng/ml desmosine gave a percentage recovery range of 103-124% with an average of 116.7% all within the acceptable range of ±25%.

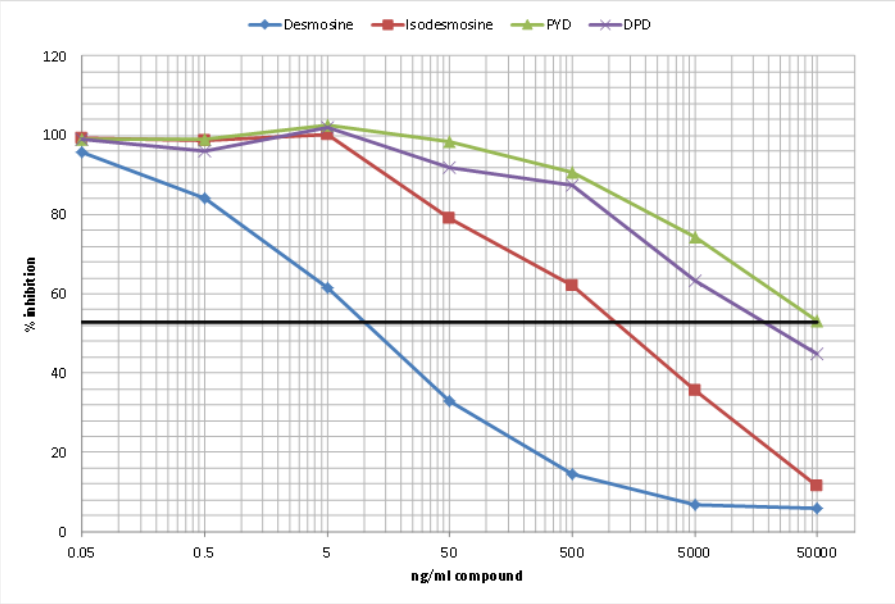
Linearity: Five urine samples with desmosine concentrations ranging from 583-710ng/ml were diluted 1 in 5, 1 in 10, 1 in 20 and 1 in 40 for the EIA linearity testing. The accuracy obtained for the 1 in 10 based on the concentrations determined from the 1 in 5 dilution ranged from 98-110%. For the 1 in 20 dilution, the range was 96-117%, for the 1 in 40 dilution it was 109-124%. For the LF assay, 4 urine samples with desmosine concentrations ranging from 668-802ng/ml were diluted 1 in 5 and 1 in 10. For the 1 in 10 dilution, the accuracy ranged from 80-94%, any greater dilution did not provide an acceptable accuracy.

Intra assay precision: For the EIA, 12 replicates of each standard ranging from 0.82ng/ml to 200ng/ml were run by one operator, the %CV obtained ranged from 1.4-3%. For the LF, 12 replicates of each standard ranging from 1.37ng/ml to 1000ng/ml produced %CV ranging from 4.1-8.9%. LF devices are prone to higher variability so it is expected to generate higher CV's then plate assays, but all CV's were within the acceptable specification of <20%.

Inter assay precision: One operator repeated the EIA assay on 3 different plates (12 replicates for each standard ranging from 0.82ng/ml to 200ng/ml). For plate 1, the %CV ranged from 3.3-7.7%, plate 2, 3.2-9.4% and for plate 3, 4.0-7.1%, all below acceptable level of 10%. The standard curve fit (r^2) for each plate was 0.9980, 0.9983 and 0.9986 respectively. For the LF assay, three separate batches were prepared, and 10 replicates of each standard were run ranging from 1.37ng/ml to 1000ng/ml, the resulting %CV for batch 1 was 4.7-8.1%, for batch 2, 3.8-11.7% and for batch 3, 3.2-8.9%. The r^2 for each batch was 0.9995, 0.9993 and 0.9940 respectively. Satisfactory precision results were obtained for both assays.

Assay specificity: Specificity of the antibody was evaluated in both the EIA and the LF assay. Figure D displays the amounts of interfering substances needed to achieve 50% of the uninhibited signal in the desmosine assays, expressed as concentrations (ng/ml). The cross reactivity of the EIA and LF to isodesmosine was 0.89 and 1.33% respectively; this is of no practical consequence for diagnosis, as the molecule fulfils essentially the same biochemical role as its isomer, desmosine. Both occur as cross-linking molecules in elastin and are effectively identical biomarkers. Actual cross-reactivities to PYD and DPD in the EIA assay were 0.02 and 0.06% respectively and the LF assay cross reactivity was 0.86 and 1.40%, respectively.

a)



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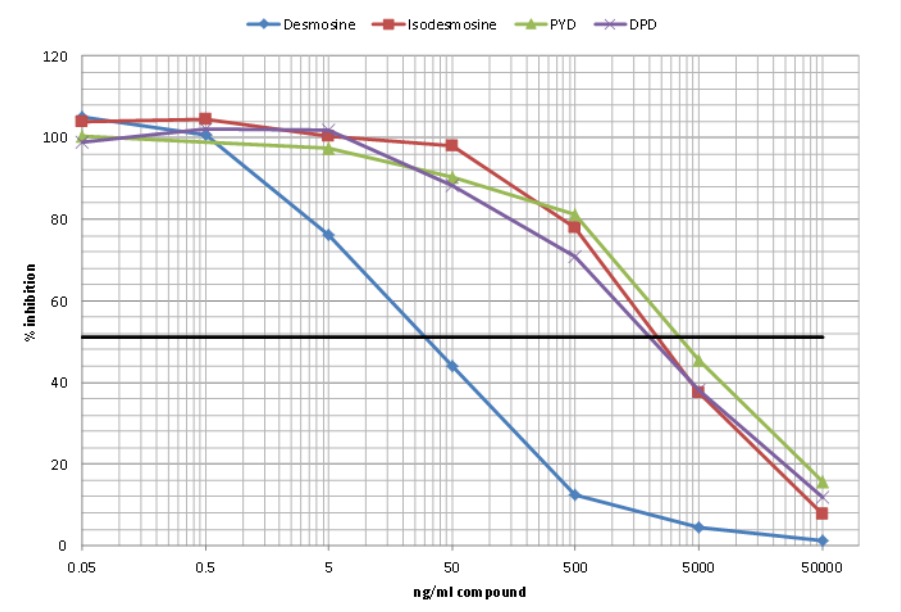
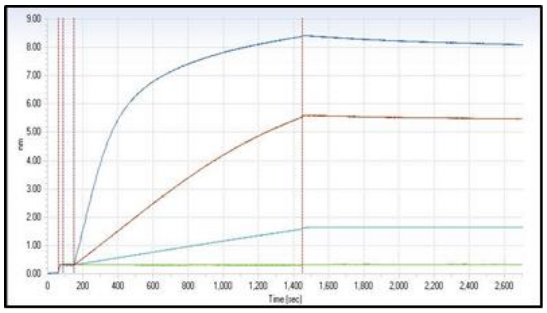


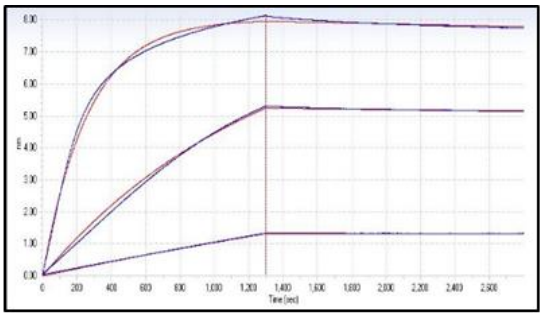
Figure D. Cross reactivity with PYD and DPD. Concentrations at 50% desmosine inhibition for each compound were determined from the graphs above and used to calculate the percentage cross reactivity (a) EIA 50% B/B0 concentration for desmosine, isodesmosine, PYD and DPD were 10, 1125, 50000 and 17500ng/ml respectively (b) LF 50% B/B0 concentration for desmosine, isodesmosine, PYD and DPD were 30, 2250, 3500 and 2000ng/ml respectively.

Further investigations of the antibody affinity and specificity were carried out by determining the binding kinetics with the Fortebio Octet biosensor. Figure Ea is the generated kinetic sensorgram for biotinylated desmosine loading (10µg/ml) to a specified biosensor with an on-rate (association) and off-rate (dissociation) binding to the antibody at various dilutions (7.5µg/ml, 1.5µg/ml and 0.3µg/ml). The raw data were processed to fit a 1:1 binding model to extract kinetics and affinity measurements (see figure Eb). The measured kinetic rates and affinities of streptavidin sensors immobilized with biotinylated desmosine, PYD and DPD resulted with on-rates of 75460 M-1 s-1, 24920 M-1 s-1 and 23740 M-1 s-1, off-rates of 1.458 x 10-5 s-1, 1.38 x 10-5 s-1 and 8.495 x 10-6 s-1 and affinity (equilibrium dissociation constants) of 19.33nM, 5.54nM and 3.58nM. These results are summarized in figure 5c. The affinity KD for desmosine was 1.9 x 10-10 which is a 28.6 fold increase over the affinity for PYD and an 18.5 fold increase over that for DPD. The results suggest that assay format (including the antigen conjugation) and procedure had a significant impact on the specificity as demonstrated by the negligible cross reactivity observed with the immunoassays.

a)



b)



c)

CF1316 (Anti Desmosine)	$K_{association}$ ($M^{-1}s^{-1}$)	$K_{dissociation}$ (s^{-1})	Affinity Kd (M)
Desmosine Biotin	7.546×10^{-4}	1.458×10^{-5}	1.933×10^{-10}
PYD Biotin	2.492×10^{-4}	1.3800×10^{-5}	5.54×10^{-9}
DPD Biotin	2.374×10^{-4}	8.495×10^{-6}	3.58×10^{-9}

Figure E. Binding affinities of anti-desmosine. a) typical raw data sensorgram collected from protein/ligand binding experiment. A sensorgram is the kinetic profile of biotinylated desmosine 10µg/ml immobilised on streptavidin sensors measuring on-rates (association) and off-rates (dissociation) of antibodies with gradient dilutions of 7.5µg/ml, 1.5µg/ml and 0.3µg/ml (top to bottom), b) example of processed data analysed to 1:1 fitting (red line). c) summary kinetic values (Kdissociation, Kassosication) and affinity (equilibrium dissociation, KD)

Validation of EIA and LF assays with the LC-MS/MS reference method. Urine samples were analysed for desmosine by 3 methods, isotope dilution LC-MS/MS (175) (chosen as the reference standard) and the two new immunoassays. Hydrolysed and non-hydrolysed samples were run for comparison in the EIA, but only non-hydrolysed samples were run on the LF tests due to limited sample volumes (Table A). Both the EIA and LF values were strongly correlated with the LC-MS/MS results. The best correlation to the reference assay was observed using the EIA with non-hydrolysed samples (Spearman's rank = 0.84, p = <0.0001) whereas hydrolysed samples had a lower correlation

(Spearman's rank = 0.79, p = <0.0001). The LF (non-hydrolysed samples) gave a Spearman's rank correlation with the LC-MS/MS and EIA (non-hydrolysed samples) of 0.78 and 0.72 respectively.

Table A. Desmosine measured in urine samples from COPD patients and healthy individuals.

	COPD		**Healthy**		**Mann-Whitney**
	Median	IQR	Median	IQR	p value
LF (non-hydrolysed)	47.8	(25.1-80.0)	28.0	(23.1-34.6)	0.0040
EIA (non-hydrolysed)	20.0	(6.5-43.0)	4.6	(2.7-6.7)	<0.0001
EIA (hydrolysed)	4.4	(1.4-9.5)	0.1	(0.1-0.4)	<0.0001
LC-MS/MS (hydrolysed)	16.4	(9.5-27.3)	5.5	(3.9-11.4)	<0.0001

PYD and DPD concentrations in the samples were measured with the LC-MS/MS giving levels ranging from 15-763ng/ml PYD and 2-288ng/ml DPD. The impact of these on the correlation was analysed by excluding those samples with high background values of PYD and DPD as measured by LC-MS/MS. Of the 120 clinical samples, 9 were found to have both PYD and DPD at elevated concentrations (PYD over 400ng/ml and DPD over 50ng/ml). A further 18 were found to have just DPD elevated above 50ng/ml. When these samples were removed from the data set, the correlations between LC-MS/MS and EIA were re-calculated to give Spearman's rank values that were negligibly different from that of the complete set for the EIA (from 0.84 to 0.86). However, a significant improvement was found in the correlation of the LF assay results, with an increase in spearman's rank from 0.78 to 0.85, making it comparable to the EIA. This is, consistent with the observation that the LF assay is more prone to cross-reactivity than the EIA with this antibody and assay format.

Clinical validation of assays. The 30 COPD patients who all donated samples at day 0 (exacerbation), were 53% male (16/30), had an average age of 67.27, median of 60 pack years (IQR 33.8-93.8), and FEV1 of 1.05 (IQR 0.76-1.36). The 20 healthy controls were mostly male (16/20; 80%) and had a mean age of 38.6 (range 22-67). Two were known to have well controlled asthma, and 1 was a current smoker. Statistically significant differences were observed between desmosine levels in urine samples from COPD patients and samples from healthy volunteers.

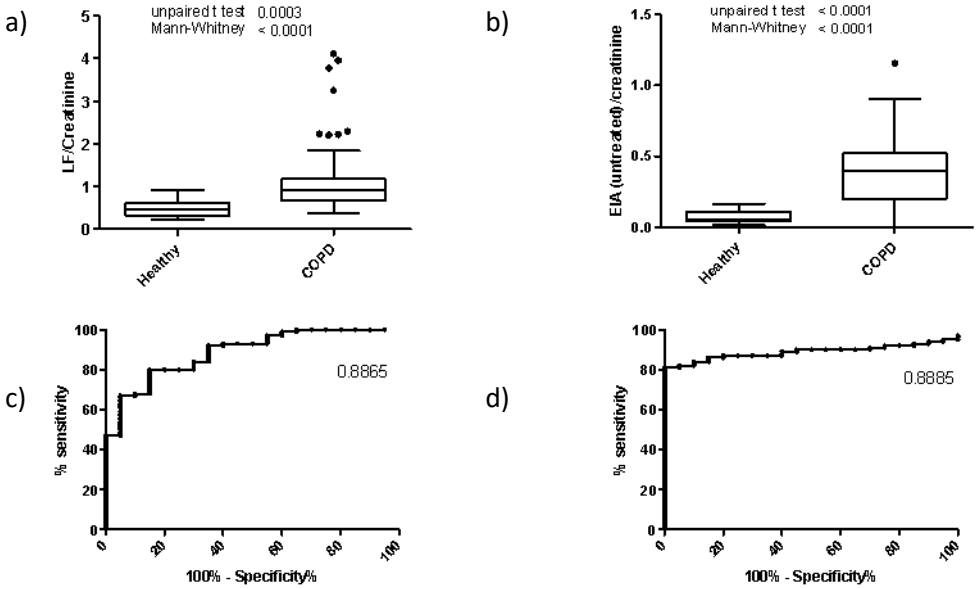


Figure F. Analysis of healthy (n=20) and COPD (n=100) groups for desmosine/creatinine ratios by 2 statistical tests; unpaired t-test Mann-Whitney test a) LF and b) EIA (both using non-hydrolysed samples) and ROC procedures c) LF and d) EIA (non-hydrolysed samples)

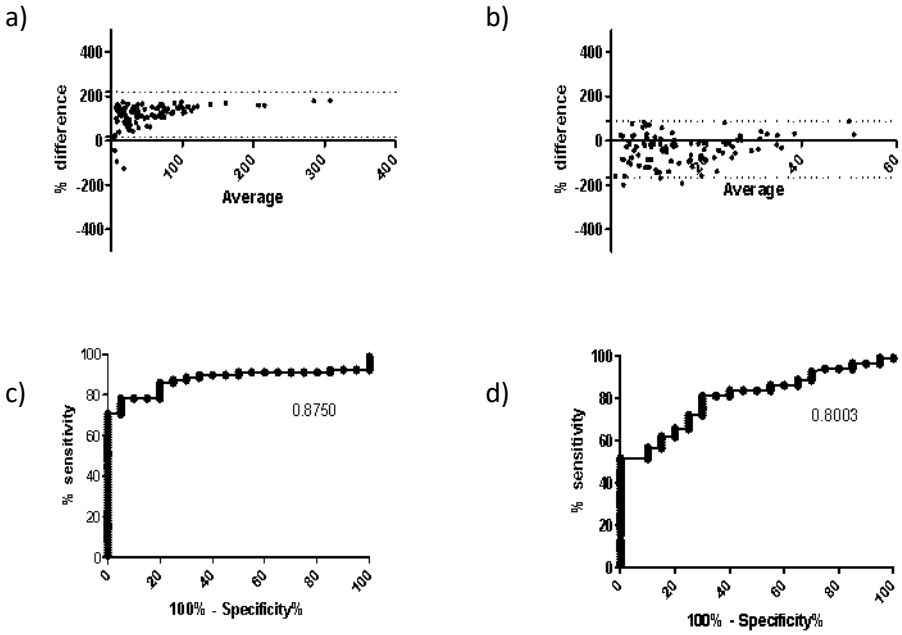


Figure G. Bland-Altman plots for LF determination of desmosine compared to standard method LC-MS/MS a) pre-refined and b) post-refined and ROC analysis between samples from healthy individuals and COPD patients c) pre-refined and d) post-refined

Refinement of assays to improve specificity. The specificity of the new antibody (pre and post purification by affinity separation) was evaluated in both the EIA and the LF assay with all 4 compounds, desmosine, isodesmosine, PYD and DPD. For the EIA version minimal improvement was observed. The PYD cross-reactivity changed from 0.033% to <0.01% (pre- to post-purification) and for DPD 0.10% to <0.01%. The pre-purified antibody LF version cross reacted with PYD and DPD at 0.56 and 1.27% respectively, but this interference was removed by the use of the refined antibodies (0.01% for both) which is consistent with the tendency for the LF assays to be more susceptible to antibody cross reactivity. LF assays and EIAs using the pre-purified and post-purified reagents were re-tested with 98 and 90 samples respectively from the original study. Prior to the refinement, a mean bias of 117.5% was observed in LF when compared to the isotope dilution LC-MS/MS method, with 95% confidence interval of limits of agreement being 16.6-218.4% (figure G). After refinement, the mean biases improved to -39% with 95% confidence interval of limits of agreement of -166.1-87.99%. The refinement did not significantly change the diagnostic performance.

In EIA (figure H), a similar bias was found in the pre-refined assay and improvement was observed with the refined assay bias from 45.8 to -18.3%. 95% confidence intervals of -69.3-160.8 was observed for the pre-refined compared to -127.9-91.2 for the post-refined. Similarly, the diagnostic performance was not significantly changed when comparing samples collected from healthy and COPD individuals.

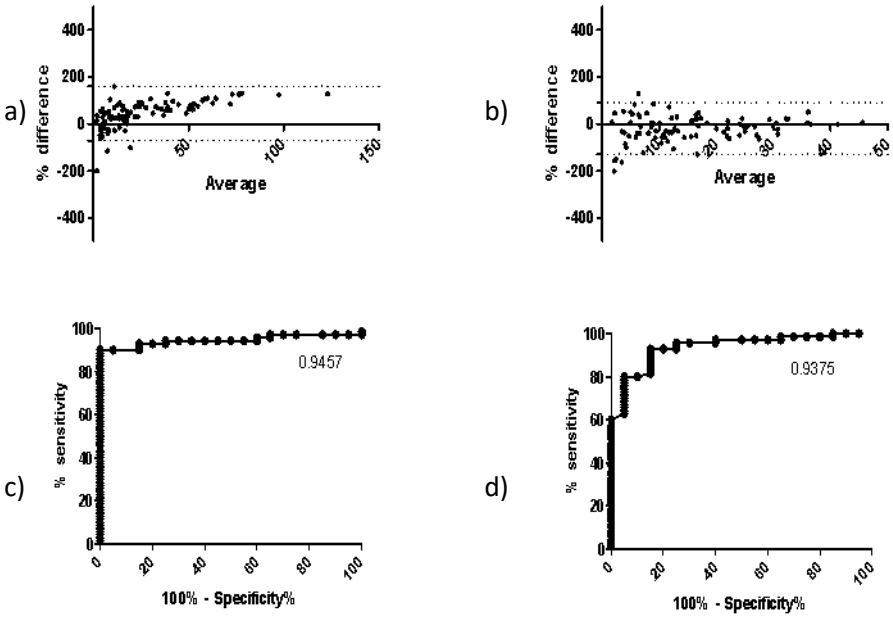


Figure H. Bland-Altman plots for EIA compared to LC-MS/MS a) pre-refined and b) post-refined and ROC procedures between healthy and COPD samples c) pre-refined and d) post-refined

I.i.iii Discussion

The heterogeneity of DES and DES-containing peptides in urine is an important factor affecting any immunoassay aimed at testing fresh, unmodified urine samples in the home or at the point of care. Currently, assays used in the laboratory for desmosine are thought to only detect the free, unattached desmosine. To estimate total elastin degradation, urine samples are generally pre-treated with an extended, aggressive acid hydrolysis at 108°C (lasting between 12 and 48 hours) to release desmosine from all the peptide forms before analysis by LC-MS/MS (97). The overall process is very slow and laborious but highly accurate due to the use of stable isotope dilution, such that it is considered as a reference assay in this study (184).

When both EIA and LF assays were evaluated in this study, it was found that intra assay, inter assay repeatability, linearity, and spike recovery all met the required acceptance criteria of FDA guidelines. The assays correlated well with the reference LC-MS/MS assay, with a Spearman's rank coefficient of 0.84 for the EIA and 0.78 for the LF test. The concentration ranges varied between the assays and, in particular, between hydrolysed and non-hydrolysed samples as measured by the EIA with a median of 6.2ng/ml and 26ng/ml respectively. This potential underestimation in hydrolysed samples could be due to differences in sample preparation prior to running the assays. Both EIA and LF (non-hydrolysed) provided higher median values than the LC-MS/MS (hydrolysed), the differences between assays could be influenced by increased susceptibility to cross reactivity. The correlation between the LF and the LC-MS/MS was improved with the omission of samples containing high levels of PYD and DPD supporting the theory that cross reactivity is responsible for this and the overestimated values generated, particularly with the LF assay. Alternatively, there are likely to be differential effects of desmosine attached to residual "stubs" of fragmented elastin. Other EIA assays previously developed have suffered from cross reactivity to isodesmosine up to 45% (185) and to PYD up to 20% (186). The EIA used in this study was based upon a polyclonal antibody with sufficient affinity and specificity, raised in sheep in response to a synthetic immunogen derived from desmosine, following a well-defined immunization protocol for difficult antigens. Although better correlation has previously been reported between EIA and HPLC (187), the samples used were aortic tissue samples and limited comparative data is available with urine. Moreover, other EIAs previously developed for urine used hydrolysed samples (133).

The EIA of this study was developed for use with non-hydrolysed urine samples, which is a 24-hour process. The LF version further reduced the assay time to just 10 minutes, without compromising the differentiation between COPD patients and healthy individuals (AUC=0.80-0.88). The ability to identify and quantify the presence of free desmosine, as well as desmosine attached to peptide

stubs in untreated urine samples, provides a step-change improvement in the value of desmosine as biomarker of inflammatory damage to tissues in which elastin is present.

The results show that extensive refinement of anti-DES antibody improved its specificity in the EIA and LF assay formats.

I.ii Measurement of MMP activity (ELTABA)

Mologic's "ELTABA" technology (enzyme linked transformation affinity binding assay) was designed to detect the protease activity "footprint" of neutrophil leukocytes. The test detects the combined enzyme **activity** of enzymes secreted by neutrophils (and others) - matrix metalloproteinases (MMPs) and neutrophil elastase (HNE) - rather than just concentration of the enzyme molecules. Three ELTABA platforms are described, each of which can be modified to measure other enzymatic activity (within limits):

- ELTABA: the 1st test developed by Mologic which was a 'negative'-read test for measuring the composite activity of MMPs and HNE. This test was developed to measure the enzyme activity in wound fluid.
- Reverse ELTABA, the 2nd test developed: a 'positive' read derivative of the original version (above). It is a more sensitive assay than ELTABA but requires a more labour-intensive sampling procedure.
- Ultimate ELTABA: an ultra-sensitive 'positive' read test for active MMPs only, this test was developed specifically to measure low concentrations of MMPs in urine.

I.ii.i ELTABA

Instead of detecting the enzyme molecules themselves, the assay detects a specially designed indicator peptide that is acted upon (cleaved) by the enzymes. The test is not specific to HNE and MMP-9 but has a strong bias towards them. This means that high concentrations of active MMP 2, for example, will also be detected. The procedure requires a short pre-incubation step of 10 minutes with the sample to allow enzyme digestion to take place. The presence of protease activity above a particular threshold results in the formation of a single visible red line (indicating the presence of significant neutrophil infiltration), while the absence of excessive neutrophil activity results in the formation of two visible red lines. The two lines appear only when the protease activity in the sample is below the detection threshold.

The principles underlying the Mologic ELTABA protease assay are set out in the diagrams and descriptions below. The indicator molecule (peptide) contains two binding domains and a region containing an amino sequence cleavable by the relevant proteases, as shown in figure I.

One of the two binding domains is recognised by a capture molecule immobilised on the test line of a lateral flow test strip, while the other is recognised by a binding partner molecule carried on gold particles. When the protease activity is low, most of the indicator peptide molecules remain intact, so enabling the formation of two lines to give reassurance that all is well with the proteolytic enzyme

balance and, hence, there being no serious infection or inflammation (Figure IA). Proteolytic enzymes in test samples with excessive proteolytic activity can cleave the peptide, so destroying the ability of the indicator molecule to form a bridge between the antibodies on the gold particles and those on the test line (Figure IB). Thus, the formation of a single line indicates the presence of a damaging neutrophil infiltration. This relationship between result and clinical condition is intuitive, for it means that a two-line result is good and just one line is bad.

I.ii.ii Reverse ELTABA

Reverse ELTABA on the other hand, gives a "positive read" (strong test line) for a positive sample (i.e. high proteolytic activity) and a "negative" (absent test line) for a negative sample (low proteolytic activity).

- In samples with normal levels of protease activity the peptide remains intact and the test line is absent.
- In samples with high protease activity the peptide is degraded, which results in the appearance of an easily visible TEST line.
- The peptide used for Reverse ELTABA the same as that used for the original ELTABA format, but it is pre-complexed with polystreptavidin (PSA).

One of the three binding domains (1st) is recognised by Pre-Absorption lines contained in a hidden capture zone, a second is recognised by a capture molecule immobilised on the test line (BSA-Biotin) of a lateral flow test strip and a third is recognised by a binding partner molecule carried on gold particles (biotin gold). When the protease activity is low, most of the indicator peptide molecules remain intact. These intact indicator molecules are captured in the hidden capture zone, resulting in an absent test line. Proteolytic enzymes in test samples with excessive proteolytic activity can cleave the peptide, releasing the PSA with the 2 epitopes to form a bridge between the antibodies on the gold particles and those on the test line (Figure JD)
Thus, the formation of a single line indicates the absence of a damaging neutrophil infiltration, and two lines indicate the presence of a damaging (pathogenic) degree of neutrophil infiltration.

I.ii.iii Ultimate ELTABA

This 'positive' read assay depends on a unique antibody that recognizes a cryptic epitope exposed only once the peptide has been cleaved by the target enzyme(s). The Ultimate ELTABA antibody was raised in response to an immunogen derived from the cleaved indicator peptide (stub). For the assay the antibody is labelled with gold particles as the visible indicator.

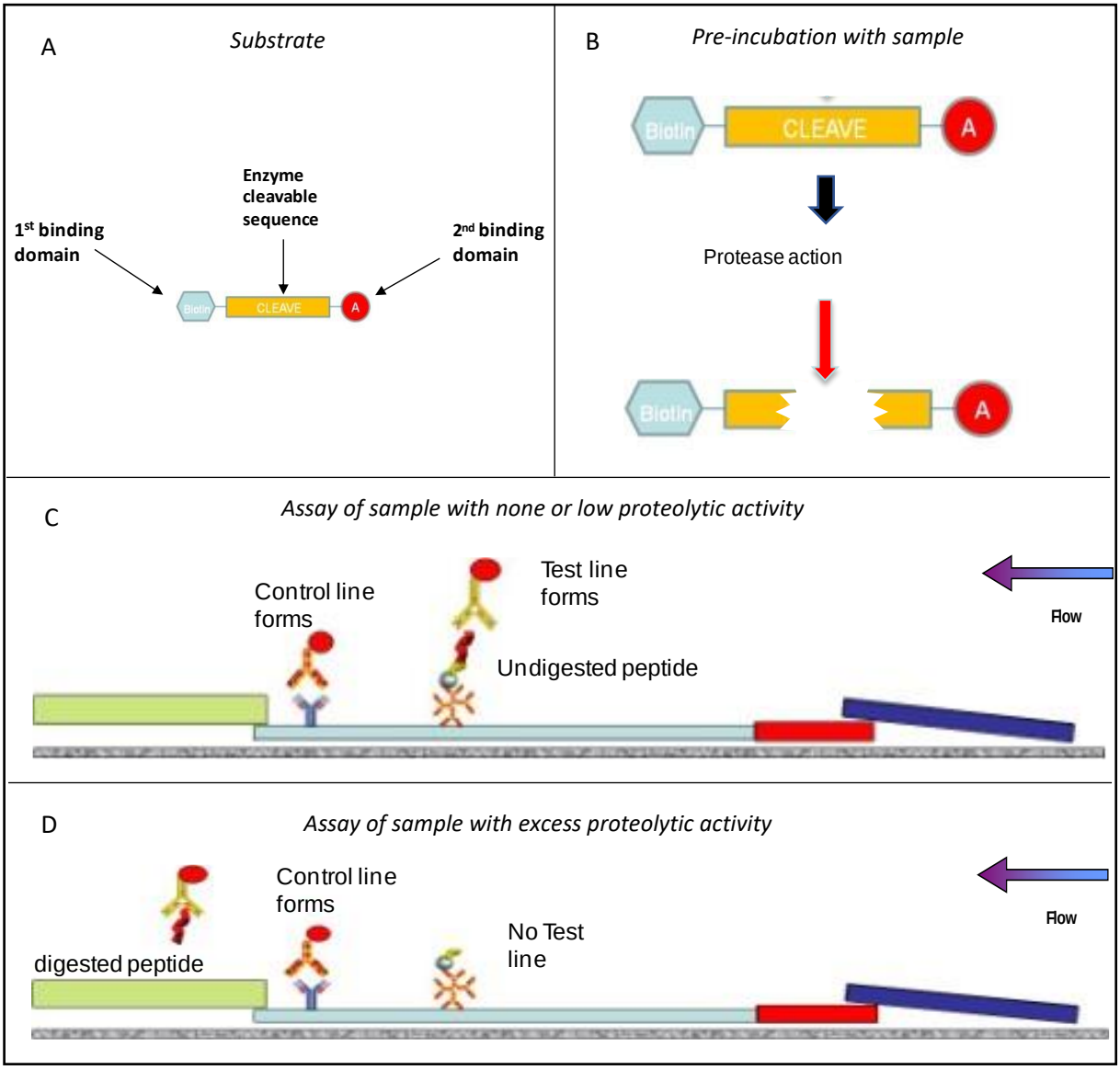


Figure I. Principle of the ELTABA MMP/HNE activity test. A) diagrammatic representation of the ELTABA indicator peptide, 1st binding domain binds to the immobilised streptavidin test line and 2nd binding domain binds to the antibody conjugated to gold B) The effect of a relevant protease on the ELTABA indicator peptide. Note that the two binding domains become separated by cleavage of the cleavable sequence. C) Diagrammatic representation of an ELTABA test on a healthy sample with a low level of relevant protease, the intact peptide remains resulting in the formation of two lines – a test line and a control line D) Diagrammatic representation of an ELTABA test on a unhealthy sample with a high level of relevant protease, the peptide sandwich is not able to form, resulting in the formation of only 1 line – the control line.

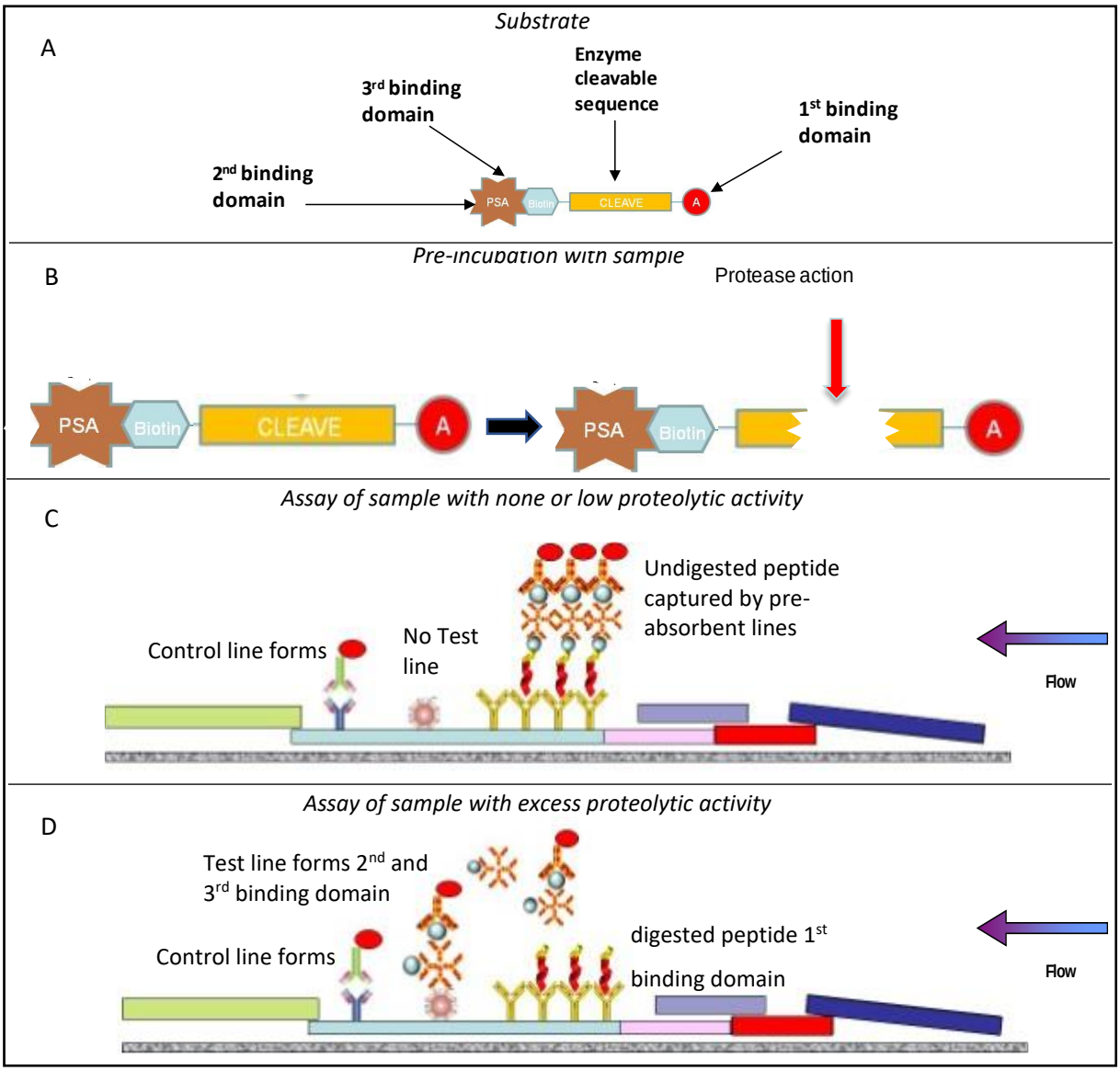


Figure J. Principle of the Reverse ELTABA MMP/HNE activity test. A) diagrammatic representation of the Reverse ELTABA indicator peptide B) The effect of a relevant protease on the indicator peptide. Note that the two binding domains become separated by cleavage of the cleavable sequence. C) Diagrammatic representation of the Reverse ELTABA test on a sample with a low level of relevant protease, resulting in the disappearance of the test line. A "test complete" or control line is included on the strip in the usual way D) Diagrammatic representation of the Reverse ELTABA test on a sample with a high level of relevant protease, resulting in the appearance of the test line. A "test complete" or control line is included on the strip in the usual way.

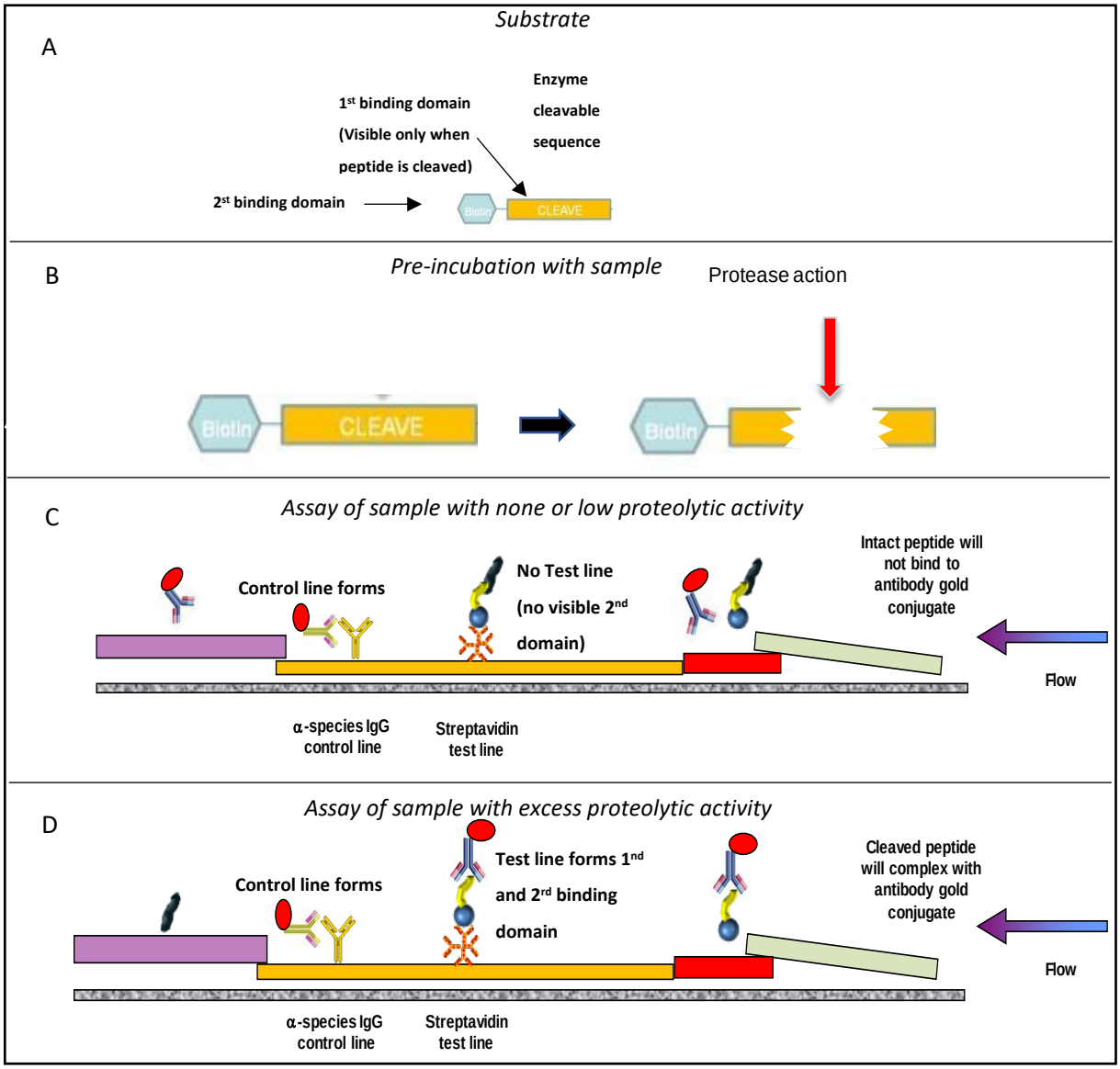


Figure K. Principle of the Ultimate ELTABA MMP activity test. A) diagrammatic representation of the Ultimate ELTABA indicator peptide B) The effect of a relevant protease on the indicator peptide. Note that the two binding domains become separated by cleavage of the cleavable sequence. C) Diagrammatic representation of the Ultimate ELTABA test on a sample with a low level of relevant protease, resulting in the disappearance of the test line. A "test complete" or control line is included on the strip in the usual way D) Diagrammatic representation of the Ultimate ELTABA test on a sample with a high level of relevant protease, resulting in the appearance of the test line. A "test complete" or control line is included on the strip in the usual way.

I.ii.iv Materials and Methods

Disposable 96-well polystyrene plates were obtained from Nunc (Maxisorp™ flat bottomed) or 96 well plate coated with PSA (Nunc, 442404). MMP-9 was supplied by Alere San Diego and activated with APMA. Anti-cleaved stub antibodies were obtained from sheep immunised with various peptides covalently attached (via glutaraldehyde cross-linking) to the carrier keyhole limpet hemocyanin (KLH) using same method as used for desmosine described above. Peptide stubs for immunisations and peptides for assay were synthesised at Mologic. The sheep Anti-cleaved stub serum was affinity purified against solid-phase immobilised peptides. The affinity-purified antibody fraction was subsequently conjugated to alkaline phosphatase (AP) using kits supplied by Innova biosciences (cat No. 702-0010). Donkey anti-sheep alkaline phosphatase was supplied by Sigma (Cat No, A5187). pNPP substrate solution was obtained from Sigma (Cat No N2765). MMP buffer (Aq. Solution of 50mM Tris, 100mM sodium chloride, 10mM Calcium Chloride, 50µM 20mM zinc chloride, 0.025% Brij 35, 0.05% sodium azide at pH 8.0). Wash buffer for plate assay (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20).

Antibody development and characterisation. Antibodies were generated to recognise a cleaved peptide sequence, the designed peptide sequence GPQGIFGQ, a target for MMP digestion is known to be cleaved between G and I, however, it was unknown if the cleaved stub GPQG or IFGQ was more immunogenic. It was also unknown whether it was better to immunise with a short peptide consisting of 4 amino acids or if it would be better to immunise with a longer peptide with the required sequence exposed. Four peptides were designed, prepared and conjugated to KLH as shown in table B for immunisations in sheep.

Table B. Details of the immunogens (sequence and ID), the sheep ID the peptides that were used to assess the titres of the antibodies.

Immunogen sequence	Immunogen Name	Sheep ID	Peptide name	Peptide sequence
KLH-CGPQG	MOL223	CF1532/CF1533	A3	B-GPQG
IFGQC-KLH	MOL224	CF1520/CF1521	A1	IFGQ-B
IFGQGPQGC-KLH	MOL225	CF1522/CF1523	A2	IFGQGPQG -B
KLH-CIFGQGPQG	MOL226	CF1524/CF1525	A4	B-IFGQGPQG

The titre of the anti-cleaved stub antibody in the serum was measured by serial dilution of the serum in sample diluent (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20 and 1% (w/v) BSA) and evaluated in a plate assay using the following protocol.

Each peptide A1-A4 (1µg/ml in PBS) was added to a 96 well plate coated with polystreptavidin (Nunc, 442404) 100µL per well, and incubated for 1 hour at room temperature with gentle agitation. The sensitised-well surfaces were blocked after the plates had been washed 3 times with wash buffer (50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20). Wash steps were carried out between the blocking step and each of the incubation steps. The blocking buffer consisted of 50mM tris buffered saline pH8, supplemented with 0.1% (v/v) Tween20 and 1% (w/v) BSA, which was left in place for 1hour at room temperature. Diluted serum samples (100µl) were added to duplicate wells and incubated for 1hour at room temperature with gentle agitation. Donkey anti-sheep IgG alkaline phosphate conjugate was diluted 1 in 30,000 in the sample diluent and added to each microtitre well (100µl), incubated for 1hour with gentle agitation. After the final plate wash, the colorimetric detection step was initiated by the addition of 100µl of pNPP solution to each well. Once colour had been allowed to develop, the absorbance was measured at 405nm using an Omega plate reader (BMG labtech, UK). Selected antibodies were affinity purified using the specific peptides they were raised and conjugated to AP against and then analysed by ELISA to determine the most appropriate assay format to give the best sensitivity.

Assay format development*:* Peptides containing the cleavable sequence (GPQGIFGQ) were synthesised with a biotin or Pegylated biotin attached to either the C-terminus (MOL038 and PCL008-A2 respectively) or the N-terminus (MOL310 and MOL378 respectively).

Table C. Details of the peptides, sequence and ID. Two peptides have been designed with each one having an alternative form with a PEG linker. Added to the 8AA sequence is the ALP sequence. Antibodies are available at Mologic that recognise the ALP sequence.

Peptide ID	Sequence
MOL038	Biotin-GPQGIFGQESIRLPGCPRGVNPVVS
PCL008-A2	Biotin-PEG-Asp -AEEAc-AEEAc- GPQGIFGQESIRLPGCPRGVNPVVS
MOL310	SIRLPGCPRGVNPVVSGPQGIFGQ- Biotin
MOL378	SIRLPGCPRGVNPVVSGPQGIFGQ-AEEAc-AEEAc- PEG-Asp Biotin

The peptide can be anchored to a solid phase either by binding to a streptavidin capture via the biotin or to sheep antibody CF1060 capture via the ALP sequence, the proposed formats described in figure L were evaluated using the following protocol.

Ultimate ELTABA Plate assay. For ALP binding to the plate, microtitre plates were sensitised overnight with 100µl per well of anti-ALP affinity purified antibody (CF1060) at 1µg/ml in PBS. The sensitised-well surfaces were blocked after the plates had been washed 3 times with wash buffer. An additional wash step was required to remove the blocking buffer before use. For biotin binding to the plate, 96 well plate coated with polystreptavidin (Nunc, 442404) were used. Active MMP-9 was diluted in MMP buffer to give concentrations between 39 and 2000ng/ml (or as required) to generate the standard curve. Peptide was incubated with the standard or undiluted urine sample for 30 minutes, at the end of the incubation period, 100µl was added per well and incubated for a further 1hour at ambient where the peptides were immobilized by the streptavidin or CF1060 bound to the plate. After a subsequent wash step, each sheep antibody conjugated to Alkaline phosphatase were added at a dilution of 1/500 (100µl/well) and incubated for 1 hour at ambient. After the final plate wash, the colour reaction was initiated with the addition of 100µL of pNPP solution to each well. Once colour had been allowed to develop, the absorbance was measured at 405nm using an Omega plate reader and the standard curve was approximated in a sigmoid 4 parameter logistic model.

Ultimate ELTABA Lateral flow assay. Antibody CF1060 or polystreptavidin was immobilised onto Sartorius CN140 nitrocellulose membrane at 1mg/ml using an Isoflow flatbed dispenser (Imagene Technology), dried in a tunnel dryer (Hedinair, UK) at 60°C and stored with desiccant prior to use. Affinity purified sheep anti-cleaved stub antibody was conjugated to 40nm gold colloid in the optimal suspension buffer. Following a 10min incubation, any unbound colloid was blocked with a final concentration of 2mg/ml BSA in PBS. The sheep anti-cleaved stub gold conjugate was sprayed onto Millipore GO41 glass fibre pads in a deposition buffer containing 3% (w/v) BSA, 5% (w/v) sucrose and 1% (v/v) Tween 20 using an Isoflow flatbed dispenser. The sprayed conjugate pads were dried in a tunnel dryer at 60°C and stored with desiccant prior to use. Both prepared membranes and conjugate pads were laminated and assembled into LF devices (VWR Cat No SLINM00810) according to an in-house protocol. Active MMP-9 was diluted in MMP buffer to give concentrations between 8 and 500ng/ml (or as required) to generate the standard curve. 12.5µl volumes of peptide at 2µg/ml was incubated with 80µl of standard or undiluted urine sample for 10 min, at the end of the incubation period, 87µl was added per well. Following a 10 min incubation the signals generated were quantified using a LF device reader (LFDR101; Forsite Diagnostics, York, UK).

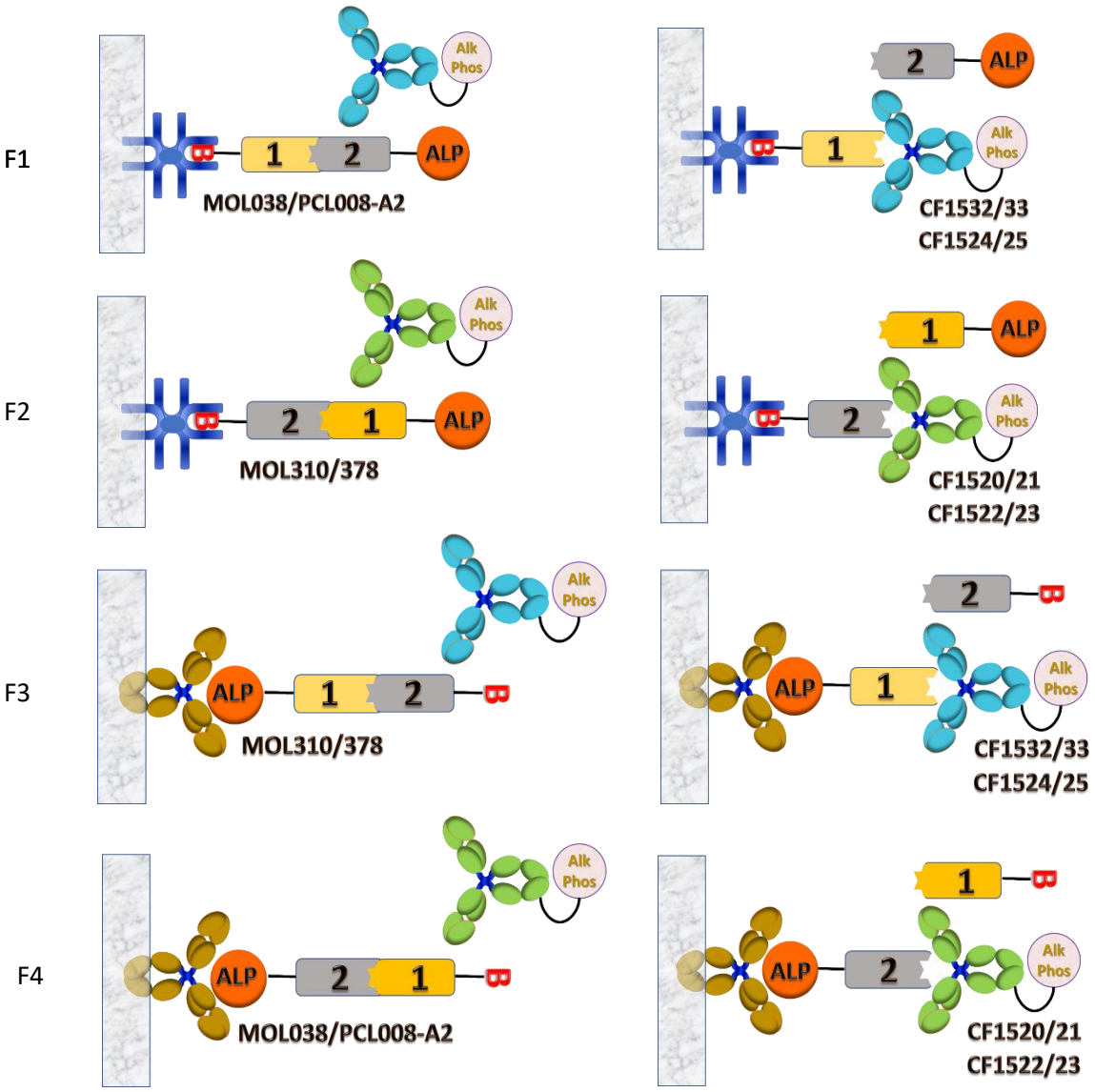


Figure L. Different formats evaluated with different combinations of peptides and antibodies. Format 1 (F1)- Format 4 (F4) are shown. Antibodies are listed that are expected to work in this format, the figure on the left demonstrate the binding of the intact peptide (i.e. no enzymatic activity) with no visible signal. The figure on the right demonstrate the binding of the antibody to the peptide when cleaved.

Comparison with a commercial assay kit. The commercial kit (Sensolyte®520 MMP-9 Fluorimetric Assay kit, AS-71155) was designed for specifically detecting MMP-9 in biologic samples such as culture medium, serum, plasma, synovial fluid, and tissue homogenate. A monoclonal anti-human MMP was used to pull down both pro and active forms of MMP from the mixture first, and then the activity of MMP-9 was quantified using fluorescence resonance energy transfer (FRET) peptide. An MMP-9 standard AMPA activated in-house was run on both the kit and a lateral flow format of the invention at a range of 250ng/ml – 4ng/ml. For the commercial assay the MMP-9 was diluted in an MMP buffer supplied in the kit and a Tris buffer saline 1% Tween20 for lateral flow devices.

Detection of enzyme activity in wound fluid and inhibition of enzyme activity. Wound samples from 18 patients were tested on the ultimate ELTABA device to measure active MMPs in this biologic matrix as there was expected to be high levels of MMP in this sample matrix. The samples were extracted from a swab (Copan, 552C.US) in MMP buffer and then frozen at -20°C until use. The addition of a chelating agent (5mM EDTA) to the sample to inhibit the protease activity was undertaken to determine the specificity of the device to calcium dependent enzymes e.g. MMPs. Each wound sample was diluted 1 in 20 in MMP buffer and 75µl was placed in a collection device with a defined amount of peptide (25ng/test). The collection device was rotated vigorously in order for the sample to mix sufficiently with the substrate solution. This reaction mixture was incubated at ambient temperature for 10 minutes before running on the assay as previously described.

Detection of enzyme activity in urine. Samples collected from people with COPD, CF and healthy state were tested on the Ultimate ELTABA devices. The respiratory samples were a mixture between exacerbation and stable samples.

Specificity of the assay to MMPs. Various MMPs (1, 2, 3, 7, 8, 9, 10, 11, 12, and 13) were tested on the Ultimate ELTABA device at 2µg/ml, 1µg/ml and 0.5µg/ml diluted in MMP buffer.

I.ii.v Results

Assay format selection. In the plate assay, 2 different formats were evaluated, with either CF1060 or polystreptavidin immobilised on the solid phase. There were four formats selected figure M with a streptavidin capture line, the selected peptide/sheep antibody parings were MOL378/CF1522 and PCL008-A2/CF1525 as predicted. Both peptides contained a PEG-Asp -AEEAc-AEEAc required to reduce any steric hindrance. With a CF1060 capture line, the selected peptide/sheep antibody pairings were MOL038/PCL008-A2/ CF1522 and MOL378/CF1525 as expected. The best combinations summarised in figure N confirmed that format 4 using sheep antibody CF1522 with

peptide MOL378 gave the best performance. This was also repeated in lateral flow format and the same combination was selected and taken forward.

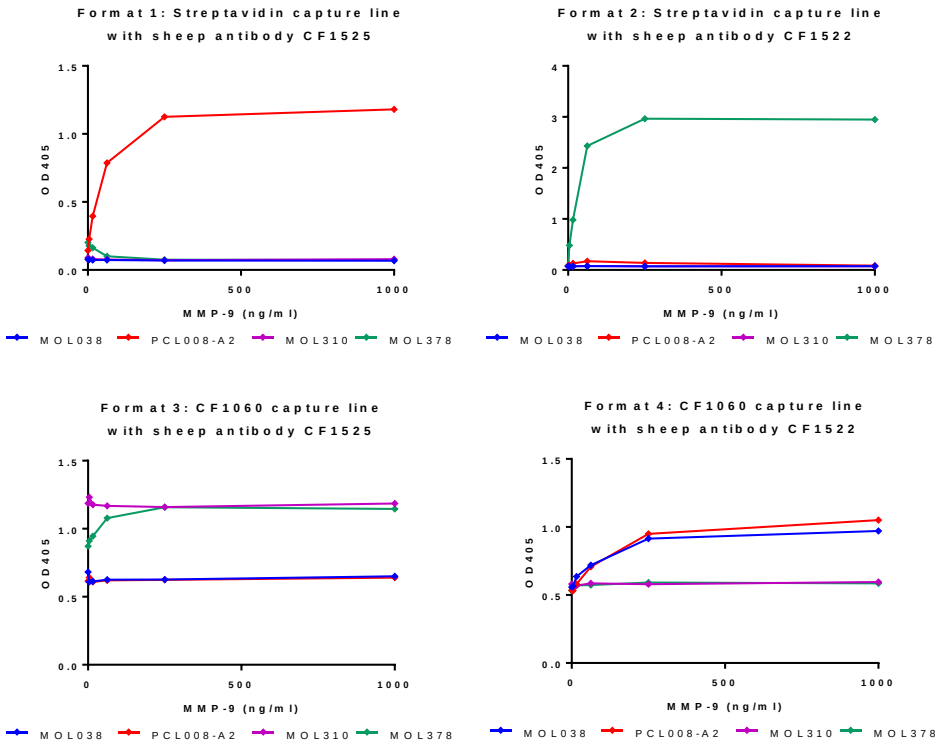


Figure M. Selection of best performing format in ELISA. Standard curves were obtained with format 1 and 2 when using peptides with a PEG linker. For formats 3 and 4 standard curves were obtained with peptides with and without PEG linkers as expected with no signals with the other peptides. NSB was obtained with MOL310 with format 3.

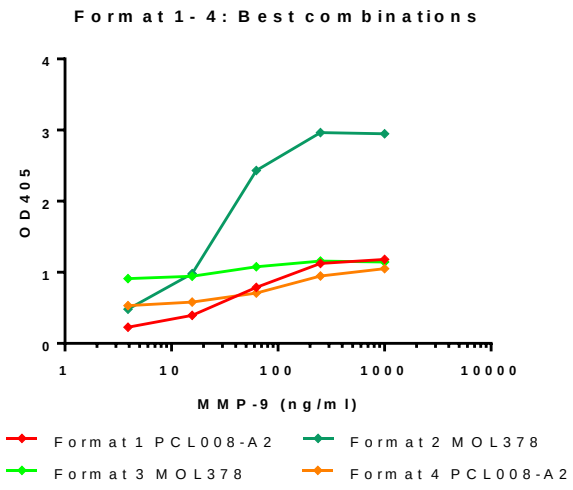


Figure N. Comparison of best performing peptide for each format. Both peptides with a PEG linker were deemed to be the most effective. Format 1 and format 2 produced standard curves with wider dynamic ranges (both used a streptavidin capture line).

Format 2 in both ELISA and lateral flow was the most optimal format.

Comparison with reference assay. The same MMP in-house standard run on both assays showed equivalence when comparing the RFU and lateral flow reader values. Both assays measure the lowest standard and have similar assay ranges. The reference assay had a run time of approximately 3 hours compared to 20 minute Ultimate ELTABA test.

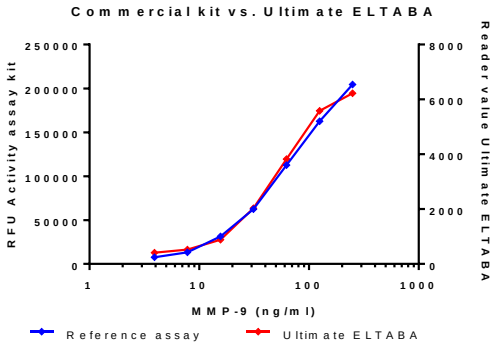


Figure O. Comparison of Ultimate ELTABA with commercial kits. A graph comparing the ability of a commercially available active MMP assay kit and Ultimate ELTABA with the same standard applied to both assays.

Detection of enzyme activity in wound fluid. MMP-9 present in the sample cleaved the indicator molecule at the cleavage site, exposing the recognisable epitope thus allowing the gold conjugate to form a complex with the cleaved stub. The lines that were formed were assessed by their relative intensities. The presence of a test line indicated that there was protease present in the test sample. A negative test line indicated a zero or low level of protease that was below the detectable limit. Stages in between these extremes indicated different levels of protease in the test sample. The intensity of the developed coloured lines was measured visually and with a Forsite Lateral flow device reader. Figure P(A) shows that addition of EDTA to the wound samples inhibits the readout, confirming the presence of MMP in the samples and also confirms that the assay is specifically measuring active MMPs.

Detection of enzyme activity in urine. Laboratory testing with reference assays have shown that protease activity is higher in urine from COPD and CF patients than in urine collected from healthy volunteers, this was confirmed with limited sample testing with Ultimate ELTABA as shown in figure P(B). Good discrimination between healthy and disease states with the lateral flow tests was shown.

Specificity of ultimate ELTABA with MMP. A bias towards MMP-13, MMP-9, MMP-2, MMP-12 and MMP-8 was observed in figure Q. The commercial MMP-9 did not behave in the same way as the other MMPs as it appeared to hook at the highest concentration. This was not reproduced with the inhouse MMP-9.

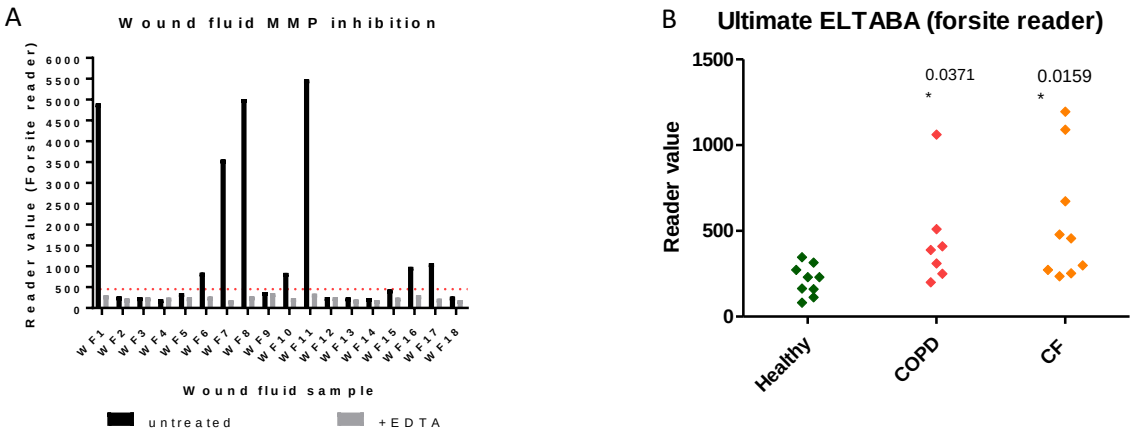


Figure P. specificity of Ultimate ELTABA with wound fluid and urine. a) Inhibition of enzyme activity in wound fluid by addition of EDTA to the sample. Red dotted line indicates the visual cut-off between positive and negative result. b) Detection of enzyme activity in urine, unpaired t test values show a significance difference between disease state and healthy with p values <0.05.

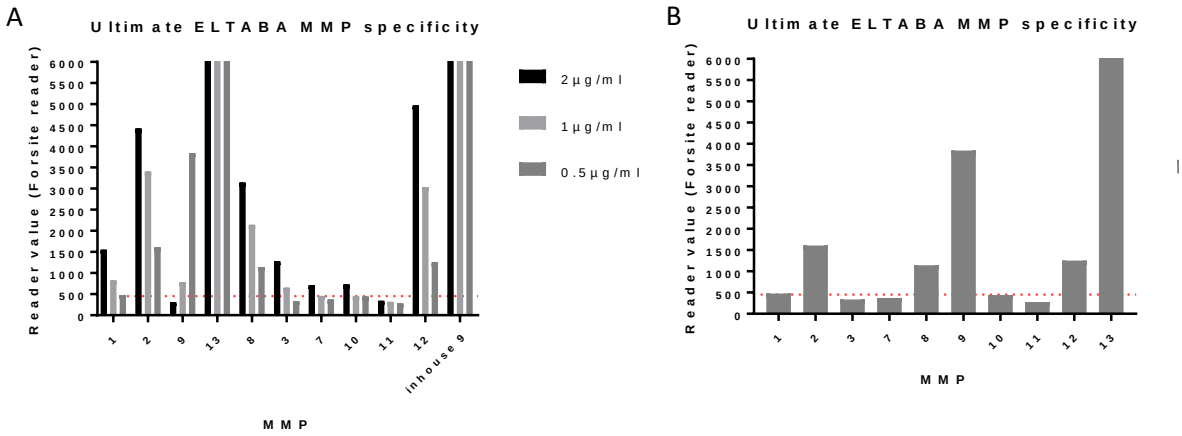


Figure Q. Ultimate ELTABA MMP selectivity. A) MMP 1-13 evaluated at 3 different concentrations b) summary of MMP's in order with a selected concentration of 2µg/ml with a bias towards MMP13, MMP-9, MMP2, MMP12 and MMP-8. Cut off value for the forsite reader is shown by the dotted line i.e. discrimination between a negative and positive visual read.

I.ii.vi Discussion

The test is deemed acceptable for measurement of MMP in urine samples. Verification experiments conclude that the assay range in in line with a commercial ELISA kit, that there is an indication that there is a difference between healthy and diseased states, conformation that the assay is measuring MMP as shown by inhibition studies and that the assay is measuring the correct MMPs believed to be present in relevant inflammatory conditions such as COPD and Cystic Fibrosis.