UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences

THE RENIN-ANGIOTENSIN SYSTEM ENZYMES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

by

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Thesis for the Doctor of Philosophy

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ABSTRACT

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Introduction: Chronic obstructive pulmonary disease (COPD) is the third leading cause of mortality in the world. As this disease is progressive in its nature, patients suffer a gradual decline in their quality of life due to a progressive deterioration in lung function.

Although there are treatments available to alleviate COPD symptoms, there is no pharmacological treatment that stops the decline in lung function within the lungs of COPD patients. Literature has suggested that angiotensin-converting enzyme inhibitors (ACEi) could potentially reverse lung decline but there is limited research into angiotensin-converting enzyme (ACE) in COPD lung. The work in this thesis investigated lung ACE as well as other enzymes associated with the renin-angiotensin system (RAS) within the lungs of COPD patients.

Methods: Lung samples were taken from subjects undergoing surgery. These subjects were classified according to their lung function into groups of COPD (n=36) or non-COPD (n=34). The lung samples were evaluated for the localisation, abundance and activity of enzymes that are associated with the production of RAS peptides: ACE, chymase, renin and ACE-2. This was by immunohistochemistry and enzymatic assays. The number of positive cells/vessels and the activity of these enzymes were measured in COPD lung and compared to subjects without COPD. The effects of ACEi prescribed to these subjects as well as their smoking status on lung enzyme expression and activity were also investigated.

Results: ACE activity was significantly lower in the lungs of COPD subjects compared to subjects without COPD. For the first time, ACE-2 staining was observed in macrophages from lung tissue as well as monocyte-derived macrophages (Mdm) and this human ACE-2 protein seemed catalytically active. ACE-2 activity and expression seemed to be higher in COPD and also in smokers compared to controls however this was not significant.

Conclusions: The activity of the RAS is overlooked as a potential risk factor for COPD. The work in this thesis has identified that the RAS enzyme ACE is linked with COPD and could be a target for pharmacological treatment of lung decline observed in COPD in the future. Another enzyme of the RAS, ACE-2 is localised to the lung macrophage, which is a cell associated with the inflammatory response. This finding could spark research into the function of ACE-2 in the macrophage and ACE-2 expression modification may be beneficial for inflammatory disease such as COPD in the future.

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DECLARATION OF AUTHORSHIP

I, Jessica Rajaram

declare that the thesis entitled

The Renin-Angiotensin System Enzymes in Chronic Obstructive Pulmonary Disease

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

this work was done wholly or mainly while in candidature for a research degree at this University;

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where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

none of this work has been published before submission,

Signed: ……..

Date:…….

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Definitions and Abbreviations

AA1	Antibody used to identify mast cell tryptase
ACE	Angiotensin-converting enzyme
ACE-2	Angiotensin-converting enzyme-2
ACEi	Angiotensin-converting enzyme inhibitors
AEC	3-Amino-9-ethylcarbazole
Ang-(1-7)	Angiotensin-(1-7)
Ang II	Angiotensin II
ARB	Angiotensin receptor blockers
ARDS	Acute respiratory distress syndrome
AT_1	Angiotensin type I receptor
AT_2	Angiotensin type II receptor
BCA	Bicinchoninic acid assay
BAL	Bronchoalveolar lavage fluid
CC1	Antibody used to identify mast cell chymase
CD68	Antibody used to identify cluster differentiation 68
COPD	Chronic Obstructive Pulmonary Disease
CSE	Cigarette smoke extract
DAB	3,3'-diaminobenzidine
DD	ACE deletion genotype
DX600	ACE-2 inhibitor
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular matrix
EN4	Antibody used to identify endothelium
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GMA	Glycol Methacrylate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GP	General Practitioners
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HBSS	Hank's balanced salt solution
HHL	Hippuryl-L-histidine-L-leucine
HRU	Histochemistry Research Unit in Southampton
HSA	Human serum albumin
ICC	Immunocytochemistry
IHC	Immunohistochemistry
II	ACE insertion genotype
IL-1β	Interleukin-1 beta
Il-6	Interleukin-6
IL-8	Interleukin-8
MAPK	Mitogen activated protein kinase
IPF	Idiopathic pulmonary fibrosis
Mca-APK(DnP)	ACE-2 substrate
MCP-1	Monocyte chemoattractant protein-1
MC_T	Tryptase positive mast cell
MC_{TC}	Chymase positive mast cell
Mdm	Monocyte-derived macrophage
MMP	Matrix metalloproteinases
NF-κB	Nuclear-factor-κB
NICE	The National Institute for Health and Clinical Excellence
O_2^-	Superoxide
PBS	Phosphate-buffered saline
rhACE-2	Recombinant human angiotensin-converting enzyme-2
RAS	Renin-angiotensin system
RFU	Relative fluorescence units
RPMI	Roswell park memorial institute media
ROS	Reactive oxygen species
ROW	Reverse osmosis water

SARS	Severe acute respiratory syndrome
TBS	Tris-buffered saline
TGF- β_1	Transforming growth factor β1
TIMP	Tissue inhibitor matrix metalloproteinase
TNF-α	Tumour necrosis factor alpha

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Chapter 1 Introduction

1.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is the progressive, limitation of airflow within the airways of the lung (GOLD, 2015). This disease affects the quality of life of many patients with its symptoms of cough, dyspnoea and wheezing, which worsens over time. In the most severe cases of COPD, patients need constant care by family or professional carers (Yohannes, 2007). Not only is the individual affected by COPD but there is also a large economic burden caused by costs of medication and hospital care. According to the Department of Health in England, COPD exacerbations are the second most common reason for hospitalisation and the cost of the acute care for COPD patients is more expensive than other diseases of the lung such as lung cancer (Department of Health, 2011).

1.1.1 Epidemiology

COPD is a global problem. The Global Burden of Disease Study has demonstrated that COPD is the third leading cause of mortality based on data collected from 50 countries between the years 1990 and 2010 (Lozano *et al.*, 2013). Approximately 900,000 people in the UK have been diagnosed with COPD (Booker *et al.*, 2004) however data from the Health Survey in England predict that a further 2.7 million people are suffering from COPD but have not been clinically diagnosed (Shahab *et al.*, 2006). COPD is more prevalent in the elderly than in the younger population (De Marco *et al.*, 2013). With an ageing population who have smoked cigarettes for a number of years, the prevalence of COPD is only going to increase in the future.

Some could argue that the incidence of COPD will not increase in the future due to a decline in the number of people who smoke cigarettes in the UK in the past 40 years (Office for National Statistics, 2013); nevertheless there is a significant proportion of the UK who still smoke cigarettes. For example in the last survey in 2013, one in five adults smoked cigarettes (HSCIC, 2015). Furthermore the proportion of people in Great Britain who smoked cigarettes in 1974 was 46 % (Office for National Statistics, 2013). This proportion of people still could develop COPD, even if they have given up cigarette smoking since Lokke *et al.*, (2006) demonstrated that 12 % of people who gave up smoking earlier on in life then went on to develop COPD.

1.1.2 Pathologies of Chronic Obstructive Pulmonary Disease

COPD is the preferred collective term for a group of conditions characterised by the reduction of airflow (NICE, 2011). The two main conditions are emphysema and chronic bronchitis, which are generally located in the alveoli of the lung, and the large airways, respectively (figure 1.1).

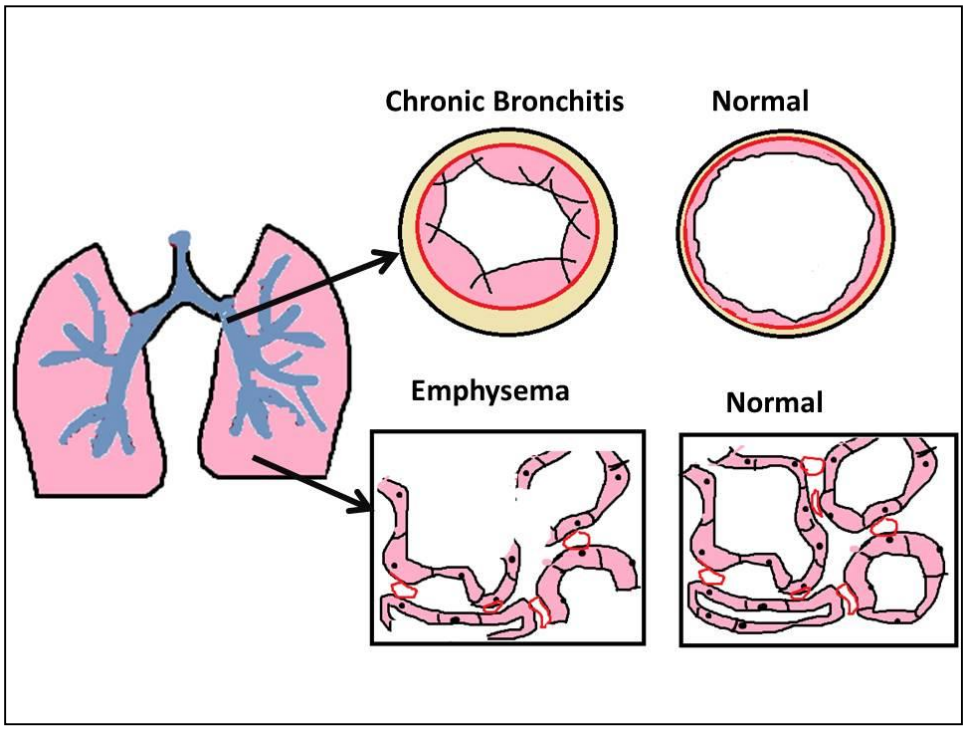

Figure 1.1 Diagram of the anatomical location of lung pathologies The pathology of chronic bronchitis is due to airway remodelling which reduces lumen size compared to normal healthy airway. This normally occurs in the lower lung within the bronchial and small airways. The pathology of emphysema involves tissue destruction and enlargement of airspaces within the alveoli. Diagrams on right represent transectional slices through the tissue. Colours denote *Blue- the bronchial trees; Yellow- smooth muscle layer; Pink- alveolar tissue; Red- small capillaries.*

It is important to note that these two pathologies: emphysema and chronic bronchitis, are considered to be two extremes across a spectrum of COPD (Kim & Criner, 2013). Some patients with bronchitis also have the pathology of emphysema, as first demonstrated by Heard (1958). This mixture of pathologies supports a heterogenous nature of COPD.

1.1.2.1 Emphysema

Emphysema is the destruction of alveolar parenchymal tissue located within the lobes of the lung (figure 1.1). Leopold & Gough, (1957) was the first group to investigate the pathogenesis of

hypertrophied lungs in emphysema and to analyse the histology of emphysematous lungs. In histology, emphysema is defined as the permanent enlargement of air spaces within the location of the alveoli with no obvious fibrosis present (Hogg *et al.*, 2004).

1.1.2.2 Chronic Bronchitis

Clinically, chronic bronchitis is defined as an expectorant cough for at least three months over two consecutive years (ATS, 1995). This is a clear clinical diagnosis for chronic bronchitis however the pathophysiology in the tissue for this disease is not as evident.

Occluded small airways situated in the lower lung (diameter < 2 mm) are the main site of the pathophysiology. Occlusion is due to a low level of inflammation leading to an overproduction of mucus as well as fibrosis of the small airway tissue (Holtzman, 2005). Due to a combination of obstructions, small airways exhibit increased resistance to airflow and as a consequence trap air within the alveolar ducts. This impairs the amount of fresh oxygenated air distributed to these air sacs resulting in inefficient gas exchange (Hogg & Timens, 2009).

1.1.3 Current Management Strategies

The current management strategies of COPD aim to alleviate the symptoms and improve the quality of the life of COPD patients. Inhalers are the most preferred route of administration for pharmacological therapies of COPD. The NICE guidelines in the UK suggest the general practitioners (GPs) should prescribe short-acting beta-$_2$ agonists such as salbutamol or short acting muscarinic antagonists such as ipratropium bromide to adults to ease the symptoms of breathlessness (CKS, NICE September 2015). These inhalers relax the smooth muscle within the airway so that the airway lumen increases in size and air can pass through. If this is not effective the GP is advised to prescribe longer acting beta-$_2$ agonists such as salmeterol or formoterol or muscarinic antagonists such as tiotropium. Moderate to severe COPD patients who are co-prescribed both of these types of long-acting bronchodilators reportedly have better lung function than each type prescribed on their own (Buhl *et al.*, 2015).

Inhaled corticosteroids are also used to alleviate the inflammation present in the airways. This is often when combination therapy of long-acting bronchodilators does not alleviate breathlessness or the patient is hospitalised due to the sustained worsening of the COPD patient's condition which is beyond normal day-to day-variations, commonly known as an exacerbation (Burge *et al.*, 2003). Corticosteroid-only inhalers are not licensed for use in the UK and it is advised that corticosteroids should only be co-prescribed with a long-acting bronchodilator (CKS, NICE September 2015). This is because the use of inhaled corticosteroid therapy alone is associated with the unpleasant side effects of pneumonia and glaucoma especially with fluticasone (Suissa

et al., 2013). Corticosteroid therapy is beneficial for some COPD patients but only with moderate severity (Gartlehner *et al.*, 2006).

Supplementary oxygen delivery is another treatment often prescribed to COPD patients who are experiencing an exacerbation or have end-stage COPD. The NICE guidelines in the UK recommend short bursts of oxygen therapy for 10-20 minutes (CKS, NICE September 2015). This only applies if the patient is not already on long-term oxygen therapy. This treatment ensures there is sufficient oxygen present within the blood and therefore tissues in the body. In Canada, the practice of the same flow rate for all people is slowly changing to titrating the oxygen flow rate to achieve an optimum oxygen saturation level. This is because titration has shown to lower the mortality rate of COPD patients on oxygen treatment significantly (Austin *et al.*, 2010).

Not just the intratracheal route is used in the pharmcological delivery of treatment for COPD; oral medications are also used such as ambroxol and carbocisteine. These drugs are classified as mucolytics and change the viscosity of the mucus in airway for easier clearance. These drugs have been shown to be more effective in preventing exacerbation within severe COPD patients rather than mild to moderate (Malerba *et al.*, 2004). One of the oldest medications taken to relieve the symptoms of COPD is theophylline. It is a phosphodiesterase inhibitor and this action leads to lowered inflammation in the lungs however it is not prescribed often since patients experience adverse effects such as vomiting diuresis and headaches. Along with these negative effects, theophylline is also a non-selective adenosine receptor antagonist, which has been known to cause cardiac arrhythmias (Barnes, 2006).

In some severe cases, surgical treatments are employed to remove excess tissue that builds up due to fibrosis and excessive air trapping. Bullectomies and lung volume reduction surgery apply pressure on the remaining tissue within the lung in order to restore surface tension (GOLD, 2015). In some cases lung transplantation is also a treatment option that is undertaken (Lahzami *et al.*, 2010).

Currently, the pharmacological treatments available such as bronchodilators and corticosteroids focus on relieving the symptoms rather than slowing the progression of lung damage in COPD patients. New potential targets are needed in order to develop treatments that can slow the decline of lung function in COPD. To find these new targets, the risk factors that cause the lung decline in COPD should be investigated.

1.1.4 Aetiology

1.1.4.1 Cigarette Smoke

The main risk factor for COPD is the inhalation of smoke particles either from cigarette smoke or the burning of biomass fuels. At least 6,000 compounds in smoke are inhaled into the lungs (Kleinstreuer *et al.,* 2013). A tenth of these compounds have been identified within the gas phase of mainstream smoke and the rest in the particulate phase. The most hazardous compounds from mainstream smoke are polycyclic aromatic hydrocarbons, mixtures of which produce tissue-damaging components such as tar and cadmium (Talhout *et al.,* 2011). Smoke particles from the burning of biomass fuels for indoor cooking has also been a contributing factor to COPD in lower-economically developed countries. (Regalado *et al*., 2006; Akhtar *et al*., 2007).

In a normal smoker, this aspiration of particulates with the potential to cause lung damage is resolved by immune responses. Smokers without COPD have a low inflammatory activity in the lung due to continuous smoking compared to smokers diagnosed with COPD (Willemse *et al*., 2005). The immune responses from a patient diagnosed with COPD are dysregulated leading to an amplification of the inflammatory response and destruction of alveolar tissue and airway remodelling. The ways in which cigarette smoke cause lung tissue damage are through the generation of reactive oxygen species (Marco van der Toorn *et al.,* 2009) and inducement of the inflammatory response.

1.1.4.1.1 Reactive Oxygen Species

Reactive oxygen species are molecules that readily oxidise components of cells and tissues within the body. Examples of these are superoxide (O_2^-) or hydrogen peroxide (H_2O_2). ROS can cause lipid peroxidation to produce malondialdehydes, which are often used as a biomarker for ROS. Zeng *et al.,* (2013) has demonstrated that people with stable COPD have significantly more malondialdehyde in their sputum compared to non-COPD subjects who were both smokers and non-smokers. This evidence suggests there is a high number of ROS that oxidise lipids in the tissue of COPD patients.

ROS that are present in the gas phase when inhaled with mainstream smoke are short-lived. In contrast, certain inorganic particulates in the particulate phase of cigarette smoke can penetrate airway epithelial cell membranes and disrupt normal mitochondrial balance (Marco van der Toorn *et al.,* 2009). This can lead to the inducement of intracellular ROS. This ROS induces the stimulation of the intracellular pathway NF kappa B (NF-κB) which increases the production of inflammatory genes by translocating to the nucleus (Dandona *et al.,* 2007).

In non-diseased individuals, the damaging effects of ROS are counteracted by antioxidants. The most common of antioxidants is superoxide dismutase - which converts superoxides into glutathione and thioredoxins. If there are an insufficient number of antioxidants present within the tissue to dampen down the oxidising nature of ROS, the tissue is said to be in a state of oxidative stress.

1.1.4.1.2 Inflammation

Besides ROS, non-diseased individuals also have a mechanism to identify and manage foreign particles. These particles are inhaled through gas and particulate phase. These are recognised by inflammatory cells such as neutrophils and macrophages that release chemokines as a signal for efficient repair. Macrophages have pattern recognition receptors on their cell surface. These recognition receptors can identify pathogens that enter the body through pathogen-associated molecular patterns and minimise any further destructive effects (Rovina *et al*., 2013).

After recognition of a pathogen, macrophages engulf the pathogen by endocytosis for intracellular destruction. The internalised pathogen fuses with granules containing lysosomes and lactoferrin that destroy the pathogen completely. Macrophages of the lung also phagocytose inhaled diesel exhaust particulate matter and dead/dying host cells in order to keep the airway free from obstruction often seen in people who smoke.

Macrophages from cigarette smokers release less of the pro-inflammatory eicosanoid, leukotriene B_4 than macrophages from subjects who have never smoked (Tardif *et al*., 1990). In addition, human lung macrophages exposed to cigarette smoke extract *in vitro* demonstrated lower production of the pro-inflammatory cytokines TNF-α and IL-6 (Dubar *et al*., 1993). In contrast, bronchalevolar lavage fluid (BAL) from current smokers had a higher concentration of pro-inflammatory cytokines and chemokines (IL-1β, IL-6, IL-8 and MCP-1) compared to non-smokers (Kuschner *et al*., 1996). There seems to be a lower level of pro-inflammatory cytokine production in the macrophages of normal smokers compared to non-smokers when isolated from BAL rather than measuring from BAL itself.

Unlike normal individuals, people diagnosed with COPD have a dysregulation in the classical defence mechanism. Macrophage phagocytosis and mobility is slowed (Taylor *et al*., 2010). Dysregulation of the mechanism leads to constant inflammation and harbouring of viral particles within the structures of the lung. This gives way to opportune infections from invading pathogens such as bacteria or viruses, exacerbating the symptoms of COPD.

1.1.4.1.2.1 Lung Macrophages and COPD

Macrophages from COPD smokers had heightened release of inflammatory cytokine IL-8 compared to smokers without COPD (Culpitt *et al*., 2003). In addition to this, lung macrophages isolated from smokers with COPD had a downregulation of genes that were associated with lowering inflammation compared to the macrophages isolated from COPD patients who did not smoke (Shaykhiev *et al.*, 2009).

Subjects with COPD also had a higher matrix metalloproteinase-9 activity in alveolar macrophages compared to those subjects who did not have COPD, regardless of whether the non-COPD subjects smoked or not (Russell *et al.,* 2002). Matrix metalloproteinases (MMPs) are proteases associated with the destruction of parenchymal tissue in emphysema (Segura-Valdez *et al.*, 2000; Demedts *et al*., 2006). There are many subtypes classified by their structural features and their specificity to act on certain proteins. A more recent paper has demonstrated higher MMP-9 release from macrophages isolated from COPD patients compared to non-COPD smokers (Chana *et al.*, 2014). Furthermore the same paper also showed a higher concentration of active MMP-9 release from lung macrophages in subjects who smoked compared to non-smokers thus suggesting that cigarette smoke is involved in inducing the destructive phenotype in macrophages.

In emphysema patients, the MMP-1 antibody stained more lung macrophages compared to the numbers in subjects without emphysema (Wallace *et al.*, 2014). Furthermore MMP-12 release from BAL isolated macrophages was higher in subjects with moderate to severe COPD compared to subjects without COPD (Molet *et al.,* 2005).

An extensive review by Churg *et al.* (2012) commented that there are many subtypes of MMPs and there is some disparity between animal and human macrophages regarding the release of these subtypes in COPD. The review brings together the many experiments investigating MMPs and COPD and the general consensus is that MMP levels and activities are increased in COPD macrophages.

In conclusion, there seems to be a higher inflammatory and MMP phenotype in macrophages isolated from subjects with COPD regardless of smoking status.

1.1.4.1.2.2 Laboratory Techniques for COPD Lung Macrophages

Generally, the method of collecting lung macrophages by BAL fluid from human subjects is an invasive process, requiring surgery or bronchoscopy. This is why many laboratories use a less invasive second technique to investigate macrophages called monocyte–derived macrophages (Mdms). Mdms are monocytes isolated from the blood of volunteers that are matured into macrophages in culture (Chana *et al.*, 2014, Staples *et al.*, 2015).

The underlying theory of this technique is that monocytes derived from the bone marrow travel through the blood as peripheral blood monocytes. In response to tissue injury, the monocytes travel to the site of injury and mature into tissue macrophages. The Mdm technique simulates this process. The treatment of these Mdms with granulocyte macrophage colony-stimulating factor (GM-CSF) is essential for maturing these monocytes into cells with a similar lung macrophage phenotype.

GM-CSF is a growth factor that promotes the proliferation of granulocytes and macrophages from bone marrow cells as first shown by Lieschke & Burgess (1992). GM-CSF treated Mdms have a similar phenotype to those lung macrophages isolated from human lung tissue or BAL. For example, the scavenger receptors used in Mdms for foreign particles to bind to are similar to the receptors used by lung macrophages (Tudhope & Finney-Hayward, 2008). Furthermore Winkler *et al.* (2008) reported that GM-CSF treated Mdms released IL-8 concentrations similar to macrophages isolated from human lung tissue. These Mdms also demonstrated a reduction in phagocytic ability in response to cigarette smoke extract (CSE) as observed in lung tissue macrophages. Laboratory techniques for the culture of macrophages are essential to understand the effect of smoke particles on the lung system.

1.1.4.2 Protease-Antiprotease Balance

Inhalation of smoke particles may not be the only risk factor for COPD. A sample of people followed for 25 years demonstrated that 12 % of people who gave up cigarette smoking earlier on in life went on to be diagnosed with COPD (Lokke *et al.*, 2006). Furthermore non-smokers make up a small proportion of COPD patients (Salvi & Barnes, 2009). An alternative risk factor to cigarette smoke is the protease-antiprotease imbalance in the lung leading to a destruction of the tissue.

In support of this, some COPD patients have a deficiency of α-1 antitrypsin protein level. α-1 antitrypsin is an antiprotease. The reduction of this antiprotease allows elevated function of the protease neutrophil elastase. Neutrophil elastase cleaves elastin within the alveolar structure leading to emphysema. When this was first discovered, researchers thought the loss of α-1 antitrypsin was the main cause of emphysema however Lieberman *et al.*, (1986) have demonstrated that α-1 antitrypsin occurs in less than 2 % of the number of emphyema patients that they studied. This suggests deficiency of α-1 antitrypsin is not the main cause of emphysema after cigarette smoke.

Another group of proteases is the MMPs. Gross (1962) was the first paper to show an enzyme, present in animal tissue, with the ability to break-down collagen thus leading to the discovery of the enzymes called MMPs. There are many different subtypes of MMPs, some are specific to

collagen (collagenases) others break-down elastin (elastases) or gelatin (gelatinases) (Churg *et al.,* 2012). The aforementioned substrates of these enzymes are collectively known as the extracellular matrix (ECM). The ECM is essential for the structure of tissues such as the airway wall and alveoli in the lung and is present in the subepithelial layer of these tissues (Makinde *et al.,* 2007).

MMPs are generally regarded in the literature as being destructive to tissue (Churg *et al.,* 2012). For example mice with the MMP-12 gene knockout did not undergo as much alveolar tissue destruction after exposure to cigarette smoke compared to wild-type controls (Hautamaki *et al.,* 1997). In COPD patients, higher protein levels of the elastase MMP-12 have also been found in sputum of COPD subjects compared to non-COPD controls (Demedts *et al.,* 2006). Segura-Valdez *et al.,* (2000) also demonstrated higher MMP-1 activity levels in COPD patients compared to non-COPD controls in BAL.

MMPs are regulated by naturally occurring proteins in the tissue called tissue inhibitor matrix metalloproteinases (TIMPs) and these anti-proteases inhibit MMP enzymes and therefore the destruction of tissue. TIMP-1 secretion is reduced in macrophages isolated from stable COPD patients compared to non-COPD controls (Pons *et al*., 2005). TIMP-1 protein levels are also lowered in the sputum of COPD patients during acute exacerbation compared to their levels before the exacerbation (Mercer *et al.,* 2005). Higher MMP activity was also observed in these patients during exacerbation. This shift of MMP/TIMP balance during the exacerbation could imply that the antiprotease balance was involved in worsening the symptoms of COPD. It is worth noting this paper only observed the differences but did not investigate a causative link between exacerbation and antiprotease balance.

The literature strongly suggests that protease-antiprotease imbalance describes a plausible pathological mechanism in COPD because of the heightened protease destruction activity in emphysematous lungs.

1.1.4.3 Fibrosis

The destruction of tissue is not always the cause for pathogenesis in the COPD lung. Peribronchiolar samples donated by COPD patients of varying severity have demonstrated an increased thickening of the small airway walls, specifically where the ECM is localised (Hogg *et al.,* 2004). This thickening was correlated with an increased severity of COPD.

The build-up of ECM is the reason for this thickening since increased collagen, a component of ECM, has been identified by immunohistochemistry in the airway wall of peribronchiolar samples from COPD patients (Hogg *et al.,* 2009).

The predominant cell type that produces ECM is the fibroblast. The fibroblast is activated after tissue damage in normal subjects, which is part of the process of repairing tissue. Fibroblast activation leads to a secretion of ECM to rebuild and remodel the destroyed tissue (Wynn, 2011). This building of new tissue is also balanced with the secretion of MMPs that break-down the ECM.

Dysregulation of this reparative process leads to fibrosis and this has been demonstrated in COPD subjects (Hogg *et al.,* 2004). The increase in ECM is predominantly associated with the cytokine transforming growth factor–β1 (TGF- β1) that differentiates fibroblasts into an activated form called a myofibroblast. Myofibroblasts have a higher ECM production rate than fibroblasts (Baarsma *et al.,* 2013). Furthermore, the TGF-β receptor is heavily involved in a transcriptional pathway within fibroblasts, which modulates the production of ECM components (van Diemen *et al.,* 2006; Zandvoort *et al.,* 2008). A longitudinal study demonstrated people who have fibrosis within the airways also have a higher expression of TGF- β1 in BAL compared to those who do not have fibrosis (Hodge *et al.,* 2009).

Hogg *et al*., (2009) has suggested that fibrosis is another form of COPD pathogenesis because fibrosis leads to a decrease in luminal radius of the small airway and partial or complete occlusion of airflow to the alveoli. Fibrosis could be another risk factor worth investigating to find new targets to slow the decline of lung function in COPD patients.

1.1.5 RAS - A Potential Target

Some of the aforementioned risk factors leading to the decline in lung function have been prevented or reversed by components associated with the renin-angiotensin system (RAS). RAS has been linked with the improved mortality rate in COPD and reduced rates of hospitalisation. A retrospective study of 11,212 COPD patients demonstrated that patients prescribed angiotensin-converting enzyme inhibitors (ACEi) and angiotensin-receptor blockers (ARB) that affect components of the RAS - were less likely to die in three months after hospitalisation compared to those who did not have this treatment (Mortensen *et al.,* 2009). Another study from a different group demonstrated that COPD patients with a risk of cardiovascular disease who were prescribed ARBs had a lower risk of hospitalisation as well as lower mortality rates (Mancini *et al.,* 2006). Furthermore, ACEi was the only medication out of 3 types (used in 1,170 subjects) to be significantly associated with an improvement in lung function compared to those subjects without ACEi medication (Petersen *et al.,* 2014).

The RAS is typically associated with the cause and treatment of cardiovascular disease and is often overlooked as a risk factor in respiratory diseases. Investigation into the RAS system and

its potential association with COPD could be worthwhile because of RAS antagonists/inhibitor benefits to COPD patients, as indicated in the papers mentioned above. However, there is limited research into the specific components of RAS in the lung and its association with the progression of COPD. Experiments exploring these RAS components in the lungs of people with COPD could potentially lead to a pharmacological target for the treatment of COPD.

1.2 Renin-Angiotensin System

RAS has been investigated mainly for the management of blood pressure ever since the ACEi discovery in the 1960s (Erdos, 2006). The RAS is comprised of many peptides cleaved by specific enzymes. The precursor peptide, angiotensinogen is produced by the liver and cleaved by the enzyme renin from the kidney into angiotensin I (Ang I), an inactive peptide. Further hydrolysis of Ang I by angiotensin-converting enzyme (ACE) within the lung generates angiotensin II (Ang II) peptide. Ang II binds to the angiotensin II type I receptor (AT_1 R), located on various types of tissues, in order to elicit vasoconstrictive, profibrotic and pro-inflammatory effects (Bernstein *et al.*, 2013). This is illustrated in figure 1.2.

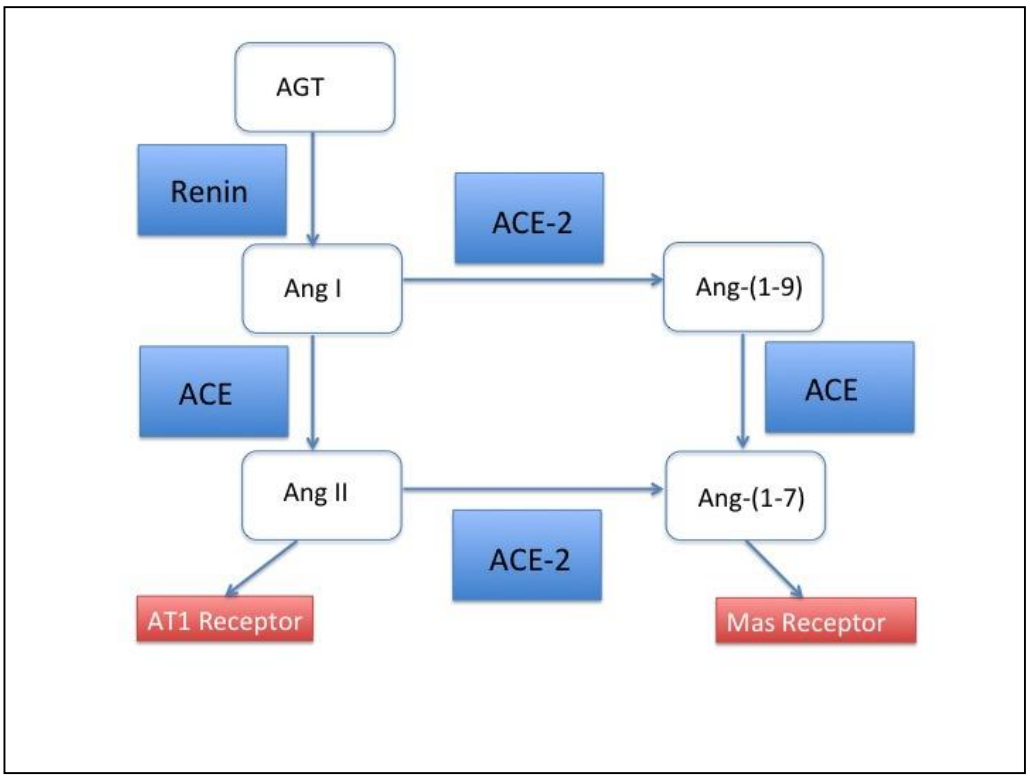

Figure 1.2 Diagram of peptides and enzymes involved in the RAS Enzymes are within the blue boxes, peptides within the white boxes *AGT Angiotensinogen ACE-2 Angiotensin-converting enzyme-2; ACE angiotensin-converting enzyme; Ang Angiotensin;* AT_1 *Angiotensin type I receptor*

This simple pathway has been accepted for some time. However, in the past 15 years, new components of the RAS have been identified rendering this system more complicated than originally thought. One of these new components was angiotensin-converting enzyme-2 (ACE-2) (Donoghue *et al*., 2000; Tipnis *et al*., 2000). This enzyme shares 42 % homology with ACE. ACE-2 preferentially cleaves Ang II over Ang I into another peptide called Ang-(1-7) which is also relatively new to the RAS (Rice *et al*., 2004). Experimental studies with rodent tissue have demonstrated that ACE-2 breaks down Ang II in the tissue into Ang-(1-7) (Herath *et al.*, 2007) and this Ang-(1-7) binds to its own receptor Mas (Shenoy *et al.*, 2010).

It has been established that there is a balance between the ACE-2/Ang-(1-7)/Mas arm of RAS and the classic ACE/Ang II/AT_1R arm as described in detail in Santos *et al.*, (2013). Since these two axes of RAS are involved with fibrotic and inflammatory effects, the careful balance could potentially be important in the control of diseases with a fibrotic or inflammatory pathological component such as COPD. As RAS consists of many peptides and a careful interplay of enzymes it is important to understand the RAS in detail. This is so that the potential target for COPD treatment that may reside in the RAS is accurately deduced from this system.

1.2.1 Peptides

Cleavage of the main precursor peptide angiotensinogen gives rise to many truncated angiotensin peptides such as Ang I, Ang II, Ang-(1-7), Ang III and Ang IV (Ribeiro-Oliveira *et al*., 2008). The two most widely studied peptides implicated in inflammation and fibrosis are Ang II and Ang-(1-7).

1.2.1.1 Angiotensin II

Ang II is an octapeptide and is formed by the cleavage of Ang I (figure 1.2). Most of the pathological effects of Ang II are due to binding to the AT_1R with subsequent intracellular cascades such as Smad and MAPK pathway (Rodríguez-Vita *et al*., 2005). As mentioned before, Ang II is responsible for the constriction of blood vessels however there are many other effects that the Ang II peptide is involved with.

Ang II plays a role in inflammation through the induction of cytokine and chemokine secretion from different cell types (Benigni *et al*., 2010). Ang II releases NF-κB for translocation by phosphorylating its regulatory protein, IκB in vascular smooth muscle cells (Zahradka, 2002). The translocation of NF-κB has been reported to cause upregulation of proinflammatory cytokines such as tumour necrosis factor-α and interleukin-1β and the perpetuation of inflammation (Dandona *et al*., 2007).

Ang II has also been reported to induce chemokines such as chemokine ligand 5 (CCL-5) in normal human lymphocytes (Jurewicz *et al*. 2007) and monocyte chemoattractant protein-1 in human macrophages (Dai *et al*., 2007). Secretion of these chemokines induced by Ang II could lead to the recruitment of other leukocytes to the site of injury.

Not only is Ang II involved in inflammation, it has also been implicated in fibrosis. The infusion of Ang II into rats led to an increase in cardiac collagen deposition compared to the non-treated controls (Sopel *et al.*, 2011). As for the lung, pre-treatment of inhibitors of ACE prevented an elevation of ECM components in the lung homogenate of rats that would have otherwise occurred in response to the administration of paraquat (Ghazi-khanasari *et al.* 2007). Furthermore, in transgenic mice with an overactive RAS, the pre-treatment of ARB did not show an accumulation of ECM within the lung compared to those without treatment (Wang *et al.*, 2015).

These examples given here mainly focus on animal models for the lung disease idiopathic pulmonary fibrosis (IPF) rather than COPD; the difference being that ECM deposition occurs in the alveolar parenchyma in IPF but not in COPD (Hogg *et al.*, 2009). Nonetheless the literature suggests that AngII is associated with an increase in ECM production. ECM is heightened in the small airways of some COPD patients so investigating Ang II could be a route to finding a target for COPD pathogenesis.

Ang II association with fibrosis is also demonstrated at a cellular level. Primary lung fibroblasts treated with Ang II induced proliferation of these cells measured by incorporation of DNA compared to non-treated (Marshall *et al.*, 2000). Ang II seems to require TGF-β to have a profibrotic effect since in the same study the fibroblast cultures treated with an antibody that neutralised the function of TGF-β did not induce a higher rate of proliferation. Ang II treatment has also induced the release of TGF-β soluble protein in primary lung fibroblasts (Uhal *et al.*, 2007) and culture rich in lung myofibroblasts isolated from patients with IPF also released Ang II, TGF-β and collagen. The literature seems to suggest that lung fibroblasts have cross-talk between Ang II and TGF-β in order for ECM accumulation.

The evidence above shows that Ang II is an important peptide of RAS associated with inflammation and fibrosis that is observed in COPD. The break-down of Ang II leads to the formation of another effector peptide:Ang-(1-7).

1.2.1.2 Angiotensin-(1-7)

The functional effects of Ang-(1-7) were characterised nearly 25 years later than its discovery in the 1980s due to the identification of its receptor Mas (Santos *et al*., 2003). Ang-(1-7) is formed by two different pathways. The first is the cleavage of Ang I peptide. The second and more

catalytically favourable pathway is the cleavage of Ang II by ACE-2 (Rice *et al.* 2004). This is the most physiologically relevant pathway according to a study done in ACE-2 KO mice where Ang-(1-7) was not formed because of the void of ACE-2 (Grobe *et al.* 2013). Ang-(1-7)-induced effects stopped the production of collagen synthesis in cardiac fibroblasts therefore suggesting an antifibrotic nature (McCollum *et al.* 2012). This paper also demonstrated a reduction of inflammatory mediators in Ang-(1-7) treated fibroblasts when stimulated with a mitogen also suggesting an anti-inflammatory nature. This effect of Ang-(1-7) is thought to oppose fibrotic and pro-inflammatory induced effects caused by Ang II (Passos-Silva *et al.,* 2013).

In the literature, the anti-inflammatory effects of this peptide have been observed in animal models. In a diabetic rat model intra-peritoneal injection of Ang-(1-7) daily for one month did not lead to elevated inflammatory mediators that were present in the diabetic non-treated rats (Renno *et al*., 2012). The same group later administered Ang-(1-7) to mice with symptoms of acute allergic asthma. This led to a fall in leukocyte recruitment in the lung compared to vehicle controls (El-Hashim *et al*., 2012). Both effects of Ang-(1-7) in the aforementioned papers were reversed by the co-administration of the Mas receptor antagonist, A779.

With regard to fibrosis, an Ang-(1-7) peptide mimetic (AVE 0991) was administered to mice modelling symptoms of chronic asthma. A subcutaneous dose of this Ang-(1-7) mimetic reduced the thickness of the airway smooth muscle that was observed during the inducement of chronic asthma (Rodrigues-Machado *et al*., 2013). Furthermore, with regards to cardiac muscle fibrosis in rats Ang-(1-7) infusion alongside exercise training attenuated the fibrosis (Shah *et al*., 2012). In summary, there seem to be many animal studies that demonstrate Ang-(1-7) instillation as being therapeutically beneficial for inflammation and fibrosis.

1.2.2 Enzymes

As described above, animal models have shown that the RAS peptides are important as Ang II and Ang-(1-7) peptides are able to influence the symptoms of animal models of inflammatory diseases. RAS enzymes tightly control the balance of these peptides.

The balance of angiotensin peptides shifts in response to insults within the body. In disease, this balance is continually dysregulated, as animal studies have demonstrated. An example of this was in animal models for Type I and II diabetes (Wysocki *et al.* 2006). Both ACE-2 and ACE protein and activity levels in the kidney were increased within these diseased mice compared to non-diseased controls. The location seems to be key since cardiac ACE and ACE-2 levels in the same mice were unchanged compared to controls thus highlighting that RAS enzyme dysregulation can be localised to particular local organs.

Another example is the experiment done by Han *et al.*, (2010). Rats were exposed to cigarette smoke for six months which induced pathologies within the tissue that were associated with pulmonary hypertension. There was an increase in ACE protein expression and a decrease in ACE-2 protein within the lung homogenate of rats exposed to cigarette smoke compared to those rats that were exposed to fresh air.

Enzymes within the lung of COPD patients have not been explored. It would be worth investigating these enzymes within the lung to see whether there is an imbalance, this may allow for a possible target for COPD treatment to be discovered. The four most important enzymes that contribute to the modulation of the Ang II and Ang-(1-7) peptides are ACE, renin, ACE-2 and the non-RAS dependent enzyme involved in Ang II production, chymase. These enzymes were chosen on the basis that they are vital for the formation and degradation of the Ang II and Ang-(1-7) peptides.

1.2.2.1 Angiotensin-Converting Enzyme

ACE was first identified as a type I transmembrane protein in 1956 (Skeggs *et al*., 1956) and is a dipeptidyl carboxypeptidase. Consistent with transmembrane proteins, ACE is composed of an extracellular N-terminus, the transmembrane structure and the C-terminal tail (figure 1.3). This N-terminal tail also accommodates two zinc-binding motifs (H-E-X-X-H) that act as catalytic domains (Natesh *et al*., 2003). There are two isoforms that exist: testicular ACE located only within sperm cells and somatic ACE which is present in endothelial cells within the rest of the body (Metzger *et al*., 2011; Hamming *et al*., 2004). As well as being present within tissue, the soluble form of ACE is detected in the blood circulation due to shedding of the ectodomain by a disintegrin and metalloproteinase (Jia *et al*., 2009; Lambert *et al*., 2005).

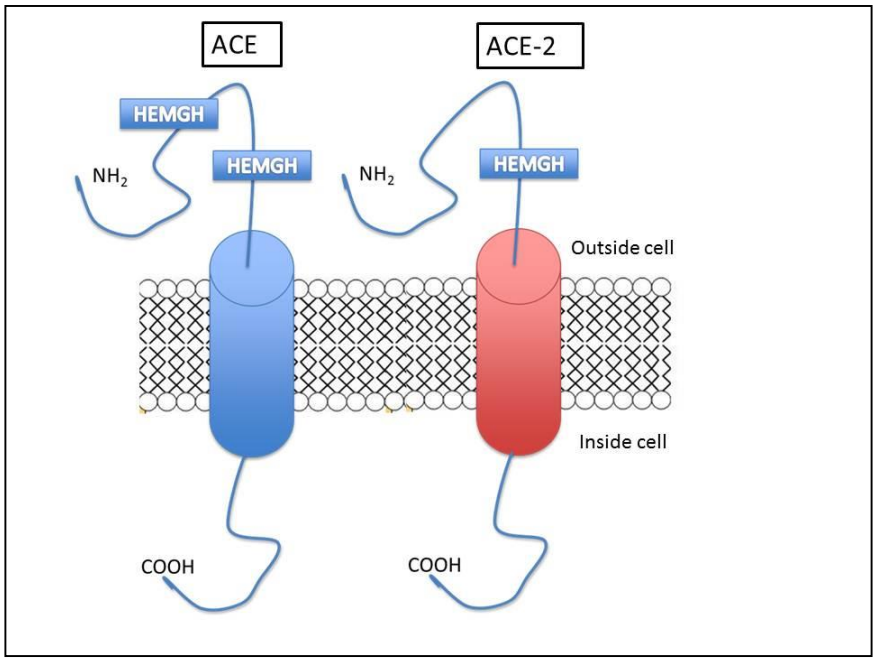

Figure 1.3 Structure of transmembrane proteins ACE and ACE-2 anchored on plasma membrane *N.B* ACE has two catalytic domains whereas ACE-2 only has one. *ACE angiotensin converting enzyme; ACE angiotensin-converting enzyme-2; HEMGH Amino acids that compose the catalytic domain*

Classically, ACE is known to cleave the Ang I peptide in the circulation by removing two amino acids from the C-terminal end to form a vasoconstrictor peptide, Ang II. Other peptides independent of RAS are also hydrolysed by ACE such as bradykinin, substance P and cholecystokinin (Fuchs *et al*., 2004). ACE function is not only limited to being catalytically active. The phosphorylation of the C-terminal tail of ACE has been demonstrated to have a role in intracellular signalling which can be affected by ACEi (Kohlstedt *et al*., 2004; Fleming, 2006). To keep the investigation simple, only the catalytic activity of ACE will be focussed on in this thesis.

ACE has been localised to vessel endothelial cells in the lung as reported by Metzger, *et al*., (2000). In this paper 12 patients who died from trauma or sudden death with no signs of pathology were sampled and the lung was the only site with consistent ACE staining of all endothelial cells compared to all other organs sampled. Another group who sampled tissue from heart-lung transplants also supported this (Orte *et al.,* 2000). The reason for death was again trauma or sudden death. The majority of the endothelium was stained for ACE except for the large preacinar vessels.

In contrast patients diagnosed with the disease pulmonary hypertension had ACE staining within the large preacinar vessels as well as in arteries that were undergoing fibrosis. ACE localisation

has not been evaluated in COPD lung therefore localising ACE in COPD lung and comparing this to COPD controls will reveal more information about whether ACE is associated with COPD or not.

ACE levels are increased in some lung diseases. The earliest paper demonstrated the serum level of ACE was elevated in patients who suffered with active sarocoidosis, a granulatamous diseases where symptoms flare primarily within the lung (Lieberman *et al.,* 1975). As a result, testing of serum ACE concentration was used as a diagnostic test for patients suspected to have active sarcoidosis (Perrin-Fayolle *et al.* 1981). Specks *et al.,* (1990) also sampled BAL fluid from 222 patients with lung damage from drug toxicity as well as diseases such as sarcoidosis and IPF. BAL ACE concentrations were higher in all lung diseases sampled compared to that of the non-smoking, healthy controls.

Although investigations have not looked into ACE activity within the lung of COPD patients, Ucar *et al.,* (1997) reported that ACE activity in the serum of COPD patients was higher than non-diseased subjects. More recent investigations into ACE and COPD have focussed on the polymorphisms of ACE in the serum as being a risk factor for COPD. The ACE gene is known to have a 287-base pair polymorphism, which is the insertion (II) genotype. Genes without this polymorphism are labelled as having the deletion (DD) genotype. Busquets *et al.,* (2007) has reported that there were more DD genotype polymorphisms in COPD smokers compared to smokers without COPD and concluded that ACE was a determining factor of COPD. More investigation into the protein expression levels in COPD compared to non-COPD is needed in order to decipher whether ACE is a target in COPD lungs as first thought of in the aforementioned ACEi clinical studies.

1.2.2.2 Chymase

Chymase, a serine protease, is also involved in the formation of Ang II (Richard *et al*. 2001). Urata, *et al*., (1990) noted that the inhibition of ACE did not sufficiently block the formation of Ang II within cardiac tissue and this group was one of the first to propose the chymase alternative pathway for Ang II conversion. Later reports suggested chymase was responsible for up to 40 % of Ang I to Ang II conversion within human tissues (Hollenberg *et al*, 1998). Chymase was reportedly able to cleave Ang I by removing aromatic residues such as tyrosine and phenylalanine (Caughey, 2007; Pejler *et al*., 2010) to leave the break-down product Ang II.

Mast cells are the only cells where chymase is stored and they are distributed throughout many organs of the body, such as skin, lung and mucosal surfaces. Mast cells degranulate when they encounter an allergen bound to the antibody immunoglobulin E during a type I hypersensitivity reaction. The degranulation releases chymase as well as other mediators such as tryptase,

histamine, cytokines, protstaglandins and leukotrienes (Pejler *et al*. 2010) for the defence against foreign allergens within the body.

This protease defines the mast cell subtype within the body. In humans, there are two types of mast cells. The first expresses the protease tryptase only (MC_T) and the second expresses both tryptase and chymase (MC_{TC}). There have been some reports that a mast cell expressing only chymase is present in the body (Weidner *et al.*, 1993). This type of mast cell has not been identified in lungs of either COPD or non-COPD subjects, confirmed using triple staining techniques (Andersson *et al*., 2009).

In lung diseases such as asthma, there seems to be a large concentration of MC_{TC} within particular areas of the lung. The small airway wall and alveolar attachments of severe asthmatics have higher numbers of MC_{TC} compared to the inner wall (Balzar *et al.*, 2005). This particular experiment was not compared to a non-diseased group; however Andersson *et al.*, (2010) did demonstrate a significant difference between asthmatic and non-diseased lungs in the number of MC_{TC} present in the alveolae of the lung. COPD patients with the most severe form of COPD also have a higher number of MC_{TC} within the lung alveolar parenchyma compared to subjects not diagnosed with COPD (Andersson *et al.*, 2009). Since MC_{TC} is increased in areas where the pathology of lung diseases occur, chymase may be cleaving Ang I in the lung and replacing the classical ACE mechanism of inducing Ang II during disease.

1.2.2.3 Angiotensin-Converting Enzyme-2

ACE-2, a homologue of ACE, is one of the most recent RAS enzymes to be discovered. ACE-2 is a zinc metalloproteinase. In the year 2000, two unrelated groups sequenced this protein from the cDNA library of ventricles from heart failure patients and also characterised its distribution within the body (Donoghue *et al*., 2000; Tipnis *et al*., 2000). Donoghue *et al*., reported ACE-2 was only conserved in the heart, kidney and testis of humans but since then it has been observed in the lung, mucosa, skin and brain (Hamming *et al.*, 2004).

The main function of ACE-2 is to convert Ang II to Ang-(1-7) peptide which has opposing effects to Ang II. ACE-2 also cleaves other peptides such as des-Arg bradykinin, apelin-13, neurotensin and dynorphin A (Vickers *et al*., 2002; Jia *et al*., 2009). Not only does ACE-2 cleave peptides but it also aids amino acid trafficking within the stomach by binding to the epithelial neutral amino acid transporter (Camargo *et al*., 2009). Furthermore ACE-2 can bind to integrins and strengthen cell-to-cell contacts (Clarke *et al*, 2012). In order to test the hypothesis of this research, this thesis will focus on ACE-2 functioning as a catalytic enzyme.

ACE-2 has been implicated mostly in the lung disease severe acute respiratory syndrome (SARS). This enzyme is identified as the protein that receives the coronavirus responsible for SARS (Wong et al. 2004). The virus binds to ACE-2 located on bronchial epithelial cells. Recombinant human ACE-2 (rhACE-2) administered to an animal model of severe acute lung injury rescued the symptoms of the disease suggesting a therapeutic role for ACE-2 in disease (Imai *et al.* 2005).

In humans, rhACE-2 has been injected into healthy volunteers to evaluate its safety and pharmacokinetics (Haschke *et al*., 2013). There seemed to be a dose dependent reduction of Ang II within 30 minutes of injection and the half-life of rhACE-2 was 10 hours. Some doses of rhACE-2 led to higher Ang-(1-7) protein levels in the blood however this increase was lowered with higher doses of rhACE-2. ACE-2 did not accumulate in the blood after repeated dosing over a week. Studies need to be carried out on patients with disease to test whether the injection of recombinant ACE-2 has any therapeutic benefit.

With regards to localisation of ACE-2, lung epithelial cells and kidney tubule epithelium are the most common locations where ACE-2 is identified (Ren *et al*., 2006). As ACE-2 is a type I transmembrane protein its structure is composed of an extracellular N-terminus and a cytoplasmic tail inside the cell (figure 1.3). This N-terminal tail also accommodates a catalytic domain. This catalytic domain shares 42 % amino acid homology with the catalytic domain of ACE (Donoghue *et al*., 2000).

The literature's exploration of ACE-2 in association with lung diseases other than SARS is limited. ACE-2 expression or activity levels in COPD lung could be at a different level compared to those lungs not diagnosed with COPD. The reason for this hypothesis is because COPD is an inflammatory disease and ACE-2 is responsible for regulating the peptides Ang II and Ang-(1-7) that have inflammatory effects. This is something that needs to be explored.

1.2.2.4 Renin

Renin, an aspartyl protease, is the oldest enzyme of the RAS and it was discovered around the year 1900 (Marks & Maxwell, 1979). Renin catalyses the conversion of the peptide angiotensinogen to Ang I. As angiotensinogen is the primary precursor protein for the formation of many RAS peptides, renin was always thought of as the rate-limiting enzyme for the production of Ang II and therefore an important influence on the RAS system.

The production and release of renin is mainly from juxtaglomerular cells lining the arterioles in the kidney. Renin is released in response to a fall in blood pressure and renin attempts to counteract this change. Often renin is first released as a preprotein form called prorenin and this

is modified to become renin in the circulation. The kidney is not the only source of renin, mast cells have also been reported to express renin protein (Silver *et al.*, 2004;Veerappan *et al.*, 2008).

In immunohistochemical experiments, the number of renin positive cells have been reported to correlate with the worsening symptoms of the lung disease COPD (Andersson *et al.*, 2010). This group investigated colocalisation of renin within lung tissue resected from subjects undergoing lobectomy. Andersson *et al.*, (2009) reported that renin was colocalised to lung mast cells in many compartments of the lung. They also followed up this finding with another paper demonstrating the number of renin positive mast cells was significantly lowered in COPD patients classified with the worst severity (GOLD IV) compared to non-COPD lung (Andersson *et al.*, 2010). Similarly Montes *et al.*, (2012) demonstrated that subjects clinically diagnosed with IPF have higher renin concentration in their lung fibroblasts when compared to non-IPF controls.

From the limited number of papers above, it seems as though renin is present within the lung and could be associated with lung diseases such as COPD.

1.2.3 Drug Treatment

Angiotensin-related medication is typically used in clinic for the treatment of blood pressure and congestive heart failure. It is associated with returning the homeostatic balance between ACE and ACE-2 and in turn the balance of the effector peptides. The two commonly used medications for the treatment of blood pressure are ACEi and ARB. Clinical studies have suggested that both medications could be beneficial for patients with COPD as mentioned previously in section 1.1.5.

1.2.3.1 Angiotensin-Converting Enzyme Inhibitors (ACEi)

ACEi are a class of oral medication encompassing captopril, benazepril and ramipril that were originally designed to treat hypertension. This was due to their main mechanism of action of blocking ACE activity (Heran *et al*., 2008). In later years, research discovered that ACEi were useful for treating diabetic nephropathy although the mechanism of action for this is unknown (NIH 2004).

There are over 25 types of ACEi prescribed in clinics in the UK. Some ACEi, have a sulfhydryl group attached to their molecular structure. Although this structure is known to cause side effects such as skin rash and neutropenia in some patients (Brown & Vaughan 1998), this entity has also been associated with reducing reactive oxygen species in blood cells (Di Napoli *et al*., 2003).

As mentioned before ACEi prescribed to patients with COPD reduced the mortality of COPD patients compared to patients not prescribed (Mortensen *et al*., 2009; Mancini *et al*., 2006). Since treatment with captopril has also been reported to lower inflammation in the mouse lung after

insult compared to no treatment (Ghosh *et al*. 2009), it suggests that the use of ACEi may resolve inflammation in the lung. ACEi therefore have the potential to resolve inflammation within the human lung disease such as COPD.

Evidence suggests that ACEi treatment seems to affect other components belonging to the RAS system. The ACEi enalapril prevented the heart failure-induced reduction of ACE-2 protein and activity in rat hearts compared to those rats which did not have ACEi treatment (Ocaranza *et al*., 2006). Furthermore, ACE-2 protein levels were elevated when the ACEi perindopril was administered to rats with liver disease compared to non-treated rats with liver disease (Huang *et al*., 2010). Not only is ACE-2 affected by ACEi but its peptide Ang-(1-7) is too. Ang-(1-7) was increased in healthy rats administered lisinopril as well as there was a reduction in Ang II protein levels in cardiac tissue (Ferrario *et al*., 2005).

1.2.3.2 Angiotensin Receptor Blockers (ARB)

ARB is another medication used to dampen the pathological effects of Ang II. Examples of this type of medication used in the clinic are losartan, telmisartan and candesartan. First available in the 1990's, these drugs competitively bind to Ang II receptors with a 10,000 higher affinity to AT_1R than angiotensin receptor type II (AT_2R) (Miura *et al*., 2011). Within the UK, 8 types of ARB are available and the all share molecular similarity based on the imidazole ring (Taylor *et al*., 2011). ARBs are prescribed to patients with hypertension, heart failure and patients with nephropathy in type II diabetes.

With regards to the lung, a study administering irebesartan to 60 COPD patients demonstrated an increase in lung capacity compared to placebo (Andreas *et al*. 2006). It was also observed that there were no differences in serum ACE activity however there seemed to be a rise in plasma concentration. In addition to this, rats pre-treated with ARB losartan followed by Ang-(1-7) treatment increased ACE-2 activity compared to vehicle controls (Wösten-van Asperen *et al*., 2011). Losartan treatment given to mice which were exposed to cigarette smoke stopped any morphological change in the lung that could be seen with no-treatment controls (Podowski *et al*., 2012). Telmisartan also reduced the proliferation and cell apoptosis in cells within lung homogenate which would have otherwise occurred without treatment (Chen *et al*. 2007; Han *et al*., 2010).

ARB seems to enhance the ACE-2/(Ang-(1-7)/ Mas axis with not much change in ACE/Ang II / AT_1 local to the site of injury in animal models. This may also occur within humans however it has not been evaluated.

In summary angiotensin-related medication seems to have beneficial effects for patients with lung diseases as well as animal models of lung disease, especially in the case of COPD. These medications affect the enzymes as well as other components of the RAS.

1.2.4 Aims and Objectives

Due to the intricate interplay of peptides of the RAS, ACEi could be affecting the formation of peptides and enzymes within the RAS network. It is these effector peptides that could be vital to the pathology process of COPD. Mediating these effector peptides are the RAS enzymes located in the lung. These enzymes have not been investigated in depth before.

This thesis will explore whether the enzymes ACE, ACE-2 renin and chymase are associated with COPD by evaluating their protein localisation, abundance and activity in human lung. This will be done in lung tissue isolated from subjects classified with COPD and compared to non-COPD controls. Any large differences between diseased and non-diseased could lead to pharmacological targets for COPD in the future.

1.2.4.1 Hypothesis

COPD patients have lower protein expression and activity levels of RAS enzymes compared to people not classified with COPD

To investigate I will attempt to answer the following research questions:

1. Is there protein localisation of RAS enzymes: ACE, ACE-2, chymase and renin in the lung of COPD patients? Are these RAS enzymes localised to the same structures that are observed in non-COPD lung?

2. Are the protein expression levels of ACE, ACE-2, chymase and renin different in lung from COPD patients compared to non-COPD patients?

3. Are the activity levels of ACE and ACE-2 different in lung of COPD patients compared to non-COPD patients?

4. Does prescription of ACEi medication or smoking affect protein expression or activity levels of ACE and ACE-2?

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Chapter 2 Materials and Methods

2.1 Immunohistochemistry

2.1.1 Materials

The following materials were used for tissue sample processing: **Acetone and inhibitors** consisted of acetone, 2 mM phenylmethylsulphonylfluoride (Sigma, Poole, UK) and 20 mM iodoacetamide (Sigma).

Cutting of sections required: **Glass microscope slides coated with poly-L-lysine 0.1 %** (Sigma) and **Ammonia:water** NH_4 (0.002 %) (Sigma). The sections on the glass slides were treated with **Endogenous peroxidase blocking solution:** sodium azide 0.1 % (Fisher Scientific, Leicestershire, UK) and hydrogen peroxide 0.3 % (Sigma), **Blocking medium** consisted of 20 % newborn calf serum in Dulbecco's Modified Eagle Medium and 1 % bovine serum albumin (Sigma).

Methyl benzoate (Fisher Scientific), **GMA Infiltration solution** consisted of 5 % methyl benzoate in glycol methacrylate solution (GMA solution JB4A) (Polysciences Inc, Northampton, UK), and GMA **Embedding solution** consisted of GMA solution A 10 mls, benzoyl peroxide 70 mg and GMA solution B 250 µl (Fisher Scientific).

Toludine blue solution Toludine blue O 1 % (Sigma) and borax 1 % (Sigma).

Primary antibodies used for immunohistochemistry were: **ACE-2** rabbit polyclonal (GR99709-4, Abcam, Cambridge, UK), **ACE** mouse monoclonal (clone 9B9, ab77990, Abcam), **AA1** mouse monoclonal (GR56194-1, Abcam), **CC1** mouse monoclonal (GR94841-2, Abcam), **EN-4 endothelial marker** mouse monoclonal (clone EN4, 60002-1, Monosan, London, UK) and macrophage marker **CD68** mouse monocolonal (clone PGM1, 00079756, Dako, Ely, UK), **Renin** mouse monocolonal (clone 2H2, AbDSerotec, Kidlington, UK) and a second **renin** mouse monoclonal (clone 7D3-E3, Abcam), **pan-cytokeratin** (clone PCK-26, Abcam) **Secondary antibodies** used were biotinylated swine anti-rabbit immunoglobulins ($F(ab')_2$ fragment) at a dilution of 1/1200 and biotinylated rabbit anti-mouse immunoglobulins ($F(ab')_2$ fragment) (Dako) at a dilution of 1/1000.

Furthermore, isotype immunoglobulin control of rabbit IgG immunoglobulins (Dako) and mouse IgG1 kappa immunoglobulin (Sigma) were used. The **VECTASTAIN® ABC complex** kit was used to amplify the signal from the secondary antibody. According to the kit instructions part A: part B: reverse osmosis water (ROW) was mixed at a ratio of 1:1:75, respectively (Vector Laboratories, Peterborough, UK).

Lastly chromogens were used; either **3,3'- diaminobenzidine (DAB)** kit (Biogenex, The Hague, Netherlands) or **3-amino-9-ethylcarbazol (AEC)** kit (Biogenex). Both chromogens were mixed according to the kit instructions. For DAB 80 μl DAB chromogen was mixed with 1 ml substrate buffer from the kit. For AEC 1 drop of AEC was added to 2.5 ml of substrate from the kit.

2.1.2 Subjects

Human lung tissue was collected from subjects (n=70) undergoing lobectomy surgery at Southampton General Hospital. Subjects were stratified into COPD and non-COPD groups by their lung function (FEV_1/FVC). FEV_1/FVC is a spirometric classification of COPD based on The Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2015). Subjects with FEV_1/FVC less than 0.69 were classified as COPD and subjects with FEV_1/FVC of 0.70 and above were classified as no COPD.

Subjects gave informed written consent. The South Central-Southampton Ethics Research Committee (reference number 08/H0502/32) gave ethical approval for this study. This enabled the study to collect lung samples from surgery, assign a participant identifier code to samples donated and use details from patient notes to analyse the data, outlined in table 2.1. The subjects included in this study were not prescribed steroids at the time of surgery and the tissue samples were taken farthest away from the site of tumour. All subjects who donated lung samples were of a severity of mild/ moderate (GOLD I/II on the severity scale).

Table 2.1 Subject characteristics of all subjects who donated lung samples in this study Lung samples donated by 70 subjects and details of these subjects are outlined *FEV_1/FVC forced expiratory volume in one second/forced vital capacity; Ex denoted subject had stopped smoking for at least one year. None never smoked; ACEi angiotensin-converting enzyme inhibitor prescribed subjects;* Data presented as mean ± s.d ****.p<0.0001 compared to non-COPD group using Mann-Whitney Test

		No COPD n=34	COPD n=36
Age (years)		66.8 ± 8.8	70.1 ± 6.57
Gender (female/male)		15 / 19	15 / 21
FEV_1 / FVC		0.78 ± 0.05	0.62 ± 0.06****
Smoking Status	**Current (%)**	26.4	41.6
	Ex (%)	50.0	50.0
	None (%)	17.6	8.3
ACEi Medication (%)		38.2	36.1

For preliminary runs to identify renin within lung tissue, paraffin-embedded renal tissue was used alongside lung tissue. The renal tissue was donated by a patient undergoing surgery at Southampton General Hospital (n=1). The subject gave informed written consent and the study was approved by the South West Hampshire Ethics Committee. The ethical approval enabled collection of sample from discarded nephrectomy specimens removed for isolated adenocarcinoma. The renal tissue was processed by the Southampton Histochemistry Research Unity team (HRU) and stored at -80^{o}C. For consistency, control lung samples - for renin staining only - were also paraffin-embedded by the HRU. Although the renal tissue was processed and immediately frozen six months before the lung tissue, freezing at -80^{o}C has been shown to maintain the quality of proteins and RNA in tissue samples for up to 5 years (Shabihkhani *et al.*, 2014). Renal tissue stored six months in the freezer therefore was not thought to be different to fresh tissue.

2.1.3 Sample Processing

2.1.3.1 Glycol Methacrylate (GMA) Embedding

Tissue samples of fresh parenchymal and bronchial lung tissue were dissected into 2 mm^3 pieces. These pieces were fixed in acetone and protease inhibitors overnight at -20^oC. The next day, the solution was replaced by fresh acetone for 15 minutes and then methyl benzoate for a further 15 minutes.

These tissue samples were then placed in the GMA infiltration solution at 4^oC for 2 hours. This infiltration solution was replaced twice at 2-hour intervals. After 6 hours of infiltration, each piece of tissue was placed into an individual flat-bottomed capsule and the embedding solution was added to this. This was polymerised at 4^oC for 48 hours. The polymerised blocks were then stored in airtight boxes at -20^oC, until required.

2.1.3.2 Check Morphology with Toludine Blue

Five GMA polymerised blocks with tissue sample were stored at -20^oC from each subject. To select the tissue sample with appropriate section morphology for immunohistochemistry the sections from each block had to be stained with toludine blue. Each block was trimmed and filed down for microtome cutting. Two sections for each block were cut from the block face, floated in water, picked up with poly-L-lysine coated glass slides and left to dry at room temperature.

Toludine blue solution was then applied to the sections on the slide and left for 30 seconds on a hot plate. The sections were then washed under cold running water and left to dry. Sections were evaluated under the microscope. Requirements for appropriate morphology of tissue samples were as follows; for lung parenchymal samples, alveolar spaces and capillaries were visible and these structures were at least 50 % of the tissue block face. For bronchial samples, an intact epithelium next to submucosa was required. Appropriate tissue samples were noted down, cut by the microtome and used for antibody staining.

2.1.3.3 Cutting GMA Sections with Microtome

Selected blocks of embedded tissue sample were cut into 2 μm sections. The Reichert Ultracut S microtome (Leica Microsystems Gmbh, Germany) was used to cut 2 μm sections of tissue from the block face. These sections were floated in ammonia:water and picked up with poly-L-lysine coated glass slides. These slides were dried at room temperature, wrapped in aluminium foil, stored at -20^oC and used within two weeks.

2.1.3.4 Antibody Staining

Slides were removed from the -20ºC freezer and allowed to reach room temperature, before proceeding with the immunohistochemical technique. Applying the endogenous peroxidase blocking solution onto slides for 30 minutes at room temperature inhibited enzyme activity within the tissue that could interfere with the technique. This was then washed off with tris-buffered saline (TBS) three consecutive times with 5-minute intervals between washes. The slides were then treated with a blocking medium for a further 30 minutes.

Primary antibodies were then applied to the slides to detect enzymes associated with RAS and they were diluted in TBS. The primary antibodies were against ACE (1/900 dilution), chymase (1/300 dilution) and ACE-2 (1/400 dilution) alongside TBS controls. In the same run antibodies detecting endothelial cells (EN4; 1/300 dilution), mast cells (AA1; 1/20,000 dilution) and macrophages (CD68; 1/100 dilution) were also applied to the slides. For primary antibodies against renin several runs were done with 2-fold dilutions of each renin antibody – 7D3 E3 clone and 2H2 clones – ranging from 1/100 - 1/2000, alongside TBS controls and pan-cytokeratin positive marker for epithelial cells (1/1000 dilution).

These primary antibodies were incubated with tissue overnight at 4ºC except for ACE-2 polyclonal, which was left over night at room temperature. Isotype controls were also applied to preliminary slides to rule out non-specific binding.

The following day, all primary antibodies were washed off with TBS and the sections were incubated with the appropriate biotin conjugated secondary antibody for 2 hours. This secondary antibody was then washed off and the VECTSTAIN ABC complex was applied to amplify secondary antibody. This remained on the slide for a further 2 hours and the excess was washed off with TBS.

To enable visualisation of the protein of interest using a light microscope, a substrate-chromogen was applied. These chosen chromogens; either DAB or AEC were incubated on slides at room temperature for 10 minutes and 20 minutes, respectively. After this, slides were rinsed under cold running water. Slides were then counterstained with Mayer's haemotoxylin. Afterwards, aqueous mounting medium was applied onto these slides and they were baked for 20 minutes at 80ºC. Slides were then left to cool before placing cover slips over the sections on slides with the aid of pertex.

Prior to analysis, to eliminate bias, the slides were coded so the subject could not be identified. Subsequently, the assessor counted ACE and EN4 positive vessels for each section. Counts were also taken for the ACE-2, CD68, AA1 and CC1 positive nucleated cells.

The area of the parenchymal tissue was then measured using the image analyser (Image Associates, Bicester, UK) using the *tolbluearea* macro within the KS400 computer software (Carl Zeiss Systems, Cambridge, UK). The macro measured a baseline white balance *i.e.* a slide without any tissue present. Tissue darker than the baseline white balance was then measured on each slide in square millimetres.

Using the image analyser was an efficient method to measure parenchymal tissue area since this type of lung tissue can be difficult due to unpredictable gaps in the alveolar tissue: air spaces for gases to move through. This measurement was then used to normalise the positive counts (per mm^2).

2.1.3.5 Statistical Analysis

All graphs were plotted with the median and range unless otherwise stated. The Kruskal-Wallis test and the Mann Whitney-*U* test were used to analyse all subject group data unless specified. The test for normality was carried out using Prism version 6.0.

2.2 Activity Assays

2.2.1 Materials

The following materials were used for the protein assay: **BCA protein assay reagent** (Fisher), **Human serum albumin standard** (Sigma).

The following materials were used for the ACE assay: **Incubation buffer** contained 0.2 M H_3BO_3 and 2.0 M NaCl at pH 8.3, **Diluent buffer** contained 0.2 M KH_2PO_4 at pH 8.3. **Hippurate standards** contained 2.5 mM benzoylaminoacetic acid $C_9H_9NO_3$ and 1 M NaOH, **Hippuryl-L-histidine-L-leucine acetate salt** 20mM (Sigma), **Termination solution** 1 M HCL, **Colour reagent** contained 0.16 M cyanuric chloride (Fisher Scientific) diluted in 1,4-dioxane (99 %) (Fisher Scientific), **Captopril** (Sigma).

The following materials were used for the ACE-2 assay: **Incubation buffer** contained 2.5 mM $ZnCl_2$ 75 mM Tris-HCl; and 1 M NaCl at pH 6.5, **Mca-APK(Dnp)** (Cambridge Bioscience, Cambridge, UK), **DX-600** (Cambridge Bioscience).

2.2.2 Homogenisation of Lung Tissue

Fresh human lung tissue samples were collected as described in section 2.1.2. These tissue samples were blotted on tissue paper for the removal of excess blood and weighed (0.5 ± 0.02 g). Samples were then stored in bijoux containers at -80ºC until required. The human lung tissue sample was thawed, finely chopped into small fragments and pulverised by using a pestle and mortar. This tissue was then mixed with an incubation buffer (2.5 ml). This suspension was transferred to a bijoux container to be blended with a hand-held electric tissue homogeniser until an even suspension was achieved. The homogenised sample was stored at -80ºC overnight and thawed the next day in order to disrupt cell components. The sample was then homogenised again and spun down at 20,000 x g (Beckman rotor JA-20). An aliquot of the supernatant was diluted in deionised water at a 1:40 dilution in order to measure the concentration of protein by the bicinchoninic acid protein assay. The remainder of the supernatant was aliquotted and stored at -80ºC until required.

2.2.3 Bicinchoninic acid (BCA) protein assay

In order to determine the protein concentration within the homogenate sample, a BCA protein assay was used. The tissue homogenate was diluted in reverse osmosis water (1:40). This sample (50 µl) was pipetted into a plate well. On the same plate, known concentration of human serum albumin (0 – 2 mg/ml) was also pipetted onto the plate. All wells were then mixed with 100 µl of BCA solution and read at a wavelength of 562 nm. The absorbances of the samples were extrapolated from the standard curve and the protein was expressed as mg/ml. Each sample /standard was done in triplicate.

2.2.4 ACE Activity Assay

ACE activity was measured using a technique adapted from Watkins *et al.*, (2008). The thawed human lung sample supernatant (50 µl) was incubated with the substrate hippuryl-L-histidine-L-leucine acetate salt (HHL) (10 µl) for 15 minutes at 37ºC. The reaction was stopped with HCl (0.5 ml), neutralised with NaOH (0.5 ml) and then diluted with KH_2PO_4 buffer (2 ml). After this, 1.5 ml of colour reagent was added to the mixture to bind to the hippurate formed. The absorbance of this mixture was detected by the spectrophotometer at a wavelength of 382 nm. A standard curve was formed using known concentrations of hippurate as the standard. The concentration of standards ranged from 0 to 200 mM with gradual increase 20 mM per time. The sample was

diluted in incubation buffer at pH 8.3. From the hippurate standard curve the amount of hippurate formed was calculated. This was then expressed as $nmol.ml^{-1}min^{-1}$.

2.2.5 ACE-2 Activity Assay

The ACE-2 activity assay for samples was based on Pedersen *et al*., (2011). The substrate used for measuring ACE-2 activity was 7-methoxycoumarin-4-yl-Ala-Pro-Lys-(2,4-dinitrophenyl) [Mca-APK(Dnp)]. Mca-APK(Dnp) is comprised of a fluorogenic peptide: 7-methoxycoumarin-4-yl (MCA). As a substrate the fluorescence of MCA is quenched by the dinitrophenyl (Dnp) moiety. Once the substrate is cleaved by ACE-2 at the amino acid Pro-Lys bond, MCA is released and fluorescence is emitted. In my experiments, a fluorescence plate reader measured this fluorescence as relative fluorescence units (RFU). The plates were read at an excitation wavelength of 320 nm and an emission spectrum wavelength of 405 nm.

Black-clear bottom microplates were used for this assay. In each well, the tissue supernatant (1500 μg protein) was incubated with the Mca-APK(Dnp) substrate (10 μM). The inhibitor of ACE-2, DX-600 (0.1 μM), was also incubated in order to demonstrate that this assay was specific to ACE-2.

2.2.6 Statistical Analyses

All experiments for ACE and ACE-2 activity in human lung were done in triplicate. For the ACE activity assay the activity was presented as 1 nmol of hippurate released from the substrate HHL per minute per mg of protein homogenate at 37°C ($nmol.min^{-1}$ mg^{-1}). For the ACE-2 assay, an arbitrary unit represented the release of the fluorescent peptide Mca-APK-OH in fluorescence units per minute per μg of protein ($RFU.min^{-1}$ μg) at 37°C. The Mann Whitney-*U* test was used to analyse all subject group data unless specified. The counts were presented as median (range) unless otherwise stated.

2.3 ACE-2 and Macrophages

2.3.1 Materials

The materials used for isolating macrophages from human lung tissue samples were as follows: **Red blood cell lysis buffer** containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA. **1 x Phosphate buffered saline** (Sigma)

The materials used for the monocyte-derived macrophage experiment were as follows: **Ficoll-Paque plus** (GE Lifesciences, Little Chalfont, UK), **anti-CD14 magnetic-activated cell sorting beads** (Miltenyi Biotech, Surrey, UK), **RPMI-1640 medium** (Gibco, Invitrogen, Paisley, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 250 ng/ml fungizone and 10 % fetal bovine serum. All of these supplements were ordered from Gibco (Invitrogen), **Cell dissociation solution non-enzymatic 1x bioreagent** (Sigma).

The materials used for the cigarette-smoke treatment of macrophages were as follows: **Viceroy dual charcoal filter cigarettes** (Brown and Williamson, Kentucky, USA), **Hank's balanced salt solution** (Life Technologies, Paisley, UK), **Sterile Acrodisc filter** (0.2 µm) (GelmanSciences, Northamptonshire, UK).

The materials used for immunocytochemistry were listed in methods and materials section 2.1.1 with the addition of **Avidin/Biotin blocking kit** (Vector Labs).

Primary antibodies used were **CD68** monoclonal (clone PGM1, 00079756, Dako) and **ACE-2** polyclonal (GR99709-4, Abcam). Secondary antibodies used were biotinylated swine anti-rabbit immunoglobulins ($F(ab')_2$ fragment) at dilution of 1/1200 and biotinylated rabbit anti-mouse immunoglobulins ($F(ab')_2$ fragment) (Dako) at dilution of 1/1000.

Incubation medium for non-specific esterase consisted of sodium phosphate dibasic (Na_2HPO_4), α-naphthyl acetate (Sigma), sodium nitrite (Fisher Scientific), pararosaniline hydrochloride (Sigma) and hydrochloric acid. For permeabilisation of cells methanol (Fisher Scientific) was used.

2.3.2 Macrophages from Lung Tissue

The isolation of lung macrophages from fresh tissue was based on the non-enzymatic method as described by Liu & Proud, (1986). Briefly, the sample of human lung parenchymal tissue (4.2 g) was cut into small fragments, placed into a sieve and rinsed with approximately 500 ml of cold sterile phosphate buffered saline (PBS) solution. The solution from the rinsed tissue was centrifuged at 400 x g (4ºC) and the pellet was resuspended with red blood cell lysis buffer solution for 5-10 minutes. The cells were centrifuged a second time and the pellet was resuspended into PBS. This resuspension was then cytocentrifuged onto poly-L-lysine coated glass slides (3×10^4 cells/slide) in preparation for immunocytochemistry.

2.3.3 Monocyte-derived Macrophages (Mdm)

2.3.3.1 Isolation of Monocytes from Blood

Volunteers who were healthy donated blood samples. They gave informed written consent. The Southampton and South West Hampshire Research Ethics Committee (reference number 08/H0504/138) gave ethical approval for this study. This enabled the study to collect blood samples from volunteers, assign a participant identifier code to samples donated and use details given in the consent form to analyse the data, outlined in table 5.1.

The Staples laboratory isolated monocytes from peripheral blood donated by healthy volunteers In brief, they used Ficoll-Paque density centrifugation to separate blood lymphocytes from other components of the blood. Monocytes were then isolated from blood lymphocytes using anti-CD14 magnetic-activated cell sorting beads (MACS). These isolated monocytes were then given to myself. I counted the cells, centrifuged them at 400 x g, at 4ºC for 5 min and resuspended the pellets into a volume of RPMI 1640 that resulted in a concentration of 1 x 10^6 cells/ml.

2.3.3.2 Maturation of Macrophages by GM-CSF

I prepared and cultured these isolated monocytes for the experiments described in this thesis. The monocytes (5 x 10^5 cells per well) were plated into 24-well plates. Maturation of these monocytes was adapted from the method that was previously described by Tudhope & Finney-Hayward (2008). Monocytes were treated with GM-CSF (2 ng/ml) for 12 days to generate macrophages with a similar phenotype to alveolar macrophages.

On days 0, 7 and 12 after plating, a proportion of cells were removed from the wells by a non-enzymatic cell dissociation solution. These cells (3 x 10^4 cells/slide) were spun using a cytocentrifuge onto poly-L-lysine coated glass slides in preparation for ACE-2 detection by immunocytochemistry. The slides were air-dried for 45 minutes before storing at - 20ºC.

2.3.4 Cigarette Smoke Extract

Cigarette smoke extract (CSE) was prepared immediately before application to the cultured macrophages. This preparation was based on the method used by Aoshiba *et al*., (2001). In brief, cigarette smoke was drawn from the mouthpiece of a cigarette into a 50 ml syringe. This cigarette smoke was then bubbled through a glass apparatus containing 20 ml of Hank's balanced salt solution equating to one puff (figure 2.1). Consecutive draws and puffs of cigarette smoke were

carried out until the cigarette was reduced to a size of 3 cm in length. Two cigarettes were passed through the media and each cigarette generated an average of ten puffs.

This media was then poured through a sterile filter (pore size 0.2 µm) and the filtrate was considered as 100 % cigarette smoke extract. This was then diluted with complete RPMI to a 10 % concentration and applied to the cultured Mdms, immediately. This CSE was applied to Mdm isolated from the blood of healthy volunteers and also applied to lung fragments donated by subjects undergoing surgery as explained above in section 2.1.2.

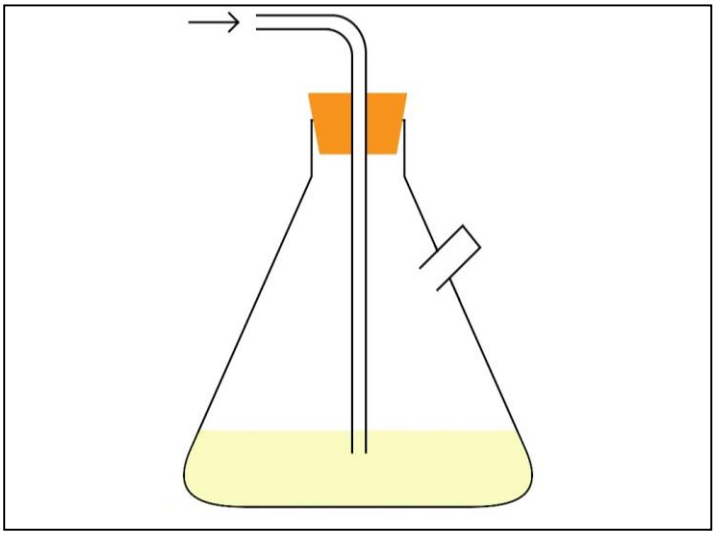

Figure 2.1 Diagram of the apparatus used to condense cigarette smoke Mainstream smoke from one cigarette collected by a 50 ml syringe was bubbled through HBSS media in the direction of the arrow.

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Chapter 3 Location of RAS Enzymes in Lung

3.1 Introduction

Assessing lung histology in a disease such as COPD is vital for understanding the mechanisms that underlie these conditions. The structure of the lung tissue in COPD can vary from patient to patient; destruction of alveolar tissue is observed in some COPD patients. Fibrosis of the small airways is apparent in others and some COPD patients exhibit a combination of the two (Kim & Criner, 2013). These types of histology that contribute to airway obstruction are due to inflammation and subsequent aberrant repair.

Ang II can cause inflammatory or fibrotic damage similar to that observed in COPD. For example, Ang II has been shown to stimulate superoxide formation causing tissue damage and subsequent inflammation (Rueckschloss *et al.*, 2002). Ang II has also been shown to stimulate the chemotaxis of human leukocytes *in vitro* suggesting its involvement in the inflammatory response (Jurewicz *et al*., 2007). Lastly, Ang II induces production of increased amounts of collagen from cardiac fibroblasts compared to those fibroblasts not treated with Ang II (Ljinjen *et al.*, 2006).

It is possible that interfering with mechanisms that cause Ang II-induced damage could modify the development of COPD. The manipulation of enzymes responsible for the production (ACE, chymase and renin) and the break-down (ACE-2) of Ang II may alter the levels of Ang II, which may lead to an improvement in COPD symptoms.

ACE protein localisation in areas of lung destruction of COPD has not been investigated previously. One study has demonstrated ACE localisation within human lung but this tissue had no histological signs of COPD (Metzger *et al.*, 2011). ACE was localised to endothelial cells of the capillaries in the lung.

This chapter aimed to assess the expression of ACE along with chymase, renin and ACE-2 within the human lung and investigate whether the expression/localisation of these enzymes differs between COPD subjects and non-COPD controls. Furthermore, it explored the effect of prescribed angiotensin-related medications due to their potential direct effect on lung RAS enzymes. The smoking status of subjects was also investigated for its effect on the expression of these lung enzymes because smoke inhalation can cause inflammation and this could affect the the protein expression levels of RAS enzymes. RAS is involved in inflammatory processes so there could be a difference of ACE, chymase, renin or ACE-2 in subjects who smoke compared to subjects who have given up smoking (ex-smoker).

3.2 Methods

3.2.1 Subjects

Human lung tissue was collected from subjects (n=63) undergoing lung tissue resection surgery at Southampton General Hospital. For some subjects, the quality of the sections when analysed for IHC were not of good quality and were therefore omitted. Subject characteristics are in table 3.1.

Table 3.1 Characteristics of subjects who donated lung samples used for IHC ACE, chymase and ACE-2 protein expression was evaluated in lung samples donated by 63 subjects. Details of these subjects are outlined in the following table. See table 2.1 for the explanation of acronyms. Some subjects did not have smoking status written in their notes (n=2 in non-COPD group). Data presented as mean ± s.d **** = $p<0.0001$ compared to non-COPD group using Mann-Whitney Test.

		No COPD n=30	COPD n=33
Age (years)		66.8 ± 9.3	69.6 ± 6.4
Gender (female/male)		14 / 16	12 / 21
FEV_1 / FVC		0.78 ± 0.05	0.62 ± 0.06****
Smoking Status	**Current (%)**	26.7	39.4
	Ex (%)	46.7	54.5
	None (%)	13.3	6.0
ACEi Medication (%)		43.3	39.4

3.2.2 Immunohistochemistry

Lung parenchymal samples were processed, checked for morphology and stained with primary antibodies against ACE, chymase (CC1), renin and ACE-2 by immunohistochemistry alongside TBS controls. For more detail refer to section 2.1. In a subpopulation of these subjects, bronchial tissue was also available (n=6). Counts were also taken for the ACE-2, CD68, AA1 and CC1 positive nucleated cells. All counts were then normalized to the area of the measured tissue (per mm^2).

3.2.3 Camera Lucida

3.2.3.1 Colocalisation

To explore colocalisation of stained cells in sequential lung sections the Camera-Lucida System was used (Leica Microsystems, Milton Keynes, UK). The camera lucida is a useful tool to investigate colocalisation of positively stained cells in tissue (Bradding *et al*. 1993).

In brief, a lung section stained for ACE-2 using immunohistochemistry was placed under the microscope. The slide under the microscope was projected onto an acetate sheet and the assessor traced around the projected image and marked the positively stained cells. Subsequent sequential slides stained for CD68 were projected onto the same acetate sheet, aligned and positive cells were marked with a different colour. For each subject, three areas were chosen from each lung sample section and traced onto three separate acetate sheets viewed using a 40 x objective. Tallies were then taken as described below.

3.2.3.2 Analysis

For each subject, the total number of single ACE-2 and CD68 positive macrophages were tallied. In addition, cells that were positive for both ACE-2 and CD68 were tallied and termed 'colocalised'. ACE-2 positive cells were also present on the alveolar epithelium and these were tallied too.

The totals of these tallies were used to calculate the percentage ratio for the number of:

ACE-2 positive cells that were colocalised to CD68 positive macrophages i.e. (colocalised total / ACE-2 total number x100)

ACE-2 positive cells present in the epithelium i.e. (ACE-2 number localised to the alveolar epithelium / ACE-2 total number x 100).

ACE-2 positive cells location unknown i.e. [ACE-2 total number – (colocalised total + ACE-2 number localised to the alveolar epithelium) / ACE-2 total number x100]

CD68 positive macrophages not colocalised with ACE-2 positive cells i.e. (colocalised total / CD68 total number x100).

CD68 positive macrophages colocalised with ACE-2 positive cells i.e. [(CD68 total number - colocalised total) / CD68 total number x100].

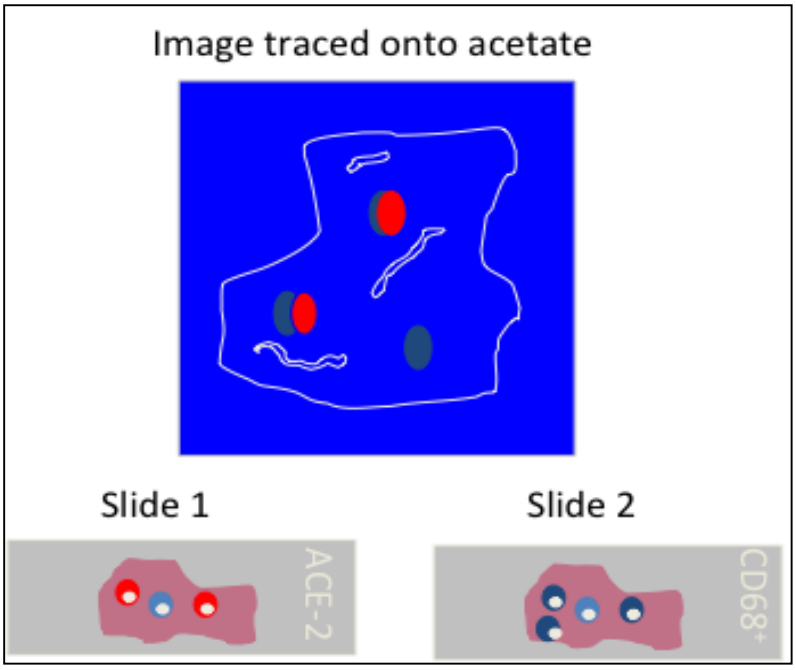

Figure 3.1 Diagram for the description of the camera lucida technique. Areas of the slides above are drawn onto the acetate sheet with the same orientation to investigate colocalisation. ACE-2 slide is the first slide with the protein of interest; CD68 second sequential slide stained with the macrophage marker

3.2.4 Statistical Analyses

All graphs were plotted with the median and range unless otherwise stated. The Kruskal-Wallis test and the Mann Whitney-*U* test were used to analyse all subject group data unless specified. The counts were presented as median with a range unless otherwise stated.

3.3 Results

3.3.1 Angiotensin-Converting Enzyme Staining

3.3.1.1 ACE staining within the human lung

ACE staining was detected in both the bronchial and alveolar parenchymal tissue excised from resected human lung. Both types of tissue sample had ACE staining localised to blood vessels (figure 3.2 & 3.3).

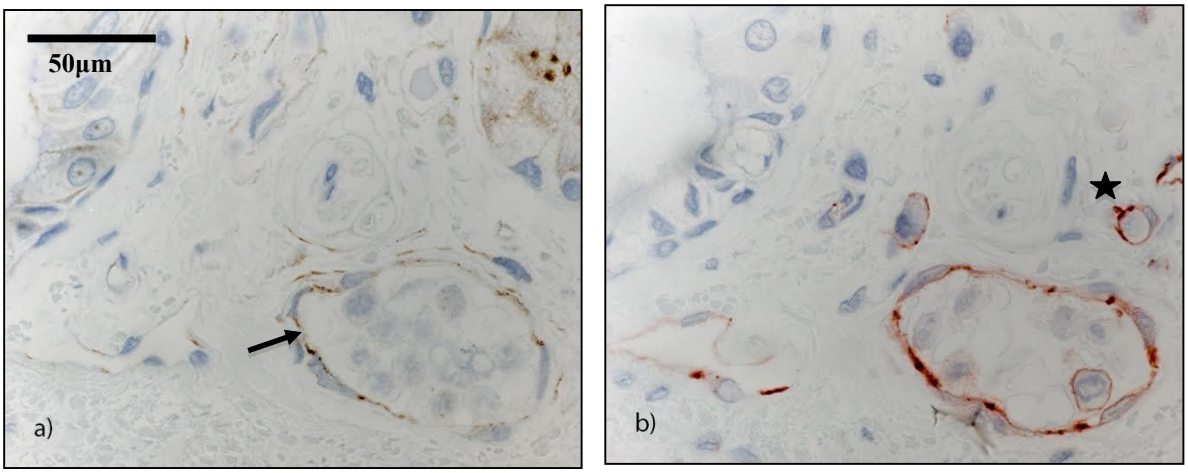

Figure 3.2 Micrograph of ACE staining in bronchial tissue Representative images stained for a) ACE using DAB b) EN4 (an endothelial marker) using chromogen AEC (red). Black arrow indicates ACE and EN4 stained vessels. Star indicates vessels stained with EN4 but not ACE.

Figure 3.2 shows ACE staining within the submucosa on blood vessels within the bronchial tissue. The EN4 marker was used to identify these blood vessels. Not all EN4 positive blood vessels present in the bronchial tissue were positive for ACE.

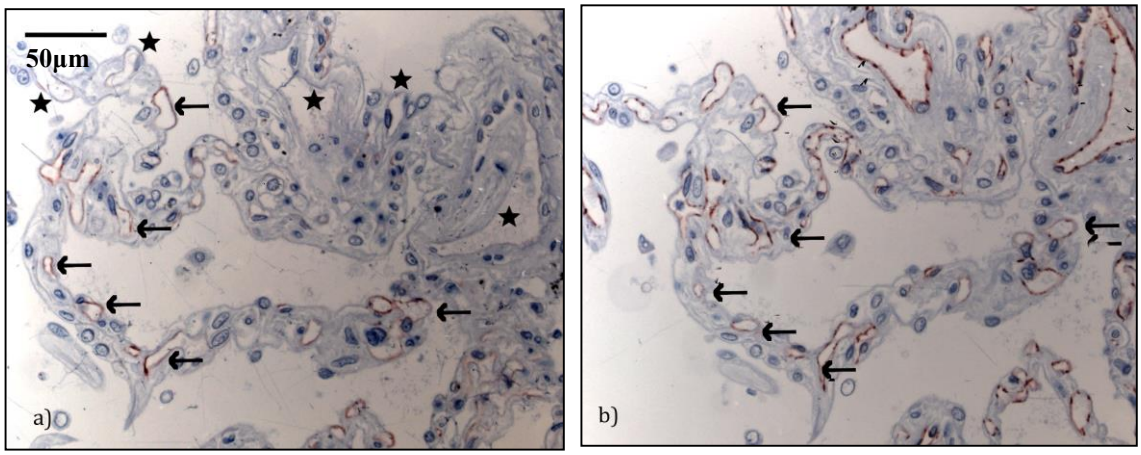

Figure 3.3 Micrograph of ACE staining in alveolar parenchymal tissue Representative images stained for a) ACE b) EN4 (an endothelial marker) using chromogen AEC (red). Black arrow indicates ACE and EN4 stained vessels. Star indicates vessels stained with EN4 but not ACE.

Figure 3.3 shows that ACE staining in the parenchyma was only limited to blood vessels. ACE staining of these alveolar capillaries and blood vessels was dense, with a similar pattern of staining to the pan-endothelial marker EN4, as shown by the black arrows in figure 3.3b. Some vessels within the lung parenchyma were not stained with ACE and these often were larger in diameter than the vessels positively stained with ACE, as shown by the black stars in figure 3.3a.

3.3.1.2 ACE staining in different lung sites

ACE staining in lung from subjects who donated both bronchial and parenchymal tissue were quantified as shown in figure 3.4. The number of ACE vessels were normalised to the number of EN4 positive vessels.

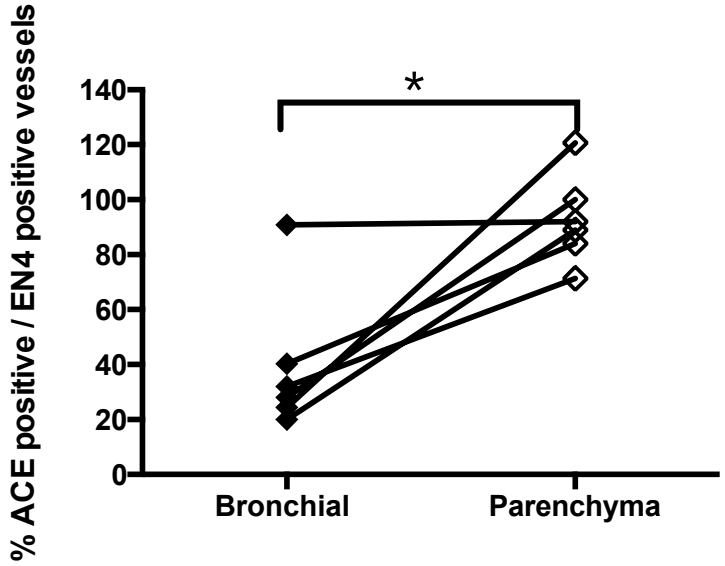

Figure 3.4 The proportion of EN4 vessel numbers stained with ACE in different locations of human lung The number of positive vessels for ACE were divided by the number of positive vessels stained with EN4 and a percentage was plotted for each subject (n=6). Each line connected the percentages from bronchial and parenchymal tissue from one subject. * $p<0.05$ Wilcoxon signed-rank

Figure 3.4 shows the proportion of EN4 positive vessels stained with ACE in the bronchial tissue was significantly lower ($p<0.05$) than in the parenchymal tissue. There was no demographic trait that differentiated the subject who had 82 % ACE positive vessels in their bronchial tissue. The subject was female, aged 64 years, a current smoker and was classified as having COPD according to their spirometry.

Due to easier availability of parenchymal tissue and the higher numbers of ACE positive vessels, the parenchymal tissue was chosen to assess the ACE staining between COPD and non-COPD subjects (figure 3.5).

3.3.1.3 ACE staining in COPD lung

All ACE staining was localised to vessels in both COPD and non-COPD lung sections analysed therefore there was no difference in localisation of ACE between these two groups.

More subjects than in the previous figure were available to provide parenchymal sections of lung and these sections were stained with the ACE antibody to investigate the protein expression levels of ACE. These subjects were classified by lung function into COPD or non-COPD groups (figure 3.5).

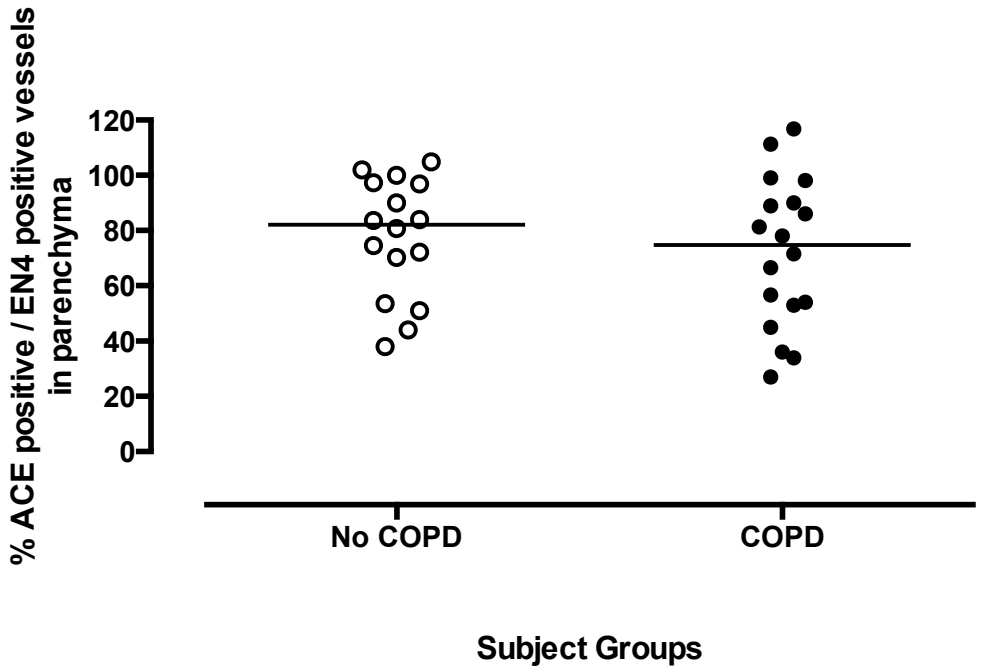

Figure 3.5 ACE staining in human lung from COPD subjects ACE positive vessel counts as a percentage of EN4 positive vessel counts in alveolar parenchymal tissue. Each plot represents one subject and subjects were classified into COPD (n=18) and non-COPD (n=16) groups. Mann Whitney Test p = 0.54 COPD chronic obstructive pulmonary disease; ACE angiotensin-converting enzyme

Between COPD and non-COPD groups, the proportion of EN4 positive ACE stained vessels were not different [COPD 74.9 (27.0 - 116.8) vs. non-COPD 82.2 (38.0 - 104.8) %].

There was also no significant difference in the counts of ACE positive vessels per mm^2 in the parenchyma between COPD and non-COPD groups [COPD 268.2 (40.0 - 612.5) vs. non-COPD 202.1 (26.0 - 696.0) per mm^2].

3.3.2 Chymase Staining

3.3.2.1 Micrographs of chymase staining within the lung

The conversion of Ang I to Ang II is a hydrolysis reaction and can be performed by other enzymes such as chymase. Mast cells expressing chymase and tryptase are termed MC_{TC} and are different from mast cells that express tryptase only (MC_T). As there were no mast cells only expressing chymase present in lung samples analysed in this study, tryptase staining was used as a marker to identify the total number of mast cells within the lung parenchyma.

Chymase staining was apparent only on mast cells. These cells were present within the submucosa of bronchial tissue samples (figure 3.6) and the alveolar parenchymal tissue (figure 3.7).

Figure 3.6 Micrograph of chymase staining in bronchial tissue Representative images stained for a) CC1 b) AA1 (a mast cell marker) using chromogen AEC (red). Black arrows indicate CC1 and AA1 stained cells.

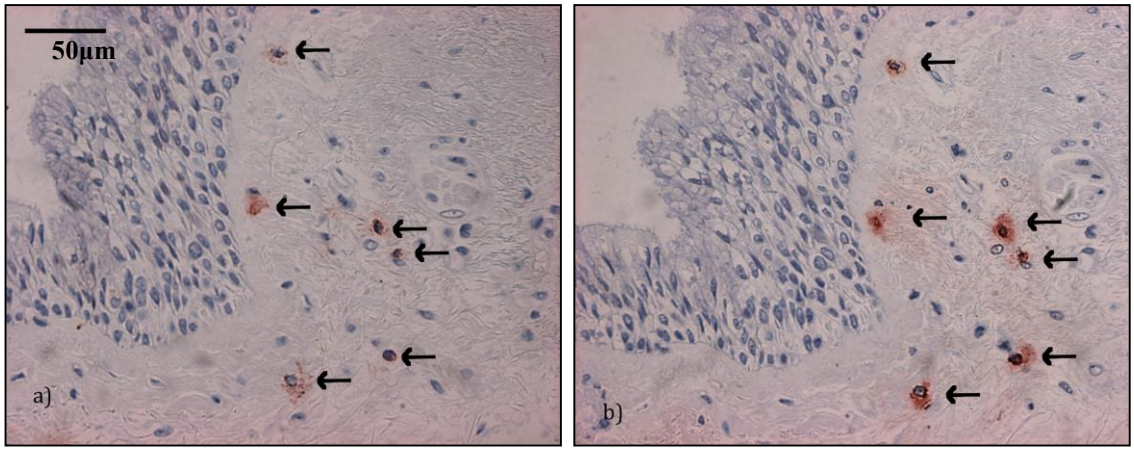

Figure 3.6 shows MC_{TC} and MC_T were localised within the submucosa. There were no mast cells identified in the intact epithelial lining of the bronchial tissue in any of the six sections belonging to the subjects analysed. MC_{TC} numbers were also evaluated in the lung parenchyma as shown in figure 3.7.

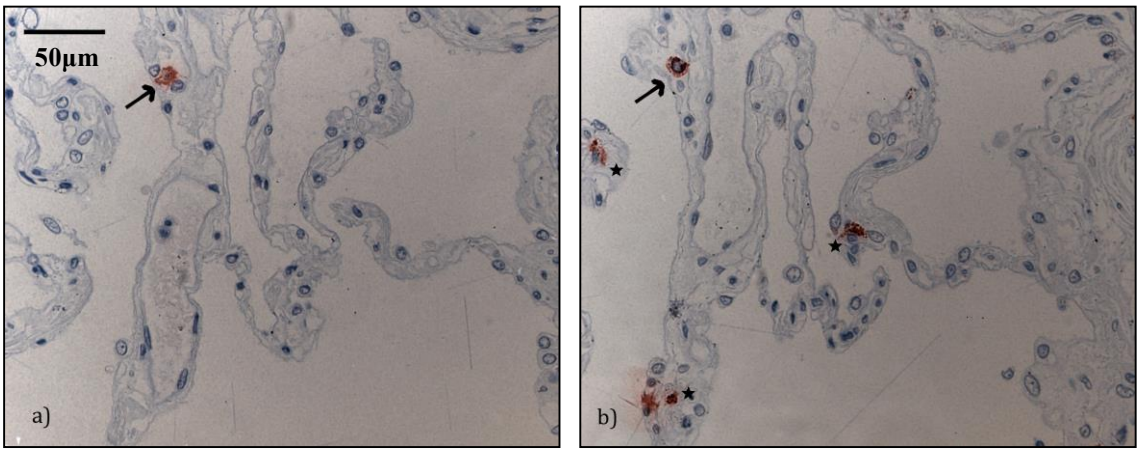

Figure 3.7 Micrograph of chymase staining in alveolar parenchymal tissue Representative images stained for a) CC1 b) AA1 (a mast cell marker) using chromogen AEC (red). Black arrow indicates CC1 and AA1 stained vessels. Star indicates cells stained with AA1 but not CC1.

Figure 3.7 shows that within the parenchyma, MC_{TC} cells were present within the alveolar walls. These cells mostly congregated around pulmonary vessels and small airways. This staining was quantified as shown in figure 3.8.

3.3.2.2 Chymase staining in COPD lung

Chymase staining was only localised to mast cells in sections from both COPD and non-COPD lung therefore there was no difference in localisation of chymase between these two groups.

MC_{TC} staining was quantified in parenchymal tissue from the same subjects who were evaluated for ACE staining, previously (figure 3.5) to investigate protein expression levels. The subjects were classified into COPD and non-COPD subject groups.

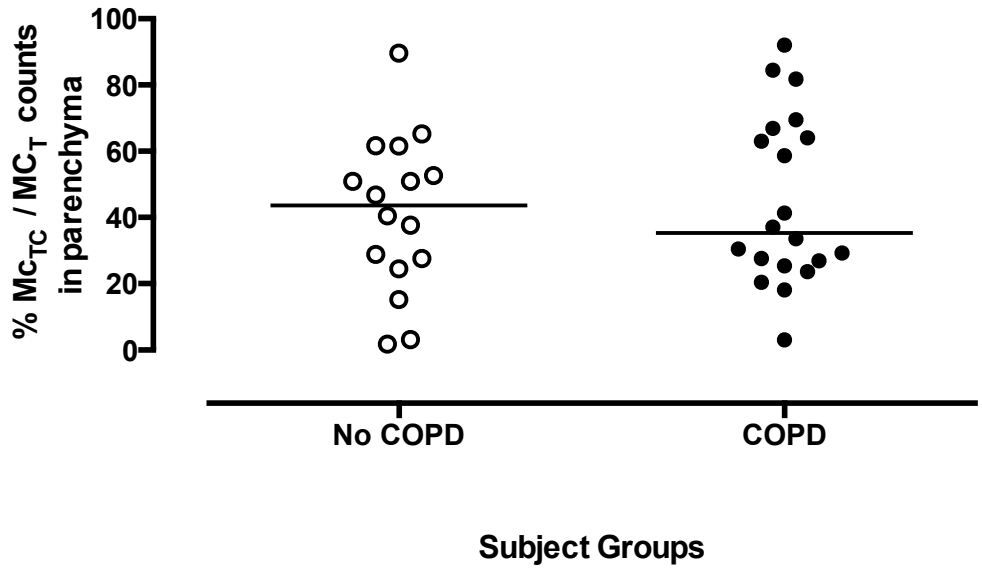

Figure 3.8 Chymase staining in human lung from COPD subjects Chymase positive mast cell counts (MC_{TC}) as a percentage of tryptase positive mast cell counts (MC_T) in the lung. Each plot represents one subject and subjects were classified into COPD (n=20) and non-COPD (n=16) groups. Mann Whitney Test p = 0.75 COPD; MC_{TC} tryptase and chymase positive mast cell; MC_T tryptase positive mast cell

Figure 3.8 shows that between COPD and non-COPD groups, the proportion of chymase positive cells (MC_{TC}) were not different [COPD 35.4 (3.0 - 92.0) vs. non-COPD 43.7 (1.8 - 89.6) %].

3.3.3 Angiotensin-Converting Enyzme-2

3.3.3.1 Micrographs of ACE-2 staining within the human lung

As with ACE and chymase (figure 3.2 & 3.6), ACE-2 was detected in both bronchial and parenchymal tissue. In bronchial tissue, dense ACE-2 staining was detected in the epithelial lining of the lumen figure 3.9.

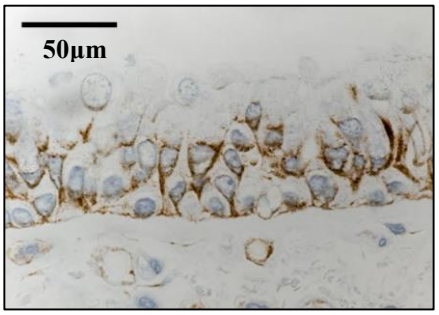

Figure 3.9 Micrograph of ACE-2 staining in human bronchial tissue A representative image of bronchial epithelium. DAB (brown) was the chromogen used to identify ACE-2.

In parenchymal tissue, ACE-2 was detected within alveolar epithelial cells, alveolar macrophages and blood vessels (figure 3.10). ACE-2 staining within blood vessels was faint so the analysis was focused on ACE-2 positive nucleated cells.

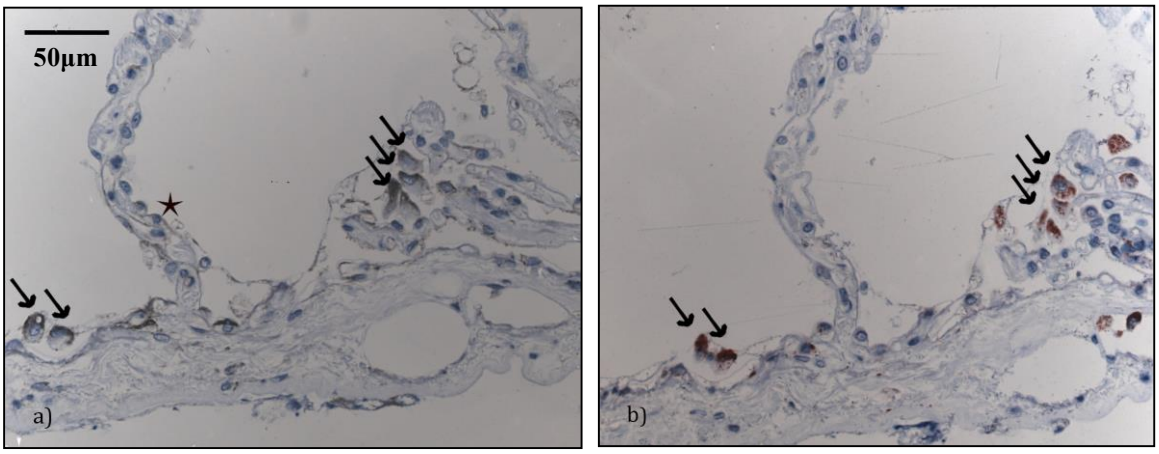

Figure 3.10 Micrograph of ACE-2 staining in alveolar parenchymal tissue a) ACE-2 staining b) CD68 staining (a macrophage marker) in another section of the alveolar parenchyma using chromogens DAB (brown) and AEC (red) respectively. ACE-2 stained macrophages are annotated in black arrows, an ACE-2 stained epithelial cell is annotated with a black star (a). In figure (b) the same ACE-2 stained macrophages are annotated with black arrows.

As shown in figure 3.10a, the majority of ACE-2 staining was within alveolar macrophages, as shown by a sequential section stained with the macrophage marker, CD68 (figure 3.10b). The total number of ACE-2 positive nucleated cells was quantified as shown in figure 3.11.

3.3.3.2 ACE-2 staining in COPD lung

ACE-2 staining was quantified in parenchymal tissue from the same subjects who were evaluated for ACE and chymase staining, previously (figures 3.5 & 3.8). Subjects were classified into COPD and non-COPD groups (figure 3.11).

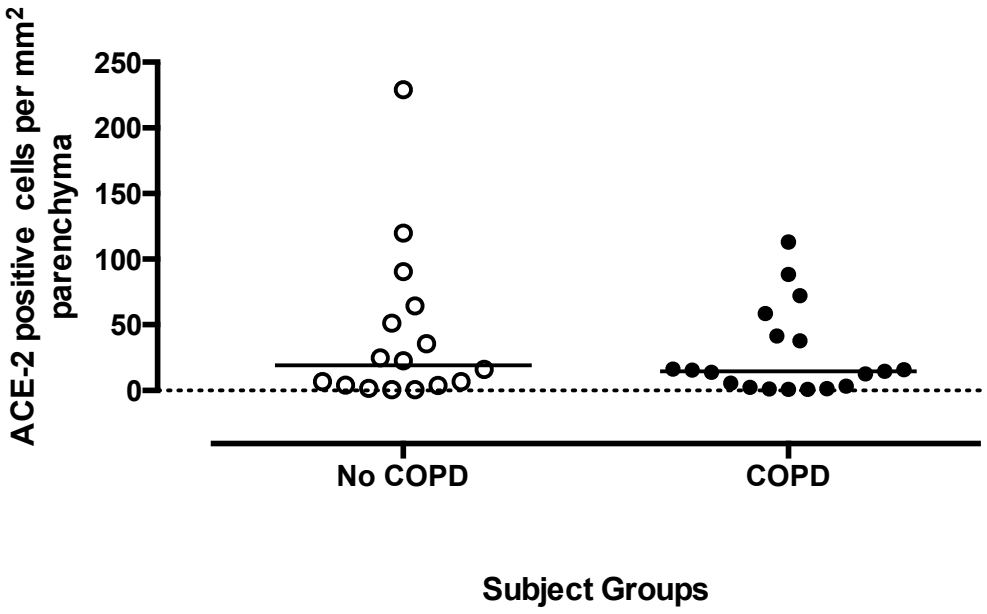

Figure 3.11 ACE-2 staining in human lung from COPD subjects ACE-2 cell counts per mm^2 of alveolar parenchyma from subjects classified with COPD (n=19) and non-COPD (n=16) controls. Mann Whitney Test p = 0.61

Figure 3.11 shows that between COPD and non-COPD groups, the number of ACE-2 positive cells was not different [COPD 14.5 (0.8 - 113.0) vs. non-COPD 19.3 (0.7 - 229.1) mm^2].

3.3.3.3 ACE-2 colocalisation in lung parenchyma

Since ACE-2 positive cells are present in many cell types in the parenchyma, the identification of these ACE-2 positive cells was assessed by colocalisation studies using the camera lucida (figure 3.12). The quantification of this location is shown in figure 3.12.

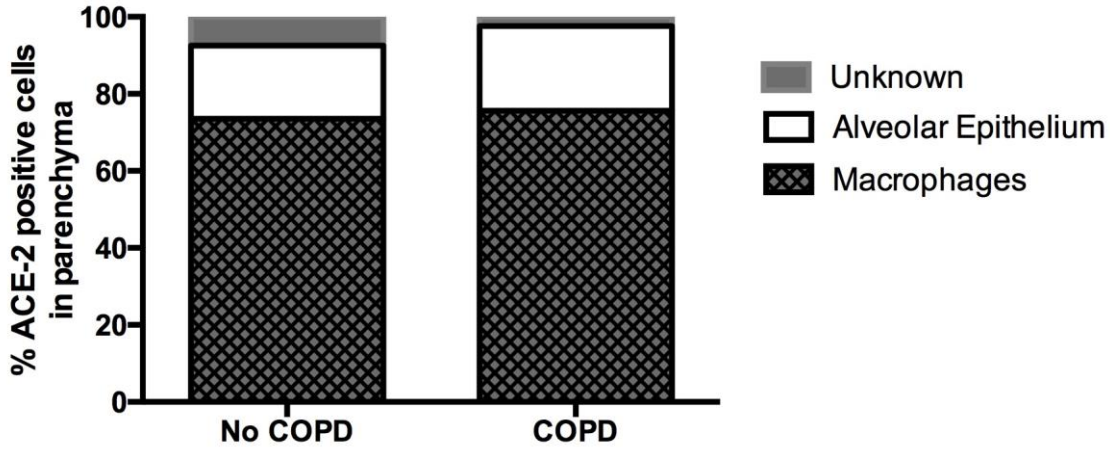

Figure 3.12 Location of ACE-2 expression within alveolar parenchymal tissue ACE-2 positive cells and CD68 positive cells were traced using the camera lucida. *Macrophages - percentage of ACE-2 positive cells identified that were also CD68 positive; Alveolar epithelium - percentage of ACE-2 positive cells located on alveolar walls.* Subjects were classified into COPD (n=4) and non-COPD (n=4) controls. Mann Whitney Test demonstrated no significance between the two groups for both alveolar epithelium and macrophages.

In figure 3.12 there were no significant differences between COPD and non-COPD groups with regards to the location of ACE-2 positive cells.

Macrophages within the alveolar air spaces were the most common cells to be stained with ACE-2 with proportions of 75.6 % and 73.5 % of ACE-2 positive cells in COPD and non-COPD groups, respectively. The second most common cells to be stained with ACE-2 were the alveolar epithelial cells with proportions of 22.0 % and 19.0 % ACE-2 positive cells for COPD and non-COPD groups, respectively. The proportion that was called unknown in figure 3.12 represents the ACE-2 positive cells that were counted in alveolar spaces but not identified in the sequential section.

CD68 positive macrophages were also evaluated for colocalisation with ACE-2 or non-ACE-2 positive cells as shown in figure 3.13.

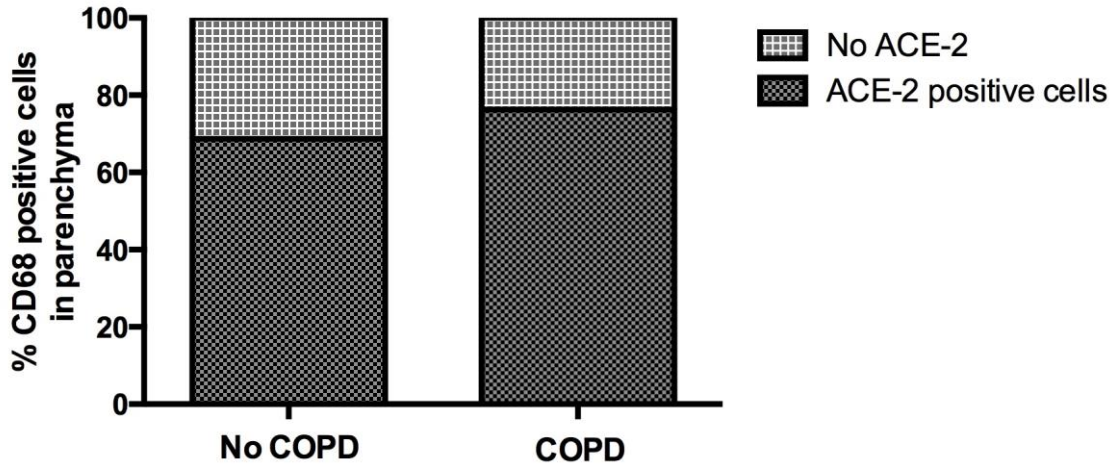

Figure 3.13 Quantification of CD68 positive macrophages colocalised with ACE-2 positive cells within alveolar parenchymal tissue CD68 and ACE-2 positive cells were traced using the camera lucida. Subjects were classified into COPD (n=4) and non-COPD (n=4) groups. Mann Whitney Test demonstrated no significance between the two groups.

Figure 3.13 demonstrates the proportion of total CD68 positive macrophages that were and were not stained with ACE-2. There were no differences in proportion of CD68 positive macrophages colocalised with ACE-2 positive cells between COPD and non-COPD groups.

CD68 stained macrophages also stained with ACE-2 were higher in percentage compared to CD68 positive macrophages that were not stained with ACE-2. Proportions of 76.3 % and 68.7 % of CD68 positive macrophages were ACE-2 positive also for COPD and non-COPD groups, respectively.

A qualitative observation revealed that CD68 stained macrophages that were not ACE-2 positive were in close vicinity to ACE-2 and CD68 colocalised cells.

3.3.3.4 Renin in lung

Renin is an enzyme that belongs to the RAS and has been reported to be present within the lung, therefore lung tissue sections were stained for renin. Two antibodies for renin were used to investigate this enzyme within the lung parenchyma. One of these antibodies have been used to identify renin in primary lung fibroblasts isolated from human lung tissue (Montes *et al.,* 2012). Nonetheless, in this experiment renin was not identified within the lung parenchyma with either antibody. A representative micrograph is shown in figure 3.14 for the second antibody. Both lung and renal samples were paraffin-embedded (see section 2.1.1.3 for more details).

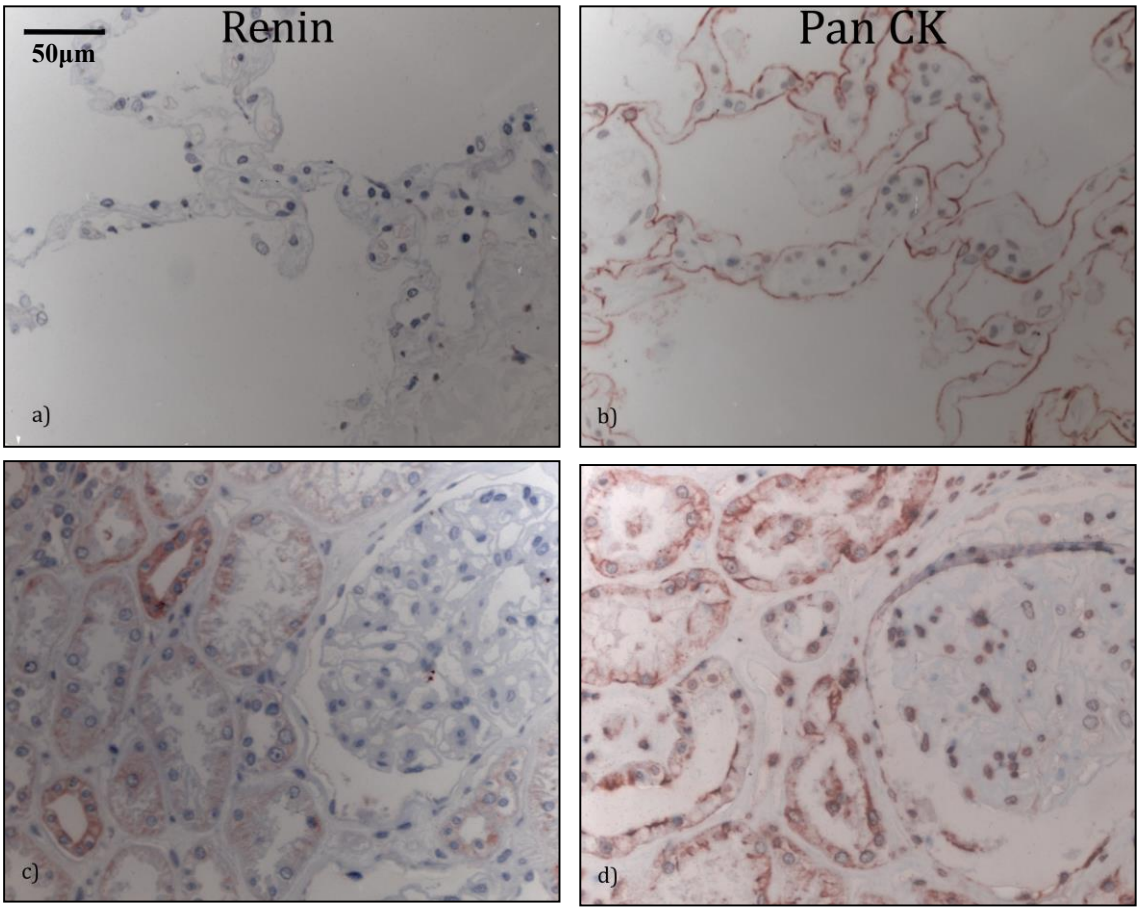

Figure 3.14 Micrograph of renin staining in alveolar parenchyma and human kidney tissue Representative images (of three runs) stained for a) c) renin (clone 2H2) b) d) pan cytokeratin (a non-specific epithelial marker) using chromogen AEC (red) respectively. Human kidney tissue was used a positive control (c) (d) for the antibody used.

Figure 3.14 shows that renin was not identified within the sections of lung parenchyma analysed. Within the same staining run, serial sections of both lung parenchyma and kidney (as a positive control) (figure 3.14c) were stained with a pan-cytokeratin antibody, a marker for all human epithelial cells. This showed that the tissue sections used were viable for the IHC staining method and that the antibody is functional.

3.3.4 ACEi Prescribed Medication

In addition to the original subjects available, there were also subjects who had been prescribed ACEi or ARB medication previously. Lung parenchyma from these subjects were stained with ACE, chymase and ACE-2 antibodies and quantified (figures 3.15, 3.16, & 3.17).

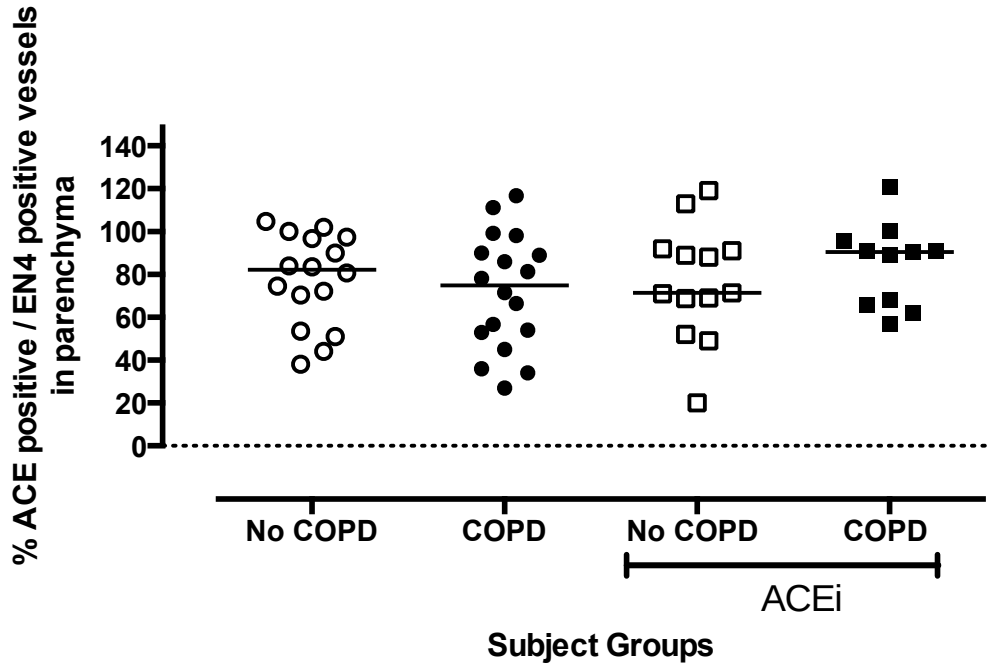

Figure 3.15 ACE staining in human lung from subjects prescribed ACEi medication ACE positive vessel counts as a percentage of EN4 positive vessel counts in alveolar parenchymal tissue. Each plot represents one subject and subjects were classified into COPD and non-COPD groups. Kruskall Wallis Test p = 0.83 *COPD chronic obstructive pulmonary disease ACEi angiotensin-converting enzyme inhibitors*

Figure 3.15 shows that the proportion of EN4 vessels stained positive for ACE was no different between ACEi, and control groups. There were also no differences between COPD and non-COPD subgroups for subjects prescribed ACEi medication.

For all groups, the qualitative ACE staining was solely localised to blood vessels as observed previously in subjects who were not prescribed ACEi (figure 3.3).

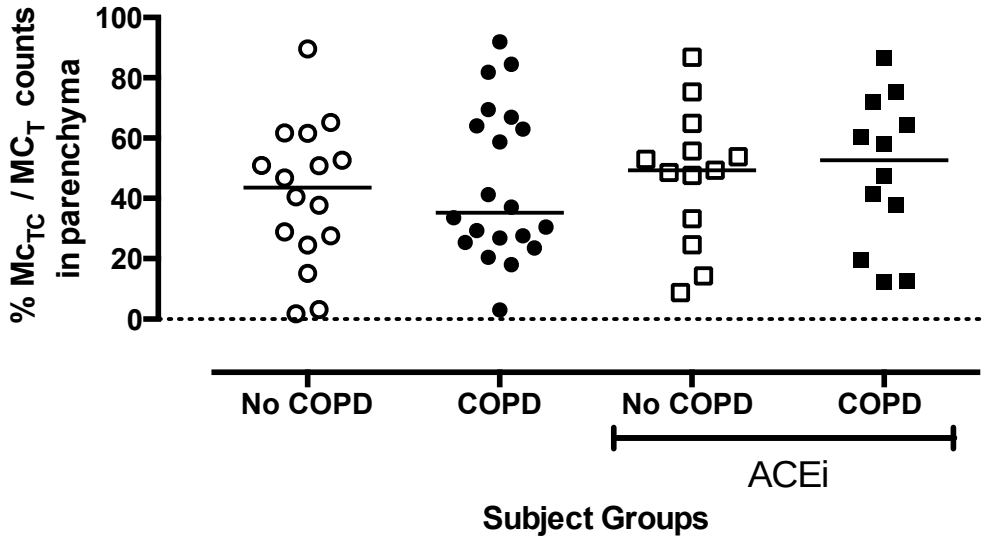

Figure 3.16 Chymase staining in human lung from subjects prescribed ACEi medication Chymase positive mast cell counts (MC_{TC}) as a percentage of tryptase positive mast cell counts (MC_T) in the lung. Each plot represents one subject and subjects were classified into COPD and non-COPD groups. Kruskall Wallis Test p = 0.90 *COPD chronic obstructive pulmonary disease ACEi angiotensin-converting enzyme inhibitors; MC_{TC} tryptase and chymase positive mast cell; MC_T chymase positive mast cell*

Figure 3.16 shows that MC_{TC} cells were not different between ACEi, and control groups. There were also no significant differences between COPD and non-COPD subgroups for subjects prescribed ACEi medication.

Qualitatively, chymase staining within the parenchyma from subjects who were prescribed ACEi medication was localised to the same mast cells that were stained in parenchyma from subjects not prescribed this medication (figure 3.7).

There was a large range within subject groups in the proportion of MC_T that were also MC_{TC} [ACEi (8.8 - 150.0) vs. no ACEi (1.8 – 92.0) %].

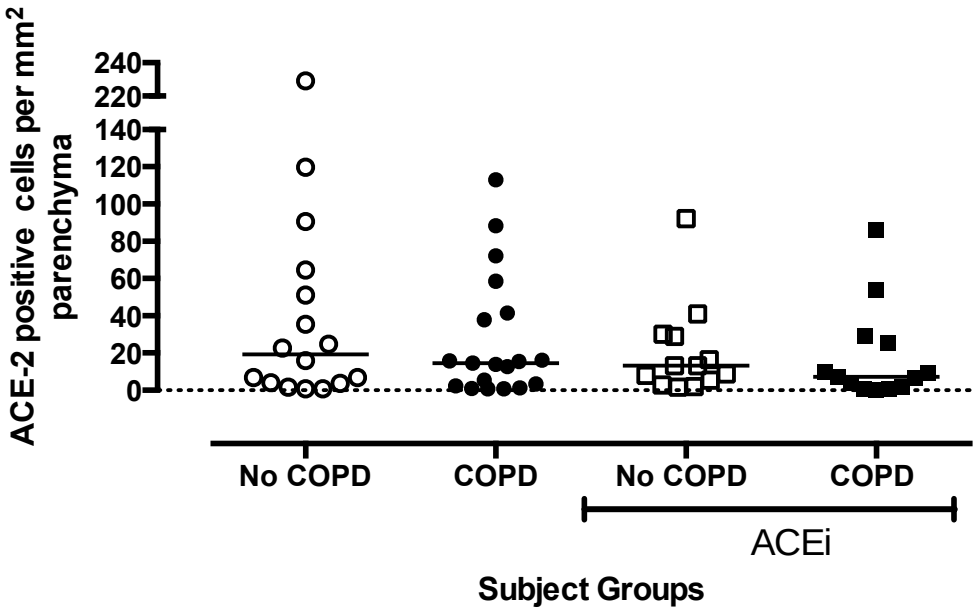

Figure 3.17 ACE-2 staining in human lung from subjects prescribed ACEi medication ACE-2 cell counts per mm^2 of lung alveolar parenchyma from subjects classified with COPD and non-COPD controls. Kruskall Wallis Test p = 0.52 *COPD chronic obstructive pulmonary disease ACEi angiotensin-converting enzyme inhibitors*

In figure 3.17 the number of ACE-2 positive cells was not different when comparing the groups ACEi and control groups. There were no differences between COPD and non-COPD subgroups for subjects prescribed ACEi medication.

Observing the staining qualitatively, the location of ACE-2 staining in parenchyma was no different in angiotensin-related medication subgroups compared to those subjects who were not prescribed this medication, as represented in figure 3.10.

3.3.5 Smoking Status

Cigarette smoking is associated with increased inflammation and therefore could affect the protein expression of ACE, chymase and ACE-2. All subjects were subdivided by their smoking status: ex-smoker or current smoker, at the time the parenchymal sample was collected. The ex-smoker group contained subjects who had stopped smoking for more than one year.

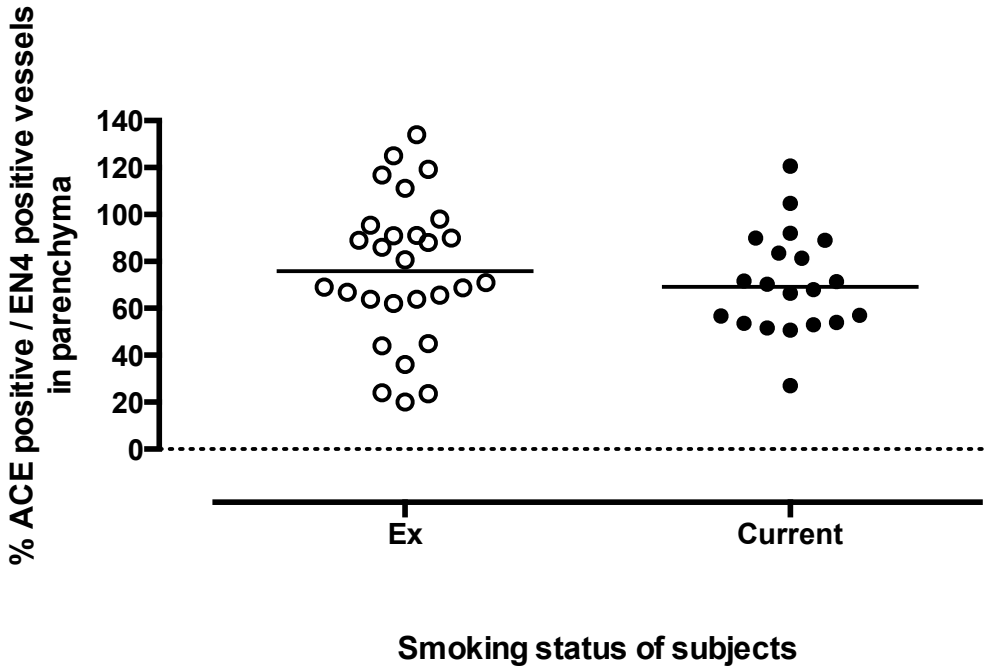

Figure 3.18 ACE staining in human lung from subjects that smoke ACE positive vessel counts as a percentage of EN4 positive vessel counts in alveolar parenchymal tissue from subjects reported to smoke tobacco (n=20) and ex-smokers (n=28). Mann Whitney Test p = 0.44 *Ex-smokers subjects who stopped smoking more than a year prior to surgery.*

Figure 3.18 shows that between the current and ex-smoking groups, the proportion of EN4 vessels also stained with ACE was not significantly different [current 69.2 (27.0 - 120.6) vs. ex 75.9 (20.0 - 134.0) %].

Vessels numbers were counted as EN4 stained vessels per mm^2. The vessel numbers of the current and ex-smoker groups were not different with medians of 249.1 and 211.5 EN4 positive vessels per mm^2, respectively.

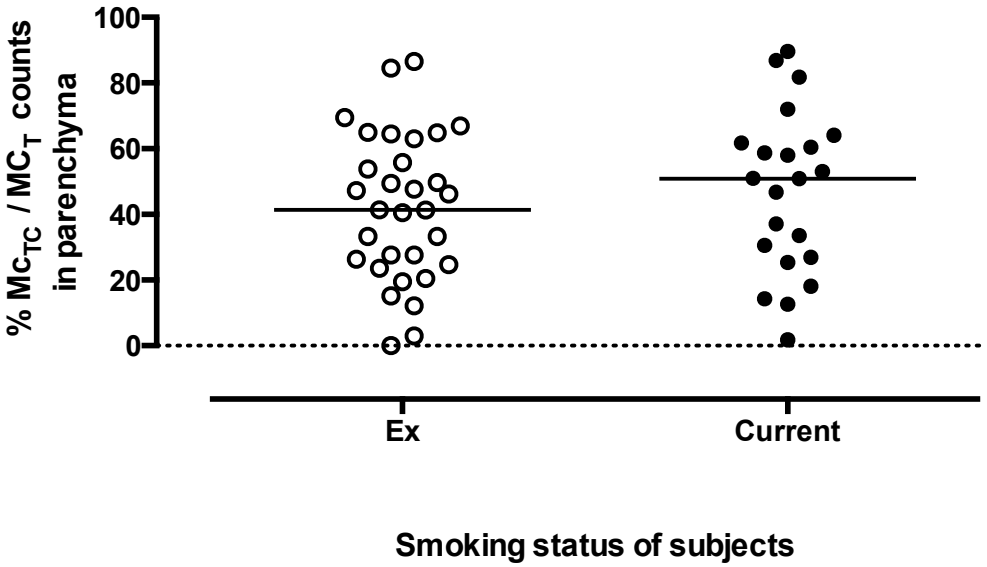

Figure 3.19 Chymase staining in human lung from subjects who smoke Chymase positive mast cell counts (MC_{TC}) as a percentage of tryptase positive mast cell counts (MC_T) in alveolar parenchyma from subjects reported to smoke tobacco (n=22) and subjects who did not smoke (n=31). Mann Whitney Test p = 0.47 *Ex-smokers subjects who stopped smoking more than a year prior to surgery.*

The proportion of MC_T cells that were MC_{TC} cells were not different between current smoker and ex-smoker groups [current 50.9 (1.8 - 89.6) vs. ex 41.4 (0.0 – 86.6) %].

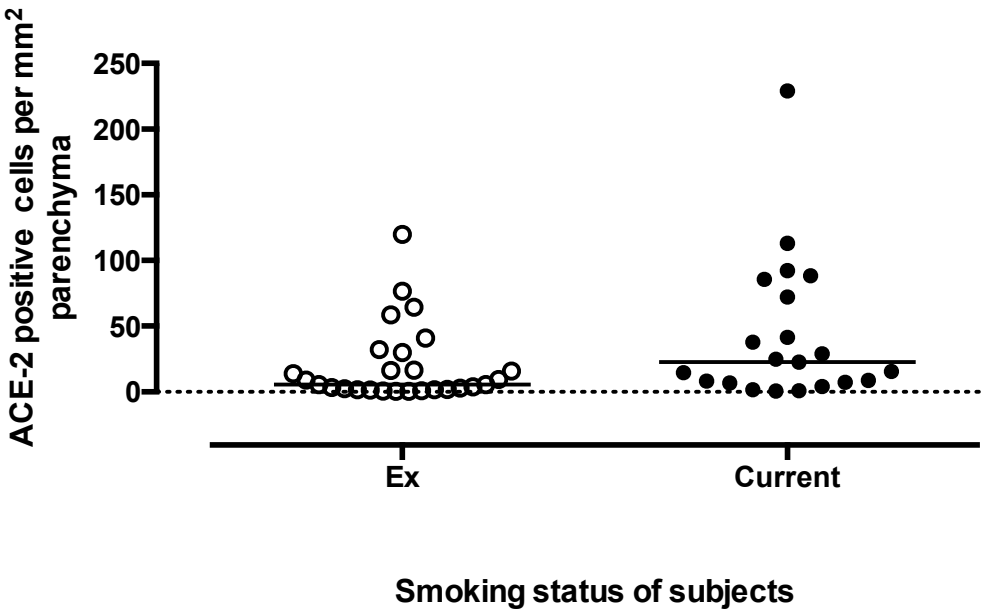

Figure 3.20 ACE-2 staining in human lung from subjects who smoke ACE-2 cell counts per mm^2 human lung alveolar parenchyma from subjects reported to smoke tobacco (n=21) and ex-smokers (n=27). Mann Whitney Test p = 0.056 *Ex-smokers subjects who stopped smoking more than a year prior to surgery.*

In figure 3.20, ACE-2 positive cells were higher in current smokers compared with ex-smokers [current 22.6 (0.6 - 229.1) vs. ex 5.4 (0.3 - 119.8) mm^2]. However, this was not statistically significant.

For the same group of subjects, the number of CD68 positive cells per mm^2 of tissue was also counted. The number of ACE-2 positive cells followed the same pattern as the number of CD68 positive cells within the parenchyma. The median of CD68 positive macrophages per smokers was higher than the current smoker group [current 43.4 (0.8 - 233.2) vs. ex 18.6 (2.4 - 242.2) mm^2] but there was no significant difference between the two groups ($p = 0.09$).

3.4 Discussion

The aim of this chapter was to identify structures of the human lung that RAS localises to and to observe any difference of localisation between COPD and non-COPD lungs. The primary intention for measuring these enzymes was to investigate if there were differences in protein expression levels between these two groups. Another objective of this chapter was to investigate the effect of ACEi medication and subject smoking status on these RAS enzymes.

IHC was chosen to identify protein expression and to observe the localisation of these enzymes to human lung tissue structures. Although other techniques were available to measure protein, for example Western Blot and ELISA techniques, protein location in reference to other structures in the lung cannot be measured by these techniques. IHC was therefore the best technique to fulfil the aims of this chapter.

The main finding of this chapter was that ACE-2 was mainly localised to macrophages within the alveolar spaces. This current study is the first to address this and to investigate its importance. Limited studies have set out to focus explicitly on ACE-2 macrophages and its possible function within the human body (Keidar *et al.* 2005; 2007).

3.4.1 Enzyme Localisation

3.4.1.1 ACE

ACE was localised to blood vessels regardless of whether it was bronchial or parenchymal tissue. It was also observed that not all vessels stained with EN4 (the endothelial marker) were positive for ACE staining in human lung tissue; most vessels that were smaller in size were positive for ACE compared to the larger-sized vessels.

These results fully support the work of Metzger *et al.,* (2011) who observed strong positive staining of ACE in human lung capillaries and less ACE staining in larger pulmonary arterioles. The lung parenchyma sampled in this paper was isolated from cadavers without any evident vessel abnormalities. Furthermore, Orte *et al.,* (2000) also identified strong ACE staining in capillaries and no staining in pulmonary veins.

3.4.1.2 Chymase

MC_{TC} were localised to the submucosa of bronchial airway. MC_{TC} staining was also present throughout the parenchyma but primarily in the lining of small airways and pulmonary vessels.

These findings support the paper of Balzar *et al.,* (2005) which reported more MC_{TC} cells within the outer lining of small airways compared to the inner walls and the parenchymal tissue adjoining alveolar ducts. Notably this was in severe asthmatics. Another paper, which sampled non-smoking subjects without asthma, found MC_{TC} lining pulmonary vessels within the lung parenchyma (Andersson *et al.,* 2009).

3.4.1.3 ACE-2

ACE-2 was present in bronchial epithelial tissue. Despite only six bronchial samples from six different subjects being sampled, the bronchial epithelial cells in all of these samples were positive for ACE-2. This confirms the work done by Ren *et al*. (2006). They demonstrated ACE-2 protein expression within the bronchial epithelium throughout the respiratory tract including the trachea, bronchioles and alveolar epithelium. ACE-2 protein expression has also been identified on the apical side of the epithelium in fresh human tracheobronchiolar tissue, this was detected by immunofluorescence (Sims *et al*., 2005).

For the alveolar parenchyma analysed in this current chapter, ACE-2 positive cells were mainly lung macrophages rather than alveolar epithelial cells. This result was surprising because many papers have focused on ACE-2 expression within epithelial cells. For example, Hamming *et al*. (2004) reported that both type I and II alveolar epithelial cells, from disease-free human lung parenchyma, were ACE-2 positive. Another group who collected lung parenchyma from subjects who died from SARS also reported alveolar epithelial cells in the parenchyma stained with ACE-2 (He *et al*., 2006). Furthermore Glowacka *et al*., (2011) reported only type II alveolar epithelial cells were positive for ACE but not type I.

As discussed above, a number of papers report ACE-2 expression in alveolar epithelial cells. These consistent reports do not suggest that ACE-2 is not present in lung macrophages but reflects the focus of the literature. At this current time, the literature is focused on ACE-2 and its relation to the SARS virus. The SARS virus is mostly present on alveolar epithelial cells in the lung (Shieh *et al.,* 2005) and this is the reason for multiple reports with regards to ACE-2 expression and epithelial cells.

3.4.1.4 Renin

Human parenchymal sections were not positive for renin staining in this study, despite using two antibody clones from different suppliers. One paper identified renin in the lung but this was in isolated mast cells from human lung tissue (Veerappan *et al.,* 2008). Another group found renin colocalised with mast cells that were still present within the human alveolar parenchyma. This

group published two papers on this (Andersson *et al.*, 2009; 2010). They used a mouse monoclonal antibody from the company Swant, which was not available at the time I carried out this current study.

In the study, two monoclonal antibodies were used to attempt to identify renin within lung tissue sections. The first identified renin in kidney sections embedded in paraffin. There were no published papers that quoted using this 7D3-E3 clone antibody to identify renin therefore another renin antibody clone 2H2 was chosen. This 2H2 clone antibody was supplied by Serotec and was used in a previous published paper to identify renin by ICC in primary lung fibroblasts (Montes *et al.*, 2012). This antibody also identified renin in the kidney sections in this current study however no staining was present in lung sections.

Given the number of papers mentioned above that have identified renin within human lung tissue, these current results of no identification of renin in human lung sections cannot firmly lead to a conclusion that renin is absent in lung tissue sections. Use of the same Swant antibody as the published papers would resolve this issue however this is not possible. Alternative methods to measure renin protein expression should be investigated such as western blot of lung homogenate.

3.4.2 Enzyme Expression Differences in COPD

ACE, chymase and ACE-2 protein localisation to structures in the human lung was not different in COPD lung and non-COPD lung nor was the protein expression levels between the two groups.

3.4.2.1 ACE

The percentage of vessels stained with ACE was not different between COPD lung and non-COPD lung. ACE protein expression in COPD lung has not been evaluated before however a number of papers have investigated the gene responsible for ACE within the blood of COPD subjects (Kanazawa *et al.*, 2003; Lee *et al.*, 2009). The ACE gene is known to have a 287-base pair polymorphism, which is the insertion (II) genotype. Genes without this polymorphism are labelled as having the deletion (DD) genotype. Higher ACE concentration in serum is associated with subjects who have the DD genotype (Rigat *et al.*, 1990).

One paper reported that ACE was associated with COPD. Busquets *et al.*, (2007) reported that subjects (n=77) with the DD genotype had a higher risk of being diagnosed with COPD compared to subjects who had the II genotype. In contrast, a larger study carried out in Denmark did not find any association between the ACE gene polymorphism and the risk of developing COPD (Lee *et al.*, 2009). Similarly, a study in Turkey did not find an association between COPD (n=66) and

the ACE genotype (Simsek *et al.*, 2013). The results of this current study aligns with the latter study, as it showed no difference in the number of ACE positive vessels between subjects classified with COPD or no COPD.

A suggested reason for finding no difference in ACE protein expression between COPD and non-COPD is that the majority of vessels were positive for ACE. It is therefore problematic to observe a clear difference if indeed there was one. Further work could investigate the activity of ACE in the lung between the two groups., which would further the understanding of the function RAS in the lung.

3.4.2.2 Chymase

As for chymase, there were no significant differences in the location and numbers of MC_{TC} between the alveolar parenchyma of COPD subjects and non-COPD controls. This is in agreement with a study that also investigated MC_{TC} numbers near the small airways in human lung. Gosman *et al.*, (2008) found that MC_{TC} numbers within the lung parenchyma, where small airways were located, did not significantly differ between subjects with severe COPD compared to non-COPD controls. Andersson *et al.*, (2010), also sampled from mild/moderate COPD subjects as done so in this current chapter and also observed no differences between those subjects and their non-COPD controls.

The experiment was carried out to address whether chymase could be the alternative enzyme for Ang II production in subjects with COPD. From these data it suggests that it is not. This was inferred because there was no difference in the number of MC_{TC} between diseased and non-diseased patients.

3.4.2.3 ACE-2

No studies to date have explored ACE-2 expression in COPD in human subjects. In this current study, ACE-2 protein expression levels were not different between COPD and no COPD subject groups. Subjects with idiopathic pulmonary fibrosis, however, have previously been sampled and their lung ACE-2 protein expression was significantly less compared to those without lung disease (Li *et al*., 2008). The same group further confirmed this with a devoid of ACE-2 staining in alveolar parenchyma of IPF subjects (Uhal *et al*., 2013). This could mean that, although ACE-2 protein expression is associated with other lung diseases, it may not be associated with COPD.

3.4.3 Angiotensin-converting Enzyme Inhibitor Medication

ACEi are frequently used to control high blood pressure. They lower blood pressure by reducing the function of ACE and because of this there may be a change to protein expression of RAS lung enzymes (ACE, chymase and ACE-2). In the current study, participants prescribed ACEi however did not have different protein expression levels of ACE, chymase and ACE-2 to those not prescribed ACEi.

3.4.3.1 ACE

ACEi have previously been shown to reduce ACE protein levels in rat lungs after 4 hours of lisinopril administration compared to baseline readings measured by autoradiography (Sakaguchi *et al*., 1988). Furthermore rats given enalapril daily for four weeks had significantly less ACE protein expression in the heart compared to vehicle controls, as measured by Western Blot (Yang *et al.*, 2013). The current chapter has shown that ACE protein expression does not differ between subjects prescribed ACEi and controls, which is in contrast with the results from the aforementioned animal studies. Animal studies are the only literature that is available as there are no human studies that have looked into ACE protein expression in the lung after treatment with ACEi.

Taking a theoretical approach, ACEi medication is designed to competitively block the active site where the substrate binds in order to be cleaved. ACEi therefore should reduce the catalytic cleaving activity of ACE rather than controlling the number of ACE positive cells within the parenchyma.

The IHC technique used in this experiment is not able to measure ACE activity. IHC can confirm whether something is present or not present,: an ‘on off’ result. This is advantageous if required to find whether the protein is present in a certain organ, as was done in this work, but does not allow the measurement of the enzyme’s function. For example, ACE and ACE-2 catalytic domains can be cleaved (shedding) leaving a part of the C-terminus attached the cell membrane without the functional catalytic domain (Lambert *et al*., 2005). Perhaps investigating ACE catalytic activity in the lung within subjects prescribed ACEi would be beneficial to explore.

3.4.3.2 Chymase

Subjects prescribed ACEi did not demonstrate any changes in MC_{TC} numbers compared to those not prescribed medication. There is no literature on the effects of ACEi on expression of chymase in human lung. One study showed that after oral administration of the ACEi captopril to mice,

there was a 14-fold increase in Ang II formation compared to vehicle controls (Wei *et al*., 2010). This paper suggested that the increase in Ang II was due to chymase. However, chymase was not explicitly measured in this paper.

3.4.3.3 ACE-2

Subjects prescribed ACEi did not differ in the number of ACE-2 positive cells in lung compared to subjects not prescribed this medication. Currently, no papers have published experiments regarding lung ACE-2 expression and the effect of ACEi prescription in humans however there have been some experiments in animal models.

ACE-2 gene expression in the mouse kidney was reduced in mice that were treated with ACEi perindopril compared to those that were not treated (Tikellis *et al.,* 2008). As ACE-2 gene expression does not always equate to the levels of protein expression the previous finding may not be a good example. Another paper demonstrated that ACE-2 protein expression measured by IHC and Western Blot technique was higher in the liver of rats that were treated with ACEi medication compared to those that were not treated (Huang *et al.,* 2010). The results in this current study do not agree with this particular paper however both the species and the organ was different in both of these studies.

3.4.4 Smoking Status

Inhaling smoke particles from cigarette smoke has been reported to damage lung tissue, specifically alveolar epithelial cells by inducing ROS and subsequent inflammation (Spira *et al.,* 2004; Thorley & Tetley, 2007). Subjects who do smoke may have differing levels of ACE, chymase and ACE-2 compared to those who do not. In this study, I found no significant difference between current smokers and ex-smokers. The ex-smoker group consisted of subjects who had smoked but had stopped for over a year before surgery (ex-smokers). There were only eight subjects who were classified as non-smokers in the sample. I chose not to analyse the non-smoker group because there were more ex-smoker subjects (n=31).

Smoking pack-year units are another standard way of evaluating smoking status in a sample of people. To calculate a pack-year it requires two pieces of information: the length of years smoked and the number of cigarettes smoked per day. This information for the subjects who donated their samples in this current study was only obtained through medical notes. The number of cigarettes that the person smoked was often missing. The most reliable information was whether they were

a current smoker or ex-smoker and the number of years that they had stopped smoking. This is why the results in this thesis were classified as these two groups instead of smoking pack-years.

3.4.4.1 ACE

The percentage of ACE positive vessels was not different between subjects who smoked and those who were ex-smokers. To my knowledge this is the only experiment that has investigated cigarette-smoking status on ACE expression.

Cigarette smoke destroys alveolar endothelial cells (Falica *et al.,* 2014), which may lead to a loss of vessels and thus fewer ACE vessels in current smokers. The literature has reported an inverse correlation between the number of vessels in the parenchyma and the severity of COPD (Matsuoka *et al.,* 2010). The subjects who were involved in this current study had a COPD severity of mild/moderate. This was calculated using the GOLD standard ranges (GOLD, 2015) explained as follows.

Mild/moderate severity is classed by spirometry measurements where the forced vital capacity measurement is less than 0.70 and the forced expiratory volume in one second (FEV_1) is higher than 50 % of predicted. This is the lowest severity rating according to the GOLD classification therefore the amount of destruction in these lungs may not be as high as the most severe COPD subjects. The destruction in mild/moderate subjects may be similar to non-COPD controls and may explain the no difference of ACE positive vessels between COPD and non-COPD controls.

The high number of COPD subjects who had a mild or moderate COPD in this study may be due to the fact that they underwent lobectomy surgery. To be eligible for surgery comorbidities should be mild in severity and under control in order for the subjects to survive and make a good recovery from the surgery. Furthermore, the non-COPD controls of this study were also undergoing lobectomy surgery and therefore may not have healthy lung tissue themselves. However, the study required an appropriate control for these subjects with COPD who were undergoing surgery. A healthy subject would not be strictly a control to these COPD subjects neither would it be practical to gain lung tissue from healthy volunteers.

3.4.4.2 Chymase

Immunostaining for chymase in smokers and ex-smokers revealed no difference in MC_{TC} counts per mm^2. Andersson *et al.,* (2010) ex-smokers and non-smokers pooled together in one group had a range of values of 0 – 50 MC_{TC} per mm^2 compared to the smokers group who had values below

10 MC_{TC} per mm^2 only. These groups were not significantly different from each other but it seemed to indicate that less MC_{TC} were identified in smokers compared to controls.

In contrast, more chymase was observed in hamsters undergoing whole body smoke exposure compared to hamsters exposed to filter air as found by IHC (Wang *et al., 2*010). A human study observed that MC_{TC} were higher in areas of tissue loss compared to intact cells (Berger *et al.,* 2003).

The technique used for identifying chymase as well as the frequency/duration of smoke exposure may be able to explain the discrepancy. The MC_{TC} per mm^2 were identified through immunostaining in Andersson *et al.,* whereas for the hamsters chymase was recorded by a radioimmunoassay rather than IHC, which may not reflect the number of chymase positive cells within the lung. Furthermore the hamsters were exposed to cigarettes twice a day for half an hour for up to four months. This is not a similar frequency pattern that would be observed in humans. The duration would not be so short.

This current study supports the findings of the human study by Andersson *et al.*, by also finding no significant differences between smokers and ex-smokers. Because of this mixture of reports it is unclear whether smoking affects MC_{TC} numbers. More studies may need to be done to further understand this.

3.4.4.3 ACE-2

The number of ACE-2 positive cells in the current smoking group was higher than in the ex-smoker group and this was close to significance (p=0.05, figure 3.20). No other studies have related smoking and ACE-2 protein expression in humans. This current work supports previous finding whereas rats exposed to cigarette smoke had higher ACE-2 protein expression compared to rats exposed to fresh air (Han *et al.*, 2010). The sparse knowledge highlights the novel aspect of this investigation.

This investigation does suggest that ACE-2 production may be affected by cigarette smoke. A new investigation into the effect of smoking status on ACE-2 activity would be valuable in understanding the bigger picture of enzyme expression and its function of activity.

3.4.5 Limitations

It is important to be aware that there are some limitations to this study. Due to the nature of the study design, this study was not able to choose whether the subjects that the lung samples were

isolated from, smoked regularly or were on a course of a particular medication. This work was reliant on willing subjects donating their lung samples while they were in hospital for surgery. This is a limitation since some of the group had low numbers. The number of subjects to be confident in the result of the statistical test was 168. This number was calculated by a sample size calculation based on the data points of this sample.

Another limitation is the assumption that ACEi were taken correctly by the subjects prescribed the medication. The side effects of ACEi such as cough, headache, rash and abnormal taste (Brown & Vaughan 1998) may contribute to subjects not taking ACEi regularly. However ACEi is generally well tolerated and most dosages require one oral tablet per day.

3.4.6 Summary

In summary, ACE-2 protein is present within alveolar macrophages and this has not been observed before in other publications. This finding is, therefore, novel and since macrophages are mainly involved in inflammation this information could be useful to manipulate the inflammatory response in COPD.

ACE is localised with the majority of vessels within the human alveolar parenchyma, chymase localised to the mast cells and ACE-2 is within alveolar macrophages. Renin was not detected within the human alveolar parenchyma sampled.

The RAS enzyme localisation was not different in subjects with COPD compared to those who were not diagnosed with COPD. Furthermore, there was no difference of enzyme protein expression levels (*i.e.* the number of positive cells/vessels) between these two groups. ACEi prescribed subjects or current smokers did not have different protein expression levels compared to their respective controls. More work is required to investigate whether these enzymes are functional in the lung and if so, whether COPD, prescription of ACEi or smoking status of subject could influence the functional activity of these enzymes both in lung tissue and isolated macrophages.

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Chapter 4 Activity of RAS Enzymes in Lung

4.1 Introduction

There is evidence to suggest that ACE activity is significantly reduced in certain lung diseases such as pulmonary hypertension and IPF. Rats modelling the lung disease pulmonary hypertension have a significantly lower ACE activity compared to non-diseased controls (Morrell *et al.,* 1995). ACE activity is also reduced in patients who suffer from acute respiratory distress syndrome (ARDS) (Orfanos *et al.*, 2000). From this paper it was reported that the more severe the lung injury, the lower the ACE activity.

The homologue of ACE, ACE-2 is also associated with lung diseases. Pre-treatment with ACE-2 was shown to attenuate symptoms of ARDS in mice compared with vehicle controls (Imai *et al*., 2005). In human lung, ACE-2 activity is significantly less in IPF patients compared to non-diseased subjects (Li *et al*., 2008). This paper was the first and only study to investigate ACE-2 activity in humans.

As the above evidence suggests, ACE and ACE-2 enzymatic activity may play an important role in the pathogenesis of some lung diseases, however, no papers have explored these two enzymes within the human lung in COPD thus far. An investigation into the natural levels of ACE and ACE-2 activity in the human lung, especially from COPD subjects could aid the understanding of the pathological mechanisms in COPD. The objective of this chapter was to measure activity of these enzymes within lung homogenate isolated from COPD subjects and compare the measurements to non-COPD controls.

In order to do this, enzymatic assays were used to evaluate the functional activity of ACE and ACE-2 in human lung samples by spectrophotometry. ACE and ACE-2 are known to cleave HHL and Mca-APK(Dnp) respectively, both of which are commonly used as synthetic substrates to investigate enzyme activity (Watkins *et al*., 2008; Pedersen *et al*., 2011). This degradation of substrates releases break-down products and these products can be measured by a spectrophotometer /fluorescence plate reader over a known period of time.

Another aim of this chapter was to investigate whether prescription of ACEi medication or smoking status of these individuals has an effect on lung ACE or ACE-2 activity.

4.2 Methods

4.2.1 Subjects

Human lung tissue was collected from subjects (n=21) undergoing lung tissue resection surgery at Southampton General Hospital. Subject characteristics are in table 4.1. The characteristics of these subjects were representative of the larger sample group investigated in chapter 3 table 3.1.

Table 4.1 Characteristics of subjects who donated lung samples used for activity assays ACE and ACE-2 activity were evaluated in lung samples donated by 21 subjects. Details of these subjects are outlined in the table. One subject was prescribed ARB however this sample was not included in the group data, for more information see 4.3.1.3. For explanation of the abbreviations please refer to the legend that accompanies table 2.1. Data presented as mean ± s.d ****p<0.0001 compared to non-COPD group using Mann-Whitney Test

		No COPD n=9	COPD n=12
Age (years)		66.0 ± 7.3	71.6 ± 7.6
Gender (female/male)		4 / 5	9 / 3
FEV_1 / FVC		0.77 ± 0.04	0.62 ± 0.05****
Smoking Status	Current (%)	22.2	66.7
	Ex (%)	66.7	25.0
	None (%)	16.7	8.3
ACEi Medication (%)		22.2	33.3

4.2.2 Processing of Tissue for Activity Assays

Fresh human lung tissue samples were collected as described in section 2.1.2. These tissue samples were blotted on tissue paper for the removal of excess blood and weighed (0.5 ± 0.02 g). Samples were then stored in bijoux containers at -80^{o}C until required.

These samples were then thawed and homogenised for activity assays (see section 2.2.2 for further details). From the homogenate, the supernatants were collected and protein concentration determined using a BCA protein assay (see section 2.2.3 for further details).

4.2.3 ACE Activity Assay

ACE activity was measured using a technique taken from Watkins *et al.,* (2008). For the purposes of validation, samples of lung tissue (0.5 ± 0.02 g) were taken from a 9-week old Sprague Dawley rat. These samples were homogenised as described section 2.2.2 for human lung tissue. After this, 10 μM of HHL substrate was incubated with the supernatant of the rat lung sample for 15 minutes and hippurate was formed. Supernatants were treated with captopril (10 μM) (ACE inhibitor) to confirm the specificity of the assay. Captopril inhibits the formation of hippurate to show assay is specific to ACE. Results were similar to Watkins *et al.,* (2008).

After validating this assay, the technique was used to measure human lung ACE activity for 21 subjects. This technique is explained in detail in section 2.2.4. In brief, the supernatant sample (50 μl) was diluted in incubation buffer at pH 8.3. Each tissue supernatant sample was then incubated with the substrate HHL (10 μM) for 15 minutes at 37^{o}C. The absorbance of this mixture was detected by the spectrophotometer at a wavelength of 382 nm as a direct measure for hippurate formation. As explained previously (section 2.2.4) the amount of hippurate formed from this technique was calculated by extrapolating from the constructed standard curve for hippurate prepared earlier. This value was corrected for protein concentration (mg/ml) and time (min). The inter-assay and intra-assay coefficient of variation was 11 % and 5 % respectively.

4.2.4 ACE-2 Activity Assay

ACE-2 activity was measured using a technique from Pedersen *et al*., (2011). The technique is explained in more detail in section 2.2.5. The substrate used to measure ACE-2 activity was 7-methoxycoumarin-4-yl-Ala-Pro-Lys-(2,4-dinitrophenyl)[Mca-APK(Dnp)].

The 7-methoxycoumarin-4-yl (MCA) part of the substrate is a fluorogenic peptide and is quenched by the dinitrophenyl (DNP) moiety. A fluorescence plate reader measured the fluorescence emitted as fluorescence units (FU). The plates were read at excitation wavelength of 320 nm and emission wavelength of 405 nm.

Mouse kidney was used in Pedersen *et al.,* 2011 therefore for the purposes of validation, mouse kidney was incubated with 10 μM of the Mca-APK(Dnp) substrate for 1 hour. These samples were homogenised as described in section 2.2.2 for human lung tissue. Treatment of these kidney supernatants with the ACE-2 inhibitor DX600 (10 μM) - and on a separate occasion ACEi captopril (10 μM) - was used to demonstrate the specificity of the ACE-2 assay. Similar results were produced to that of the Pedersen *et al*., (2011) therefore the same technique was also applied

to human lung tissue. The inter-assay and intra-assay coefficient of variation was 8 % and 4 % respectively.

4.2.5 Statistical Analyses and Presentation of Data

All experiments were done in triplicates for both ACE and ACE-2 activity measurements. For the ACE activity assay the activity was presented as nmol.min $^{-1}$mg $^{-1}$. This is translated as 1 nanomole of hippurate released from the substrate HHL per minute per mg of protein homogenate at 37^{o}C (nmol.min^{-1} mg^{-1}). For the ACE-2 assay, the activity was presented as FU.min^{1}.μg^{-1} protein. This was an arbitrary unit that represented the release of the fluorescent peptide Mca-AP-OH from the Mca-APK(Dnp) substrate per minute per μg of protein at 37^{o}C. All data are presented as median (range) unless otherwise stated. Groups were compared using the Mann Whitney-*U* test unless otherwise specified.

4.3 Results

4.3.1 ACE Activity in Human Lung Tissue

4.3.1.1 ACE activity in rat and human lung tissue

The method of Watkins *et al.*, (2008) was used to measure ACE activity in human lung tissue homogenate. These investigators used rat lung homogenate as a positive control so this was included in the validation steps as shown in figure 4.1.

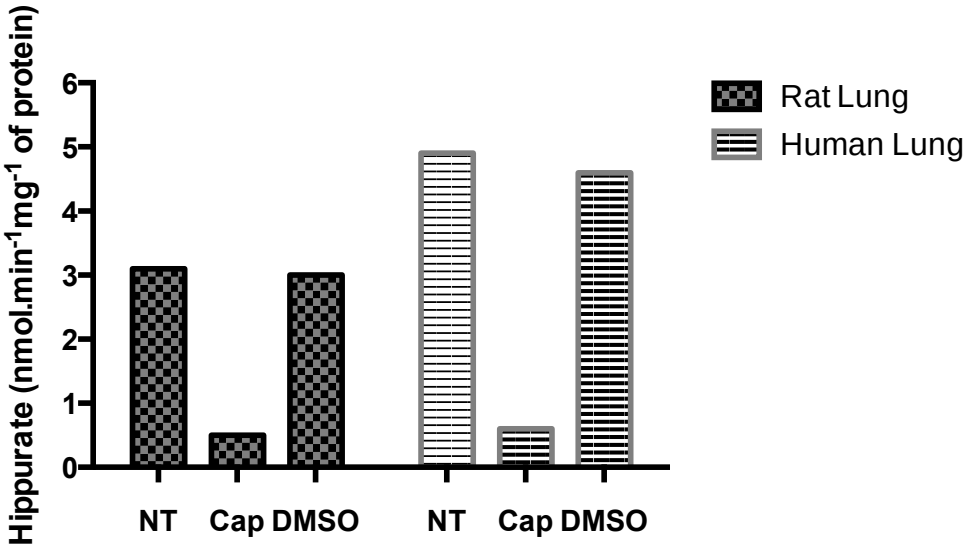


Figure 4.1 Lung ACE activity in rat and human samples The hippurate rate generated by lung ACE when substrate HHL was mixed with lung tissue homogenate. Reactions were done in triplicate during one experiment only. Each bar represents the mean value of these triplicates. *NT no treatment; DMSO Dimethyl sulfoxide (0.05%); Cap Captopril (10 ㎛M)*

Figure 4.1 shows rat and human lung homogenate samples that produced a hippurate production rate of 3.1 and 4.9 nmol.min^{-1}mg^{-1} of protein, respectively. Captopril treated rat lung samples gave a rate that was 87.8 % less than the non-treated. For human lung samples captopril treated samples was 98.3 % less than non-treated.

Captopril was dissolved in 0.05 % DMSO therefore a DMSO control was included and gave results similar to no treatment controls.

4.3.1.2 ACE activity in subjects prescribed ACE inhibitors

Figure 4.1 showed that the direct application of ACEi captopril onto lung tissue homogenate reduced the hippurate production rate in one subject sample. The experiment to investigate the

effect of the prescription of ACEi medication to subjects on lung ACE activity was plotted in figure 4.2.

Human lung samples were collected from 21 subjects and ACE activity was measured by the same technique as used for the rodent/human samples in the previous figure. Subjects were divided into whether they were prescribed ACEi medication or not as plotted in figure 4.2.

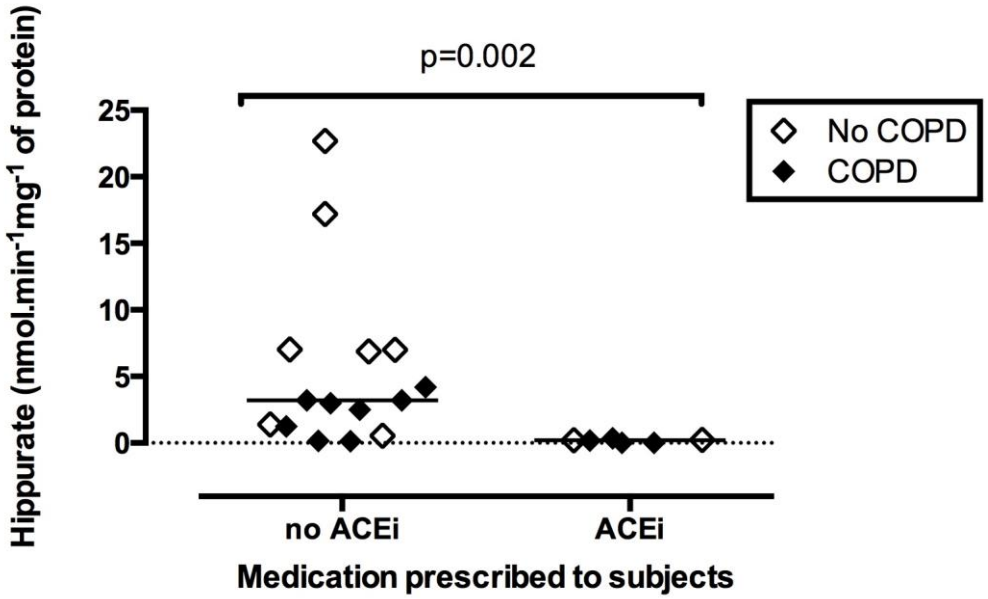


Figure 4.2 Human lung ACE activity of all subjects sampled Subjects were grouped by whether they were prescribed ACEi medication (n=6) or not (n=15) and the hippurate production rate was measured. Triplicates were done in each experiment and the mean of three independent experiments was plotted. Each plot represents one subject. Lines represent median value Mann-Whitney Test p=0.002

As shown in figure 4.2, subjects prescribed ACEi had a hippurate production rate that was significantly less than subjects not prescribed ACEi, [ACEi 0.20 (0.00 - 0.35 vs. no ACEi 3.20 (0.13 - 22.70) nmol.min^{-1}mg^{-1}] (p = 0.002).

Since all subjects who were prescribed ACEi had hippurate production rate that was negligible, the remaining 15 subjects who were not prescribed ACEi were classified into COPD and non-COPD groups according to their lung function as plotted in figure 4.3.

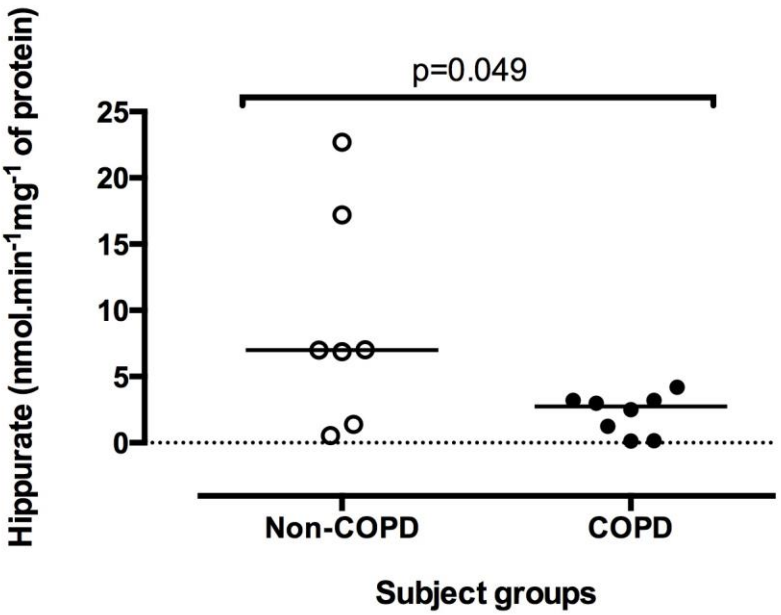


Figure 4.3 ACE activity within lung homogenate from subjects not prescribed ACEi For the subjects who were not prescribed ACEi medication (in figure 4.2) their data were stratified by whether these subjects had COPD (n=8) or no COPD (n=7). Triplicates were done in each experiment and a mean of three independent experiments was plotted for each subject. Lines represent median value. Mann-Whitney Test p=0.049.

As shown in figure 4.3, median ACE activity was significantly lower in the COPD group compared to the non-COPD group [COPD 2.74 (0.13 - 4.20) vs. non-COPD 7.00 (0.54 - 22.70) $nmol.min^{-1}mg^{-1}$] (p = 0.049).

4.3.1.3 ACE activity in subject prescribed with ARB

There was one subject who was prescribed ARB medication out of the collection of subjects sampled. The sample from this subject was treated with increasing concentrations of the ACEi captopril during the ACE activity assay. Data are shown in figure 4.4.

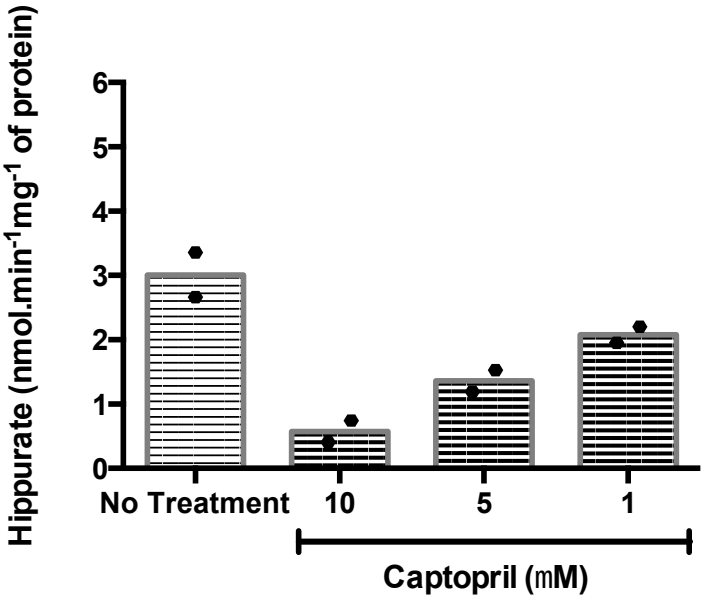


Figure 4.4 Human lung ACE activity of one subject prescribed the ARB candesartan The hippurate production rate of lung sample from one subject prescribed candesartan, treated with a range of captopril concentrations. For one subject two experiments were done and these were done in triplicates. Each circle plotted represents one independent experiment. Each bar represents the mean value of these two independent experiments.

The hippurate rate of production for the non-treated sample was 3.0 nmol.min^{-1}mg^{-1}. The captopril (10 μM) treated sample was 80.0 % less than non-treated sample. Treating the sample with different captopril concentrations such as 5 μM and 1 μM generated hippurate rates that were 54.7 % and 31.1 % less than the non-treated sample, respectively. The captopril treatment seemed to have a dose-dependent effect on the hippurate rate. This subject did not have COPD according to their spirometry results.

This experiment was also done in parallel with another experiment using a sample from a subject who was prescribed ACEi as plotted in figure 4.5.

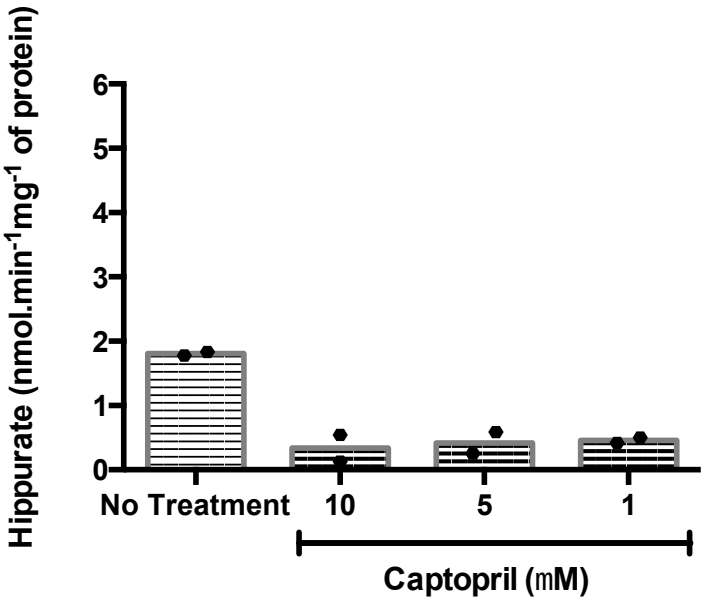


Figure 4.5 Human lung ACE activity of one subject prescribed ACEi treated with a range of captopril concentrations. Reactions were done in triplicate for each experiment. Each circle plotted represents measurement from one independent experiment. Each bar represents the mean value of these two independent experiments

The rate of hippurate production for the non-treated sample was 1.8 nmol.min^{-1}mg^{-1}. The captopril (10 μM) treated sample was 81.4 % less than the non-treated sample. Treating the sample with different captopril concentrations such as 5 μM and 1 μM generated a hippurate rate that was 76.7 % and 74.5 % less than the non-treated sample, respectively. Captopril did not have a dose-dependent effect on hippurate production rate for this subject. This subject did not have COPD according to their spirometry results.

4.3.1.4 ACE activity and ACE protein expression

Lung ACE activity and ACE protein expression measured by IHC may be associated with each other. Data from a subgroup of 13 subjects whose samples underwent both the ACE activity assay and IHC in the lung parenchyma were compared. Results from both assays for each subject were plotted in figure 4.6.

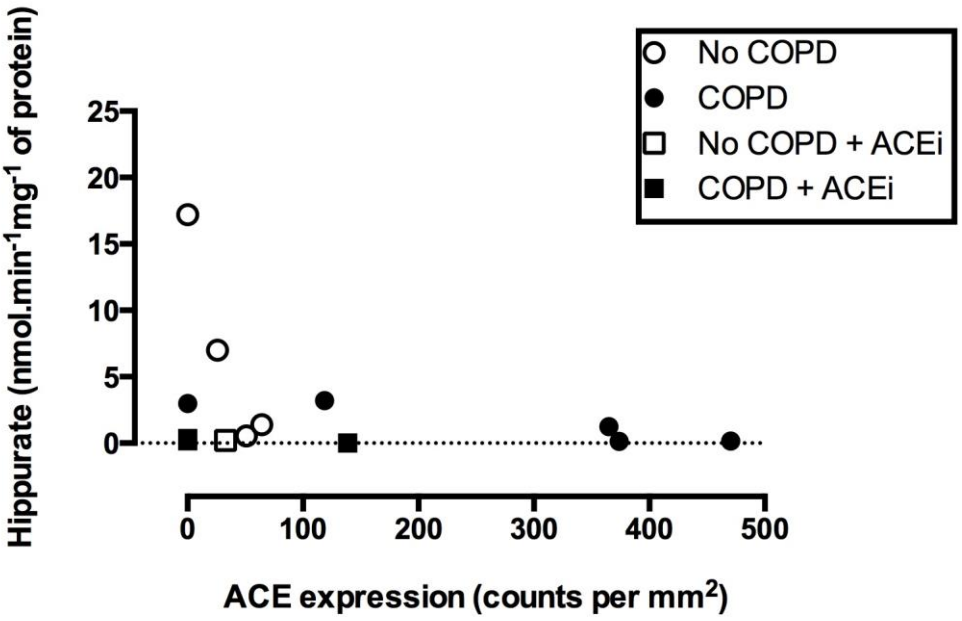


Figure 4.6 Association between lung ACE activity and ACE IHC protein expression Paired data from a subgroup of subjects evaluated for ACE activity and the number of vessels stained with ACE as representative of protein expression (n=13). ACE activity plots represent the means for each subject from three independent experiments. ACE expression was expressed as the number of positively stained ACE vessels per mm^2 for each subject. Parametric correlation analysis was done and no association was reported.

In figure 4.6, there was no association between the activity of ACE in the lung and the number of ACE vessels stained during immunohistochemistry.

4.3.1.5 ACE activity and smoking status

Lung ACE activity could also be affected by cigarette smoke. Smoke particles inhaled could travel to the alveoli where the ACE protein is located. To investigate these data for current smokers at the time of surgery and those who had previously smoked (ex-smokers) was compared. Data were

shown in figure 4.7. Again, the subjects prescribed ACEi were taken out of this analysis because of their negligible hippurate rate formation.

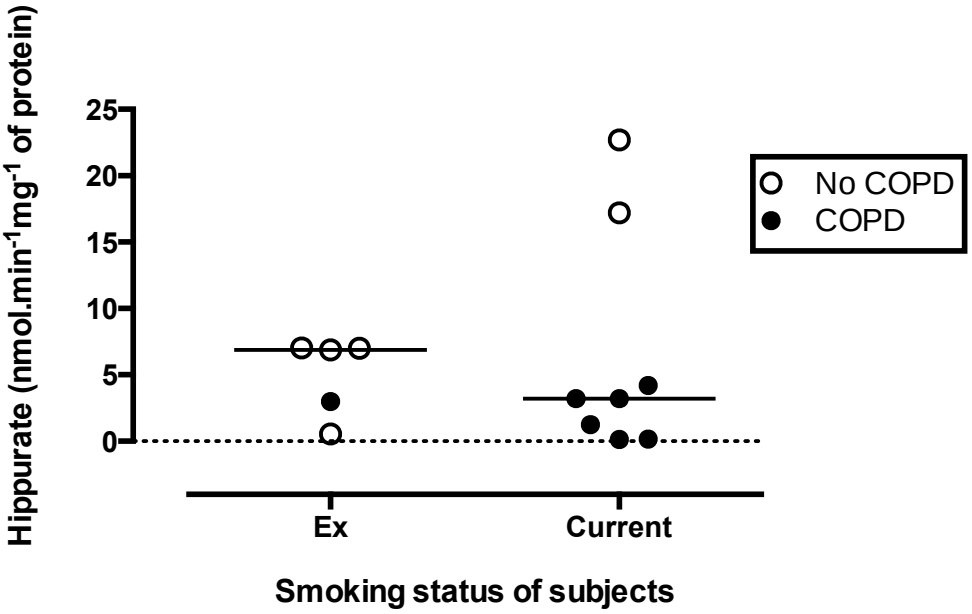


Figure 4.7 Subject smoking status and human lung ACE activity Data from figure 4.3 were reworked to evaluate ex-smokers (n=5) and current smokers (n=8). The line represents the median. One subject did not disclose their smoking status and one was a non-smoker therefore not plotted on this graph Mann-Whitney Test p=0.83

Figure 4.7 shows the hippurate rate of production did not differ between ex and current smoker groups [ex-smoker 6.88 (0.54 - 7.00) vs. current smoker 3.2 (0.13 - 22.7) nmol.min^{-1}mg^{-1}].

There seems to be a divide in the hippurate rates of the eight subjects within the current smoker group. Six subjects presented with means that were at the lower end of the scale for hippurate formation and all six had COPD. The second population consisted of two subjects with means that were at the higher end of the scale for hippurate formation. These remaining subjects did not have COPD.

In contrast there was no divide in the hippurate production rate in the ex-smoker group. Only one subject in the ex-smoker group was classified with COPD.

4.3.2 ACE-2 Activity in Human Lung Tissue

4.3.2.1 ACE-2 activity in mouse and human tissue

The method of Pedersen *et al*., (2011) was used to measure ACE-2 activity in human lung tissue. These investigators used mouse kidney homogenate as a positive control and so this was included in the validation steps as shown in figure 4.8.

a)

b)

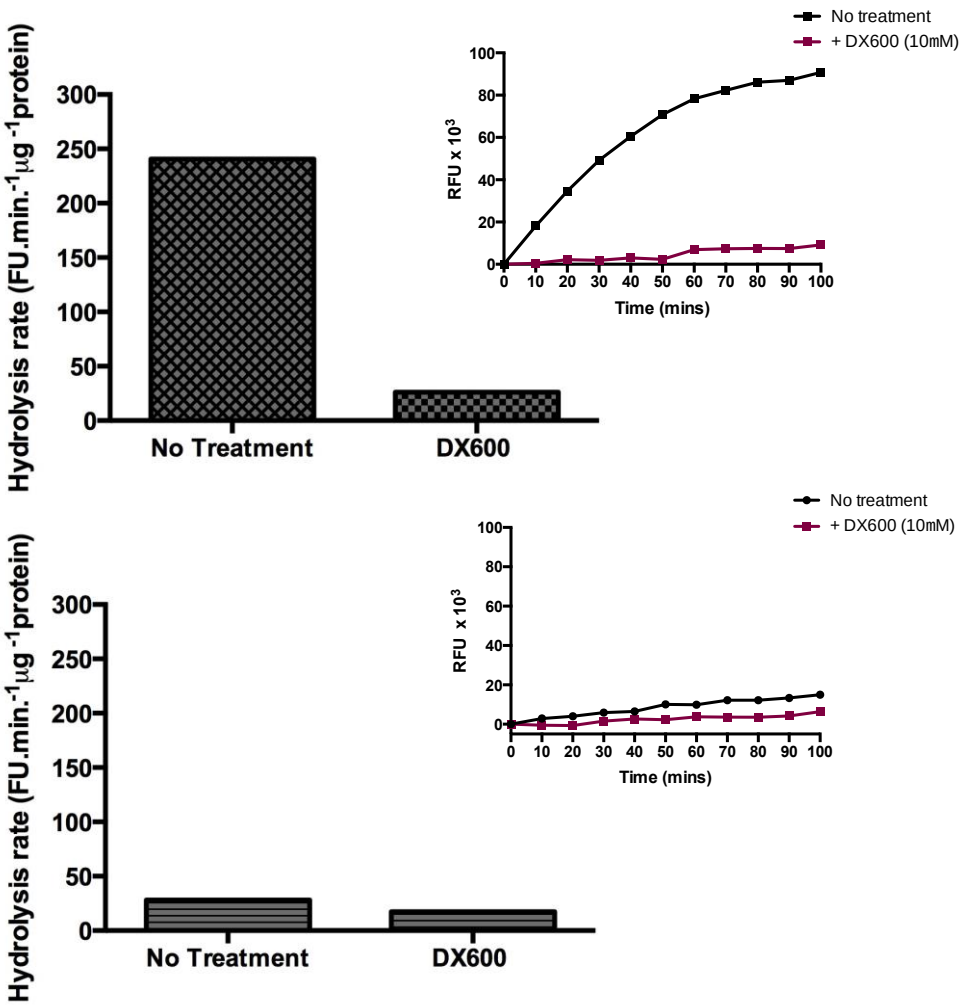


Figure 4.8 ACE-2 activity in mouse kidney and human lung Hydrolysis of fluoropeptide Mca-APK(Dnp) normalised to the protein concentration. Tissues isolated from a) C57Bl/6J mouse kidney or b) human lung in the presence and absence of the ACE-2 inhibitor, DX600 (10 µM). Bars are representative of the result from one experiment. Inset: both demonstrate the relative fluorescence units over time for each tissue

In figure 4.8 shows the hydrolysis rate of the mouse kidney was 240.53 $FU.min^{-1}\mu g^{-1}$. The mouse kidney sample treated with the ACE-2 inhibitor DX600 was almost 90 % less than that of the non-treated control.

The hydrolysis rate for human lung was 28.0 $FU.min^{-1}\mu g^{-1}$, this value was approximately 10-fold less than the hydrolysis rate observed in mouse kidney. When the human lung sample was treated with DX600 it produced an ACE-2 hydrolysis rate that was 38.2 % less than the non-treated control.

4.3.2.2 ACE-2 activity in COPD lung

The same human lung samples collected from 21 subjects for ACE activity (figure 4.2) were also evaluated for ACE-2 activity. In the same order that the ACE activity experiments were analysed (section 4.3.1.2), subjects were first divided into whether they were prescribed ACEi medication or not as plotted in figure 4.9.

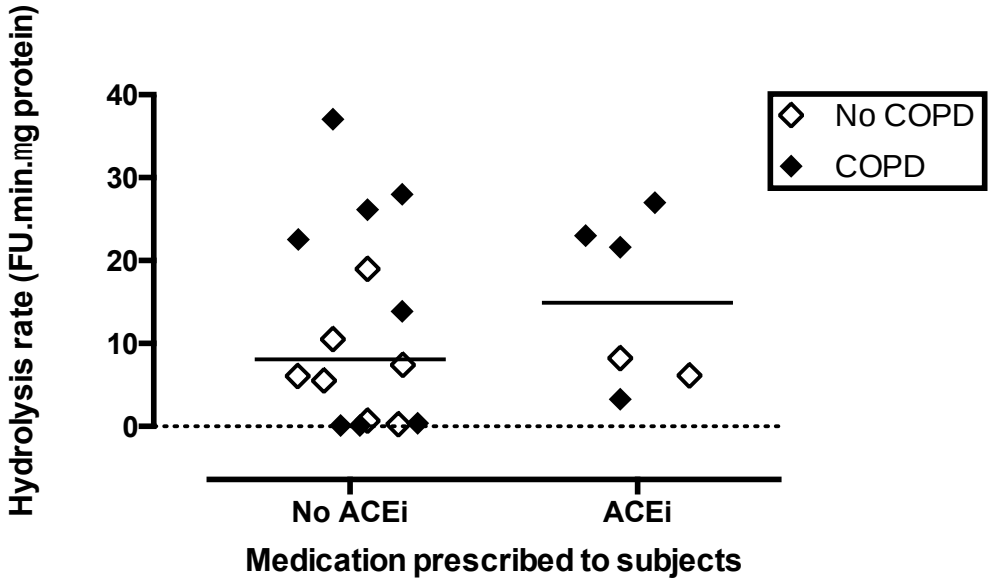


Figure 4.9 ACE-2 activities of subjects sampled Hydrolysis of fluoropeptide Mca-APK(DnP) in lung homogenate samples. Subjects were grouped by whether they were prescribed ACEi medication (n=6) or not (n=15). Triplicates were done in each experiment and a mean of three independent experiments was plotted for each subject. Lines represent median value. Mann Whitney Test p=0.41

In figure 4.9 the hydrolysis rate was not different between subjects prescribed ACEi compared to those who were not prescribed ACEi medication [ACEi 14.91 (3.29 - 26.99) vs. no ACEi 8.0 (0.10 - 37.04) $FU.min^{-1}\mu g^{-1}$].

These same subjects in figure 4.9 were classified by lung function into COPD and non-COPD groups. Data are shown in 4.10.

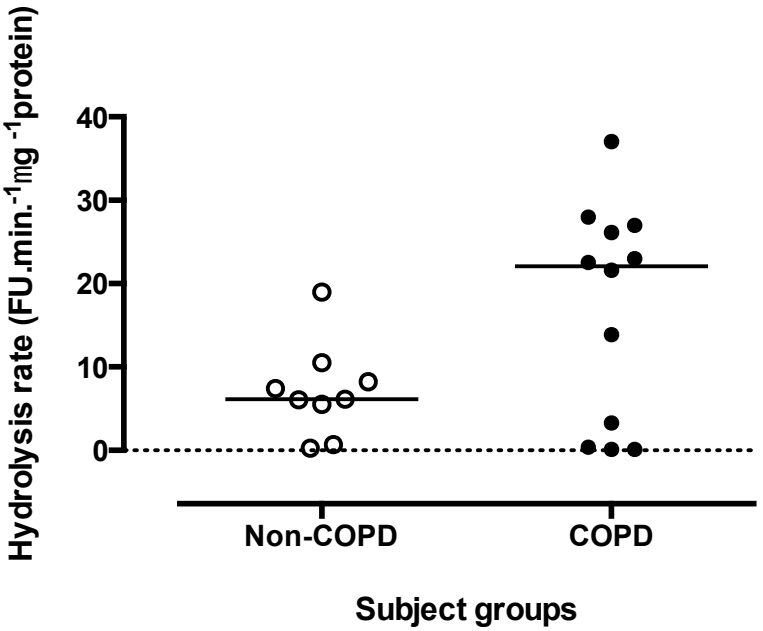


Figure 4.10 ACE-2 activity in human lung from subjects classified with COPD Data from figure 4.10 was reworked to evaluate the hydrolysis rate of subjects with COPD (n=12) or non-COPD (n=9). Triplicates were done in each experiment and a mean of three independent experiments was plotted for each subject. Lines represent median value. Mann Whitney Test p=0.16

As shown in figure 4.10 the hydrolysis rate for ACE-2 did not differ between COPD and non-COPD groups, [COPD 18.2 (0.10 – 37.04) vs. non-COPD 6.15 (0.24 – 18.96) $FU.min^{-1}\mu g^{-1}$]. The COPD group had a 3-fold higher median value compared to the non-COPD group but this was not statistically significant. Three subjects in the COPD group and two subjects in the non-COPD group had no hydrolysis rate detected in their samples.

4.3.2.3 ACE-2 activity and ACE-2 protein expression

A subgroup of subjects plotted in figure 4.11 was sampled for both ACE-2 activity and ACE-2 protein expression. Results from both assays for each subject were plotted on the same graph as shown in figure 4.11.

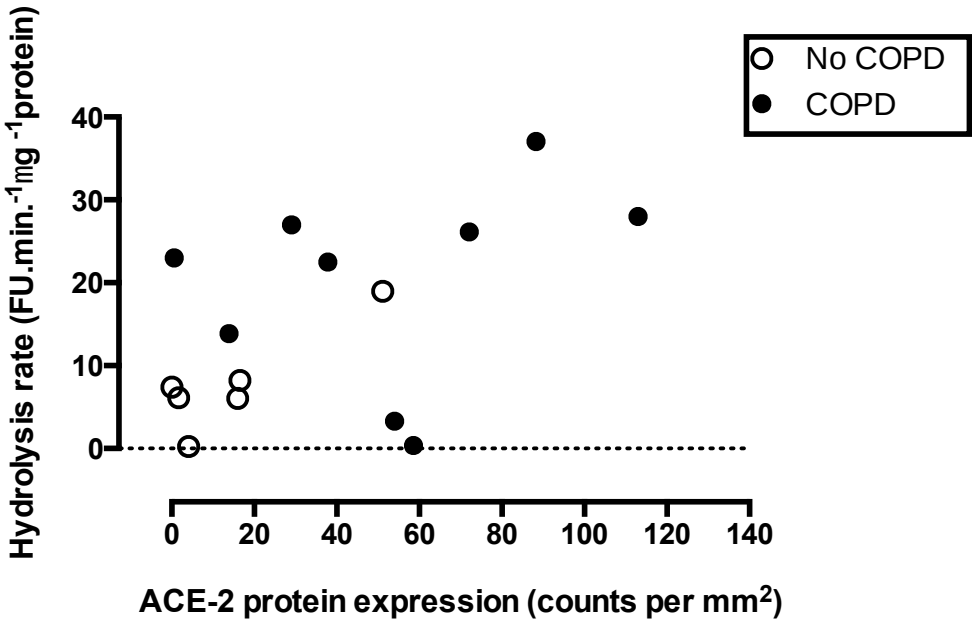


Figure 4.11 Association between ACE-2 activity and ACE-2 protein expression Paired data from a subgroup of subjects whose samples underwent ACE-2 activity and IHC of lung parenchyma (n=15). ACE-2 activity plots represent the means for each subject from three independent experiments. ACE-2 expression was expressed as the number of positively stained ACE-2 cells per mm^2 for each subject Parametric correlation analysis. Pearson r = 0.55, p= 0.035.

Figure 4.11 shows that ACE-2 activity and ACE-2 protein expressions were positively correlated in this cohort of subjects (r = 0.55, p= 0.035).

4.3.2.4 ACE-2 activity and smoking status

ACE-2 activity could also be affected by cigarette smoke therefore the data for current smokers at the time of surgery and those who had previously smoked (ex-smokers) was compared. Data are shown in figure 4.12.

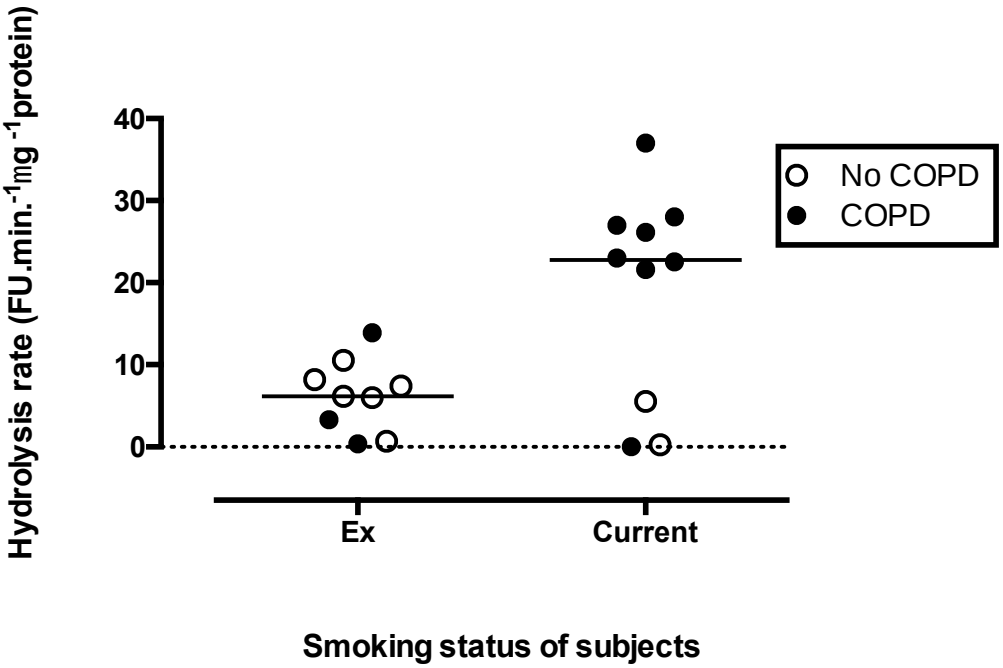


Figure 4.12 Subject smoking status and human lung ACE-2 activity Data from figure 4.10 reworked to evaluate hydrolysis of fluoropeptide Mca-APK(DnP) rate in lung tissue homogenates from ex-smokers (n=9) and current smokers (n=10). Triplicates were done in each experiment and a mean of three independent experiments was plotted for each subject. Lines represent median value. Mann-Whitney Test p=0.053

In figure 4.12 there was a trend of a lower hydrolysis rate in ex-smoker compared to current smoker groups [ex-smoker 6.15 (0.36 - 13.90) vs. current smoker 22.78 (0.00 - 37.8) $FU.min^{-1}\mu g^{-1}$]. The current smoker group had a 3.5-fold higher median value compared to the ex-smoker group and although the p value nearly reached significance this was not statistically significant (p=0.053). A power calculation was also done for this data set and it was over 0.80. This power calculation result demonstrates that there is a sufficient number of samples tested in this experiment for the result of the Mann-Whitney test to be true.

The hydrolysis rate of the current smokers group seemed to have two populations. Seven subjects were at the top half of the plot. These subjects had COPD. The remaining three subjects were on the lower end of the scale. Only one out of the three subjects had COPD.

4.4 Discussion

The aim of this chapter was to measure ACE and ACE-2 activities within the lungs of subjects with COPD – classified according to their lung function - and compare these measurements to subjects who were not classified with COPD. Other objectives were to evaluate whether angiotensin-related medication and smoking status of the subjects affected lung ACE or ACE-2 activity.

The main finding of this chapter was ACE activity in the lungs of COPD subjects was significantly lower than non-COPD control subjects. This finding is novel and informative since ACE activity has not been investigated within the lung of COPD subjects before. One finding to note was ACE-2 activity seemed to be higher in COPD subjects as measured by a three-fold higher median compared to controls but this was not statistically significant. Furthermore ACE-2 activity was also trending higher in current smokers compared to ex-smoker controls and this reached a near significance (p=0.053).

4.4.1 Method Rationale

The nature of the experiments described in this chapter meant that only a small group of subjects - a subset of the larger cohort (table 2.1) - were examined (table 4.1). Comparison of this subset with the whole cohort demonstrated that this smaller group is representative of the whole cohort.

4.4.1.1 ACE

The methods used to measure ACE activity were chosen because it had been used many times in the literature before. The measurement of ACE activity in human lung tissue has been published in detail within two papers (Friedland *et al*., 1981;Takeuchi & Shimizu, 1989). Both groups used techniques that required a substantial amount of human tissue sample of which was not feasible for this investigation. This was because only small samples were available from lung surgery.

Rat lung had also been sampled for ACE activity (Watkins *et al*., 2008). Rat lung has a similar weight to the small human samples received from surgery in this current study. The same technique used in Watkins *et al.,* was therefore used to measure the ACE activity in human samples for this current study.

4.4.1.2 ACE-2

For the ACE-2 activity assay, the method used by Pedersen *et al*., (2011) was chosen because enough detail was explained in the publication to reproduce their findings. Most of their experiments were done in mouse kidney so validation for this ACE-2 activity assay was first carried out in the current study on mouse kidney before it was used on human lung samples. There has been a paper that measured ACE-2 activity in human lung tissue (Li *et al.*, 2008) however there was not enough detail described in their methods to carry out the experiment so their method was not used.

4.4.2 RAS Activity in COPD

4.4.2.1 ACE activity

As mentioned before, ACE activity in lung is significantly less in COPD subjects compared to those subjects without COPD. There are two previous publications that have measured ACE activity in COPD subjects however these two studies measured serum ACE activity rather than lung ACE activity. The first observed no significant difference between the 35 COPD subjects and the 63 non-COPD controls (Silverstein *et al*., 1975). The second study revealed that the ACE activity in serum was higher in 26 COPD subjects compared to 32 control subjects (Brice *et al*., 1995).

The latter paper seems to contrast the observation from this current study's finding: a significantly lower ACE activity in COPD subjects compared to controls. However, it could be that both findings are true. ACE in the lung is mostly anchored to the endothelial membrane whereas ACE in serum is free to circulate. COPD may lead to an increase in shedding of N-terminal ACE from the lung (English *et al.*, 2012; Hermanns *et al.*, 2014) thus lowering ACE activity, which may increase the ACE activity within the circulation.

Other lung diseases such as pulmonary hypertension have significantly lower ACE activity compared to non-diseased controls (Morrell *et al.*, 1995). Furthermore, patients with ARDS have reduced ACE activity (Orfanos *et al.*, 2000). The results of the current study support the reports of lower activity in patients with lung diseases.

4.4.2.2 ACE-2 activity

Unlike ACE activity in the lung, the ACE-2 assay revealed a 3-fold higher ACE-2 activity median in COPD subjects compared to subjects without COPD however this did not reach statistical

significance. This may be due to a number of subjects that had no hydryolysis rates and therefore lowering the median for the COPD group.

As mentioned before, this current study is the first to investigate ACE-2 activity in the lung of COPD subjects. This study disagrees with the only paper that has reported ACE-2 levels in the context of COPD (Xue *et al*., 2014). In this paper, COPD rats had significantly lower ACE-2 gene and protein expression levels compared to non-COPD controls. Li *et al.*, (2008) reported ACE-2 activity in human lung but not lung with COPD. They found significantly lower ACE-2 activity in subjects with IPF compared to non-IPF controls.

It seems from the literature, a lower ACE-2 activity within the COPD lung is to be expected however the current study reports that ACE-2 activity seems higher in COPD compared to non-COPD subjects but this is not significant. Reasons for this discrepancy may be due to pathophysiological development of COPD being different to that of IPF and therefore ACE-2 activity is not reduced. As for the rat model of COPD, Xue *et al,* (2014) exposed rats to a high amount of cigarette smoke (8-15 cigarettes smoked over a 30 minute period twice a day) over a short period of time (12 weeks). This does not simulate the development of COPD in humans since humans typically smoke fewer cigarettes per kilogram of body weight and also smoke over a longer period of time before they are diagnosed with COPD (Lokke *et al.,* 2006). This may be a reason for the differences between low ACE-2 levels in disease as reported in the literature and the higher ACE-2 activity in this current study.

4.4.3 Incubation of ACE and ACE-2 Inhibitors

Direct application of captopril to the lung homogenate samples reduced the hippurate production rate compared to those samples not treated. This reduction was steep but it was similar to that observed in Watkins *et al*., (2008) and supports the notion that ACE activity is being measured in this assay. Furthermore figure 4.4 demonstrates dose-dependent inhibition of the hippurate production rate with increasing concentrations of captopril. This is further evidence that captopril is inhibiting ACE activity.

In the same way, ACE-2 inhibitor DX600 was used to test the ACE-2 assay, as in Pedersen *et al.,* (2011). At first glance, it seems as though the ACE-2 inhibitor DX600 was not as efficacious in human lung tissue compared to the mouse kidney tissue (figure 4.8). There is a possibility that other enzymes could have cleaved the alanine-proline bond or the proline-lysine bond in the substrate Mca-APK(Dnp). For example trypsin-like enzymes prefer the amino acid lysine (Evnin

et al., 1990) and trypsin-like enzymes such as trypsin are present in the human lung mast cell (Pesci *et al.*, 1998).

Another possibility could be due to a low basal hydrolysis rate observed in human lung samples. Any attenuation in ACE-2 activity with DX600 may be masked by the signal to noise ratio therefore the true level of inhibition with DX600 may be much higher than that observed in this study. The use of more sensitive techniques may be needed to allow accurate characterisation of ACE-2 activity in the human lung.

As mentioned before, one group did measure ACE-2 activity within the IPF human lung however they did not quote raw hydrolysis rates (Li *et al.,* 2008). Instead, they plotted the percentage change of hydrolysis from non-diseased control samples. It is therefore difficult to know whether these values are normal for ACE-2 activity in human tissue or not.

4.4.4 Angiotensin–related Medication

4.4.4.1 ACE activity

Subjects who were prescribed ACEi medication had negligible ACE activity compared to subjects who were not prescribed ACEi medication (figure 4.2). This effect overrides any differences of ACE activity between COPD and non-COPD groups by bringing the ACE activity down to negligible amounts. This logically fits with the mechanism of action of ACEi where ACE activity in the plasma is blocked at the active site of ACE. ACEi in the plasma have a half-life of 11- 17 hours (EMC, 2015).

For ARB medication, there was only one subject prescribed ARB out of all subject samples available therefore this sample was experimented with to explore the lung RAS in detail. To do this, I asked the following questions: what is the ACE activity in a subject already prescribed ARB? And secondly, would the activity change in this sample once incubated with varying concentrations of the ACEi captopril? Results demonstrated that the ARB prescribed subject had an ACE activity of 3.0 $nmol.min^{-1}mg^{-1}$. This was similar to the median value of subjects belonging to the group that were not prescribed angiotensin-related medication (3.2 $nmol.min^{-1}mg^{-1}$) (figure 4.2). This was expected as candesartan is known to block the AT_1 (Ang II receptor) and is not associated with the enzyme ACE (Belz *et al.,* 1999) and there was a dose response to varying concentration of captopril.

As a control sample, a lung sample was taken from surgery and the same techniques were applied to it. The measurement of ACE activity was also done in parallel with the ARB prescribed subject

sample. As before, this new sample was incubated with various concentrations of captopril, however, in contrast to the other results there was no dose response to these concentrations. It was later discovered that this subject was prescribed the ACEi perindopril.

The reason for the no dose response could be due to the oral ACEi perindopril inhibiting ACE activity long after it had been ingested. Perindopril when converted to its active form perindoprilat shortly after administration has a half-life of approximately 17 hours (EMC, 2015 - perindopril) due to its slow dissociation from ACE. The remaining ACEi in samples of this current study could be lowering the ACE activity and overriding any direct application of the ACEi captopril.

4.4.4.2 ACE-2 activity

In contrast to ACE activity, ACE-2 activity was not affected by ACEi prescription in the current study (figure 4.9). Tipnis *et al.,* (2000) discovered the ACE-2 molecule and they reported that ACEi did not have an effect on ACE-2 activity. This study confirms this finding. More recent studies seem to suggest that ACEi do have an effect on ACE-2 activity. Ocaranza *et al*., (2006) reported the ACEi enalapril administered to rats with heart failure prevented the reduction of ACE-2 protein and activity compared to those rats that did not have ACEi treatment. Furthermore, the ACEi perindopril was administered to rats with liver disease and the investigators observed an increase in liver ACE-2 expression levels compared to those who were not administered (Huang *et al*., 2010). The aforementioned experiments were done in animals; normal levels of ACE-2 activity have not been deduced in humans. The current study is the first to analyse the effect of ACEi/ARB prescription on ACE-2 activity lung tissue.

4.4.5 Correlations between Activity and IHC Protein Expression

4.4.5.1 ACE activity

ACE activity is not correlated to IHC ACE protein expression in the lung. The antibody used to detect ACE protein expression in IHC recognises the N-terminal tail that also accommodates the enzymatic catalytic domains. These catalytic domains are responsible for cleaving peptides therefore the number of ACE positive vessels should correlate with ACE activity accurately.

The disparity between ACE activity and protein expression may be due to the variation of ACE activity from subject to subject. Some subjects may have high activity. Other subjects with the same protein expression may have less ACE activity leading to no correlation. Serum ACE concentration is known to be higher in subjects with the DD polymorphism of the ACE gene.

Polymorphisms are frequent, out of 10,049 subjects 9,034 of them had a homologous II or DD polymorphism (Lee *et al.*, 2009) so this may be the reason for no correlation. Another suggestion could be that ACE activity could be graded. The reasoning being that there are two catalytic domains belonging to the N-terminus of the ACE molecule. If only one catalytic domain is functional it could lead to lower activity than a subject who had both catalytic domains in working order. This could lead to variations in the ACE activity between subjects.

4.4.5.2 ACE-2 activity

Unlike ACE activity, ACE-2 activity is positively associated with protein expression in the parenchyma as measured by IHC. The antibody used for ACE-2 recognises 17 amino acids at the C-terminal tail. As there is positive correlation between ACE-2 activity and protein expression it suggests that the C-terminus, identified by the antibody used in IHC, is still attached to the N-terminal tail because subjects with high ACE-2 activity also have high number of ACE-2 positive cells.

4.4.6 Activity and Smoking Status

4.4.6.1 ACE activity

Lung homogenate from current smokers did not differ in ACE activity compared to ex-smokers. Subjects prescribed ACEi were excluded from this assay because they had negligible ACE activity as established in figure 4.2. The remaining subjects [ex-smoker (n=5) current smokers (n=8)] were low in numbers and therefore this analysis had insufficient power to find out whether there was a significant difference between the two groups. To gain appropriate power, based on these preliminary results, the analysis would require 46 subjects in total. That is double the number that was sampled in this chapter.

The current smokers group had a divide in the hippurate formation values plotted (figure 4.7). Six current smokers had hippurate values that were at the lower end of the scale and were COPD subjects. The remainder were at the higher end of the scale and these subjects did not have COPD. The divide between COPD and non-COPD subjects was to be expected because COPD subjects, previously in this chapter, had a significantly lower ACE activity as shown in figure 4.2. As COPD is a confounding variable for this experiment it overrides any difference that may occur between current and ex-smokers.

It is important to state that current smoking could be associated with COPD. The two factors could be related, which could affect the ACE activity. In this particular set of results, the number

of subjects was not enough to perform a chi-square test to test whether the factors were affected by each other or not.

With regards to interpreting the effect of smoking status on ACE activity, it was helpful to focus on subjects not classified with COPD. Here, the current smokers have higher ACE activity compared to ex-smokers (figure 4.3), which may be due to the inhalation of smoke and tissue ACE destruction. As a result, current smokers have raised ACE activity to compensate for this. This speculation is based on an experiment with a low number of subjects therefore more samples would be needed in order to conclude this.

4.4.6.2 ACE-2 activity

There was evidence that current smokers had higher ACE-2 activity compared to ex–smokers. This comparison nearly achieved significance with a p value of 0.053. ACE-2 activity could be enhanced by the state of inflammation in the tissue of current smokers and an increase of ACE-2 activity was required to protect the tissue.

Studies have focused on ACE-2 protein levels rather than activity in animal models of chronic smoking. Two publications have observed a lower ACE-2 protein level within the lung parenchyma of animals exposed chronically to cigarette smoke (Han *et al*., 2010; Xue *et al*., 2014). They also showed that gene transfer of ACE-2 before cigarette smoke exposure, prevented the reduction in ACE-2 protein levels. The above literature seemed to disagree with the experimental results that were found in this study.

It is important to note that the ex-smoker control group in this current chapter was not the same as the control group used in the two aforementioned papers. The two papers used subjects who had ‘never smoked’ as controls whereas the current study used subjects who had smoked before but had stopped for more than one year at the time of surgery. This may be the reason as to why there is a difference between the literature and the current study in addition to the fact that the literature papers measured protein levels rather than the activity that was measured in this current study.

Hypothetically, never smoking allows ACE-2 protein levels to stay high in the lung whereas smoking and then stopping may defect ACE-2 activity. Smoking reverses this defect and allows ACE-2 activity to be higher than ex-smokers. These hypothetical arguments are interesting to debate however it is evident that more subjects are required to understand fully whether smoking status influences lung ACE-2 activity.

Limitations

The status of smokers was self-reported. Ex-smokers were grouped according to whether they had stopped smoking for more than one year. This could be a limitation because subjects could estimate inaccurately when they had stopped smoking leading to subjects being grouped into the wrong group. For this study the accuracy of stopping smoking may not be such a limitation since the average number of years an ex-smoker group had stopped smoking was approximately 10 -15 years. This is a lot longer than the one year cut off that was thought to lead to inaccuracy with regards to group classification.

Furthermore, patients may not be honest with the clinician or nurse with regards to their smoking status as the answer may have implications on their clinical treatment this could be another limitation to the study. Subjects could also be continually exposed to smoke through passive smoking at their place of work but are considered an ex-smoker. More rigorous controls for smoking need to be established rather than relying on self-reported behaviour. One reliable and objective method for measuring whether the subjects were current cigarette smokers is to measure the cotoline levels in saliva samples, which is a metabolite of nicotine. If present, this indicates that the subject has smoked for up to the last three days (Cope *et al.*, 2012). Unfortunately the study did not have ethics approval to take saliva samples from the subjects before surgery therefore cotoline levels could not be measured.

Another limitation is the freezing of the human lung tissue before processing into a homogenate for ACE and ACE-2 activity assays. The human lung tissue was not treated in the same way as the animal tissue samples used to validate the activity assays. These animal samples were processed as fresh *i.e.* the samples were not frozen. Nevertheless, Shabihkhani *et al.*, (2014) has reviewed various papers that have demonstrated freezing and thawing samples of fresh tissue. There was a general consensus that one freeze-thaw cycle had no effect on the integrity of RNA or protein however repeated freeze-thaw cycles of three or more did have an effect on the composition of the tissue. Since human lung samples in this study underwent freezing and thawing at a maximum of two cycles the ACE and ACE-2 proteins should not have been affected. To confirm this, a validation experiment where different samples from same participant are frozen and thawed a varying number of times for should be done. These samples should then have their ACE and ACE-2 activity measured to see if there is any variation in activity and therefore whether the number of freeze-thaw cycles affects the activity within the tissue.

4.4.7 Summary

The work described in this chapter has demonstrated that lung from COPD subjects has a significantly lower ACE activity compared to subjects not classified with COPD. On the other hand, ACE-2 activity seems to be higher in COPD subjects compare to controls, however, this is not significant. ACEi prescription lowers lung ACE activity compared to subjects who were not prescribed ACEi, as expected, but ACEi do not affect ACE-2 activity. In contrast, ACE activity was unaffected by smoking status of the subjects but ACE-2 activity seemed to be affected demonstrating a higher activity in current smokers compared to ex-smokers but this was not significant

Chapter 5 ACE-2 and Macrophages

5.1 Introduction

From the last two chapters, it has been observed that the ACE-2 identified in the lung is located within the lung macrophages and ACE-2 activity seems to be higher in COPD lung compared to controls. In the literature, the few papers that have investigated ACE-2 expression in the body demonstrate that it is associated with anti-inflammatory activity. For example, recombinant ACE-2 injected into rats with symptoms of the inflammatory disease ARDS lowered both the symptoms and the severity of the disease (Imai *et al.*, 2005). Furthermore, ACE-2 protein expression and activity is significantly lowered in severe inflammatory diseases such as IPF or diabetic nephropathy (Tikellis *et al.*, 2008; Li *et al.*, 2008). The literature suggests that ACE-2 could be required for the management of inflammatory diseases and may have an integral role in inflammation.

The aim of this chapter was to investigate factors that may affect ACE-2 protein expression levels in lung macrophages. The rationale being that factors or conditions that do change ACE-2 expression could, in the future, be applied to animal models of inflammatory disease and attenuate the symptoms of the modelled disease.

The factors investigated in this chapter were chosen because of observations that were made earlier on in this thesis as well as in the literature. One of the observations being that only a proportion of lung macrophages observed in chapter 3 were ACE-2 positive (figure 3.13). The reason why some macrophages were positively stained and other macrophages were not is not clear. As a hypothesis ACE-2 protein expression could be affected by maturation. This is an idea from a paper that demonstrated an increase in the concentration of MMP, a protease similar to ACE-2, during maturation of macrophages *in vitro* (Ghorpade *et al.*, 2001).

To investigate maturation as a factor in this current chapter, peripheral blood monocytes were isolated from volunteers and were matured into macrophages *in vitro.* This process is called the monocyte-derived macrophage (Mdm) model. ACE-2 protein expression was measured by immunocytochemistry (ICC) at regular intervals during maturation.

Another observation, found in chapter 3 and 4, was that the current smokers had a trend of more ACE-2 positive cells (figure 3.20) and a higher ACE-2 activity (figure 4.12) compared to ex-

smokers. This led to another hypothesis, that cigarette smoke exposure and subsequent oxidative stress and inflammation influences ACE-2 expression in the lung macrophage. To investigate cigarette smoke as a factor, cigarette smoke extract (CSE) was applied to the Mdms as well as fresh lung tissue fragments. ACE-2 expression and activity was measured after CSE exposure.

5.2 Methods

5.2.1 Macrophages from Lung Tissue

As a positive control for ACE-2 staining in Mdms, macrophages were isolated from fresh lung tissue by the non-enzymatic method as described by Liu & Proud, (1986). This fresh lung tissue was taken from a COPD subject (GOLD II) who smoked at the time of surgery. These isolated lung macrophages were stained with antibodies against CD68 and ACE-2 using ICC. Their non-specific esterase activity was also evaluated along with the activity of ACE-2.

5.2.2 Monocyte to Macrophage Maturation

Blood samples were taken from volunteers and peripheral blood monocytes were isolated. See section 2.3.3 for more details. Volunteers gave informed written consent. The Southampton and South West Hampshire Research Ethics Committee (reference number 08/H0504/138) gave ethical approval for this study. The ethics only approved minimal details *i.e.* the age, gender and smoking status of the volunteers to be used in analysis.

Table 5.1 Subject characteristics of healthy volunteers who donated their blood monocytes Blood samples were donated by 6 volunteers who had no known disease. Their blood monocytes were then isolated from the blood samples. Details of these volunteers are outlined in the table below; *Mdm – monocyte derived macrophage sample. Ex denoted subject had stopped smoking for at least one year. None - never smoked;* Data presented as raw values.

Subject	Gender	Age (years)	Smoking Status (Current, Ex, None)
Mdm1	Female	28	None
Mdm2	Female	42	Current
Mdm3	Male	45	None
Mdm4	Male	36	Ex
Mdm5	Male	31	None
Mdm6	Female	33	None

Monocytes were plated onto a 24-well plate at a cell concentration of 5 x 10^5 cells/well. Monocytes were treated with GM-CSF (2 ng/ml) for 12 days to generate macrophages with a similar phenotype to lung macrophages as previously described (Tudhope & Finney-Hayward, 2008).

On the same day of plating (day 0), a proportion of cells were removed and prepared for ACE-2 ICC detection. These cells (3 x 10^4 cells/slide) were spun using a cytocentrifuge onto poly-L-lysine coated glass slides. These slides were air-dried for 45 minutes before storing at -20^{o}C. On days 3,5,7,10 and 12 after plating, cells were also removed from culture using a non-enzymatic cell dissociation solution and cytospins were made.

5.2.2.1 Cigarette Smoke Extract

On day 7 after plating, a proportion of the Mdms cultured in the plates were treated with fresh 10 % CSE for 6 hours. The length of time and concentration of CSE was based on the experiments reported in Aoshiba *et al.* (2001). They used varying concentrations of CSE on lung macrophages and their technique was used in my experiments because the toxicity of the chosen concentration of 10 % CSE for 6 hours was demonstrated in the paper to be minimal. Preparation of CSE is described in the materials and methods chapter (section 2.3.4). CSE was then removed and the cells were given fresh complete RPMI media without CSE. The no treatment controls on day 7 were incubated in complete RPMI for 6 hours. The aforementioned cells were cultured for a 5 further days before they were cytocentrifuged onto poly-L-lysine coated slides (day 12) and stored at -20^{o}C.

The reason for treating with CSE once and then leaving for 5 days was to observe whether the intervention of CSE had any effect on the morphology of the cells at day 10 and day 12 after plating. The preliminary experiment explained above was based on a monocyte derived macrophage experiment from a particular paper. The paper investigated inducing an inflammatory response through the treatment of lipopolysaccharide on Mdms at day 7 of maturation (Guo *et al.*, 2009). This was to investigate the levels of cytokines released by monocytes and Mdms. The Mdms in my current study were treated at a similar time point (day 7 of maturation) to investigate whether ACE -2 protein expression was different with the treatment of CSE compared to those not treated. This preliminary experiment was done with the intention of using more samples however this was not carried out due to insufficient time.

5.2.2.2 Immunocytochemistry

Cytospins were removed from storage (-20^{o}C) and thawed at room temperature for 5 minutes. The slides were then fixed in methanol for 10 minutes. Endogenous peroxidase blocking solution was applied to the slides for 30 minutes at room temperature to inhibit the enzyme activity within the tissue that could interfere with the technique, as in materials and methods chapter (section 2.1). All solutions were applied for 30 minutes each and washed off with TBS between the solution applications.

The subsequent process was similar to the antibody staining explained in chapter 2 (section 2.1.3.4). In brief, primary antibodies for ACE-2 (1/100 dilution), and CD68 (1/100 dilution), were applied for 1 hour each. These were washed off and appropriate secondary antibodies were applied for 1 hour also. After the secondary antibodies were washed off, a VECTSTAIN ABC complex was applied in order to amplify the signal of the antibody binding. This was rinsed off and replaced with the chromogen for detection. The chromogens used to detect ACE-2 and CD68 were DAB and AEC, respectively. Isotype controls were used to rule out non-specific binding.

5.2.2.3 ACE-2 activity

Isolated lung macrophages (10 - 90 x 10^3 cells) from fresh tissue were sampled for ACE-2 activity using the ACE-2 activity assay as described earlier (chapter 2 section 2.2.5). Cells were immersed in the incubation buffer and mixed with the substrate Mca-APK(Dnp) (25 μM) at 37^{o}C for 60 minutes. ACE-2 activity was also measured in Mdms at day 12 (see 5.2.2 for more details about Mdms) and lung fragments (see 5.2.4). For ACE-2 activity units, an arbitrary unit of relative fluorescence units (RFU) represented the release of the fluorescent peptide Mca-AP-OH from the substrate by ACE-2 cleavage. To calculate the rate of ACE-2 activity per minute (RFU.min^{-1}), the

change in RFU over a known period of time in minutes was measured. This RFU was divided by the number of minutes.

5.2.3 Non-specific Esterase Activity

Isolated lung macrophages were also stained to demonstrate non-specific esterase activity. Slides of macrophages isolated from lung tissue were incubated in methanol for 10 minutes at room temperature to permeabilise the cells and transferred into a chamber with TBS to stop the slides from drying out. The slides were then placed into a coplin jar along with incubating medium at 37^{o}C for 10 minutes and subsequently washed with running water for 1 minute. The cells were then counterstained with methyl green for 2 minutes at room temperature and washed again under running water. The slides were then dehydrated rapidly using fresh alcohol and after this with clearene. Slides were viewed under a light microscope. Brown staining represented non-specific esterase and green staining represented the nuclei.

This same procedure was also used for the Mdm samples.

5.2.4 Lung Fragments

Lung fragments were small pieces of fresh human lung tissue donated by one subject. One subject was intended to be a preliminary experiment with lung fragments from a number of subjects was planned. However due to time constraints this was not possible. The validity of this preliminary experiment was low because sampling from one subject only may not give a good representative of lung fragments in a population of people. Nonetheless, it is important that these experiments are carried out in order to demonstrate what experiments can measure.

Lung tissue wet weight of 0.5 g was cut into 2mm^3 pieces. Fragments were treated with freshly prepared 10 % CSE for 30 minutes at room temperature as described in methods 2.3.4. Lung fragments that were not treated with CSE were treated with fresh HBSS as a control. After this treatment, fragments were washed twice with TBS and homogenised manually as described in methods section 2.2.2 in preparation for the ACE-2 activity assay.

5.2.5 Statistical Test and Representation of Data

For the ACE-2 assay, an arbitrary unit represented the release of the fluorescent peptide Mca-AP-OH in relative fluorescence units per minute (RFU.min^{-1}). For lung fragments ACE-2 activity was

measured in RFU over time and plotted like this to compare the treatments over time. No statistical tests were carried out because of the low number of repeats for each experiment.

5.3 Results

5.3.1 ACE-2 in Fresh Lung Macrophages

To act as a positive control for ACE-2 identification by ICC, macrophages were isolated from fresh lung tissue and ACE-2 positive cells was assessed as observed in figure 5.1.

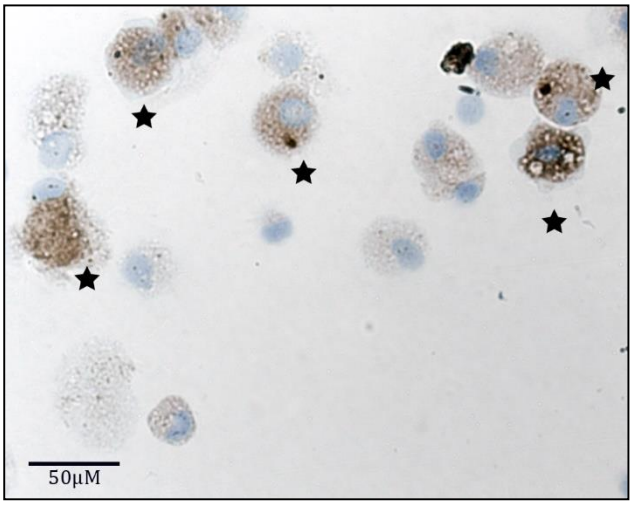


Figure 5.1 Micrograph of ACE-2 staining within human lung macrophages Macrophages were isolated from fresh resected human lung parenchymal tissue and cytocentrifuged onto glass slides. Slides were incubated with ACE-2 and chromogen DAB (brown) was used. Stars denote ACE-2 positive macrophages

Qualitatively, ACE-2 positive cells were a proportion of total macrophages as represented in figure 5.1. These lung macrophages were also evaluated for functional ACE-2 activity as plotted in figure 5.2.

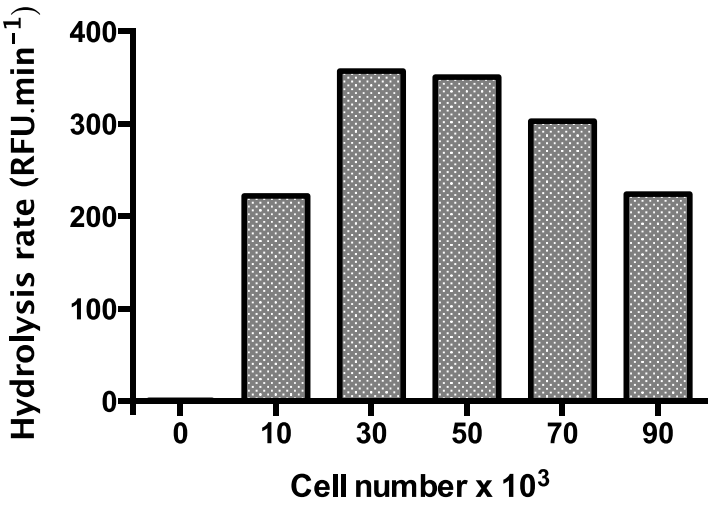


Figure 5.2 ACE-2 activity of lung macrophages isolated from fresh lung tissue Hydrolysis of fluoropeptide Mca-APK(Dnp) from varying numbers of lung macrophages. Data presented as raw values from one experiment.

In figure 5.2 there seems to be a biphasic response of ACE-2 activity with varying numbers of lung macrophages. As the numbers of macrophages increased, a higher rate of substrate hydrolysis was recorded. This observation applied to the first three sample numbers: between 0 - 10 x 10^3 macrophages there was an increase of 222 RFU.min^{-1} and between 10 - 30 x 10^3 macrophages there was a further increase of 135 RFU.min^{-1}.

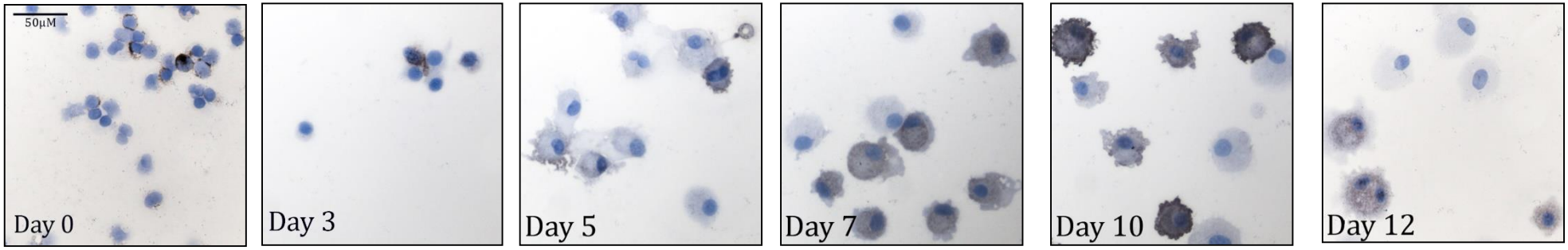
At 30 x 10^3 macrophages the hydrolysis rates stabilised. After this point any samples with higher numbers of macrophages had decreasing hydrolysis rates.

5.3.2 ACE-2 in Monocyte-Derived Macrophages

Since ICC had confirmed ACE-2 was present in lung macrophages, this enabled exploration of the factors that may influence the presence of ACE-2 protein expression in macrophages.

The first factor that was explored was the maturation of the macrophage using the Mdm model as shown in figure 5.3. Peripheral monocytes were isolated from the blood of volunteers and cultured. These monocytes were treated with GM-CSF. This treatment was used to mature these monocytes into macrophages with a similar phenotype to lung macrophages. See section 5.2.2 for the characteristics of the volunteers who donated their blood monocytes for this study.

a)



b)

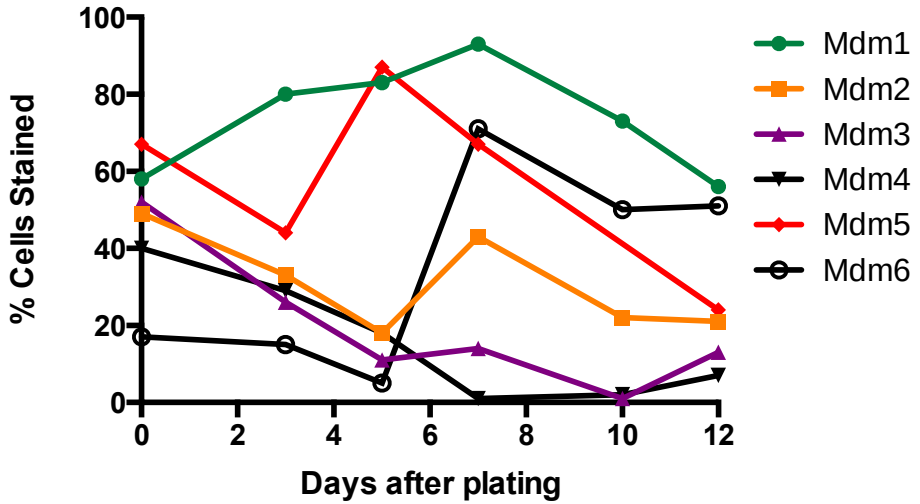


Figure 5.3 ACE-2 staining in monocyte-derived macrophages (Mdm) throughout maturation Over this 12-day period cells were treated with GM-CSF to initiate and maintain maturation a) Representative images from one subject (Mdm6) over time. *Brown staining denotes ACE-2 presence due to the DAB chromogen used. b)* Graph of percentage of total cells stained with ACE-2 over time. *Each line denotes a different subject, six in total*

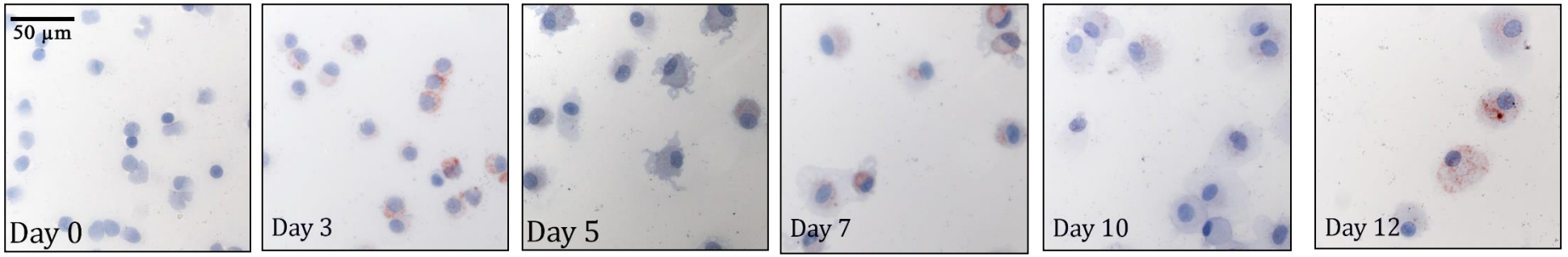
Figure 5.3 shows ACE-2 staining at regular time points during macrophage maturation. The six samples of isolated monocytes from peripheral blood of six different subjects were cultured *in vitro* into mature macrophages (figure 5.3b).

At the beginning of the experiment, day 0, ACE-2 staining was present in monocytes isolated from the blood. The median of ACE-2 positive cells for all subjects was 50 (17 - 67) %, collectively. At time points, 3 and 5 there was a depletion of ACE-2 positive cells in the sample for five out of six subjects. By day 7, there was a rapid increase of ACE-2 positive cells within three of these five subjects (Mdm samples 2, 5 and 6). Mdm6 had the highest increase from 5 to 71 % between day 5 and 7.

In contrast, for the first seven days of maturation Mdm1 demonstrated a steady increase in ACE-2 positive cells within the sample, which returned to its baseline percentage by day 12. Mdm1 was also the subject sample with the highest percentage of ACE-2 positive cells throughout all the time points (except for day 5) compared to the other subjects analysed.

By the end of the 12 days, the proportion of ACE-2 positive cells for all six subjects was 22.5 (7.0 - 56.0) %. Alongside staining for ACE-2, CD68 was used as a macrophage marker to confirm identification of macrophages. This was quantified in figure 5.4.

a)



b)

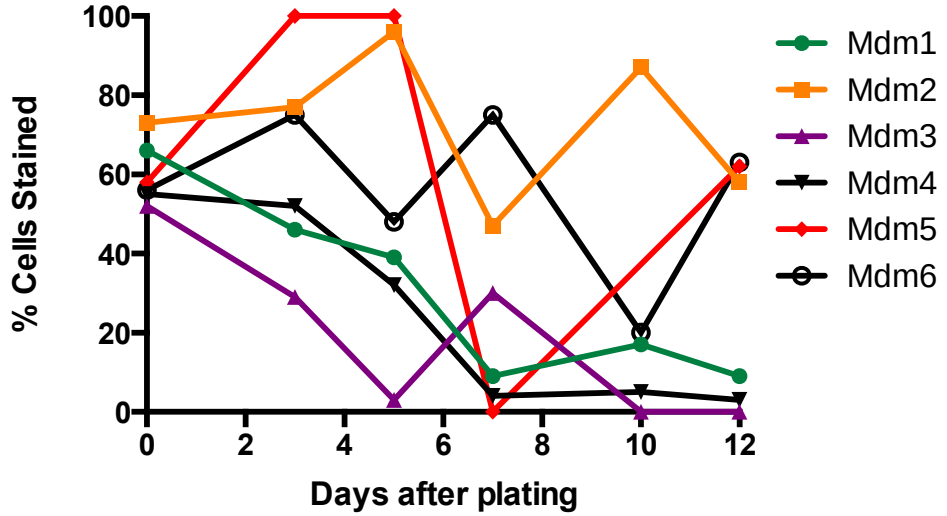


Figure 5.4 CD68 staining in Mdms during maturation Over this 12-day period cells were treated with GM-CSF to stimulate and maintain maturation a) Representative images from one subject (Mdm6) over the time course *Red staining denotes CD68 presence due to the AEC chromogen used*. b) Graph plotting the percentage of total cells stained with CD68. Each line denotes a different subject, six in total. N.B. Mdm5 on day 10 of maturation was not measured

Figure 5.4 shows CD68 staining at regular time points during maturation of the macrophages derived from peripheral blood monocytes. Samples from the same six subjects from figure 5.3 were used in this experiment.

At the beginning of the experiment (day 0), CD68 was present in monocytes isolated from the blood of these six subjects. The median of CD68 positive cells of the subjects was 57 (52 - 73) % macrophages positive for CD68.

Mdm3 had a reduction in the number of CD68 positive macrophages from day 0 to day 5 but this increased markedly by day 7 after plating. Only for this subject did the proportion of CD68 stained cells have a similar pattern to that of ACE-2 positive cells. The percentage of CD68 positive macrophages then fell to less than 1 % by day 12 after plating.

For the remaining five subjects, there was no discernible pattern for the percentages of CD68 positive macrophages throughout the 12 days of maturation. For example, Mdm2 and Mdm6 increased at the first time point and then decreased at every time point after. Mdm 5 had a steady increase in the beginning of the experiment but plummeted to no cells stained positively by day 7.

As a result of these fluctuations, another technique was introduced to confirm the identity of cells in these samples. This new technique used non-specific esterase as a marker for mature macrophages. Lung macrophages that were isolated from fresh lung tissue were used to optimise this technique. Micrographs are shown in figure 5.5.

p

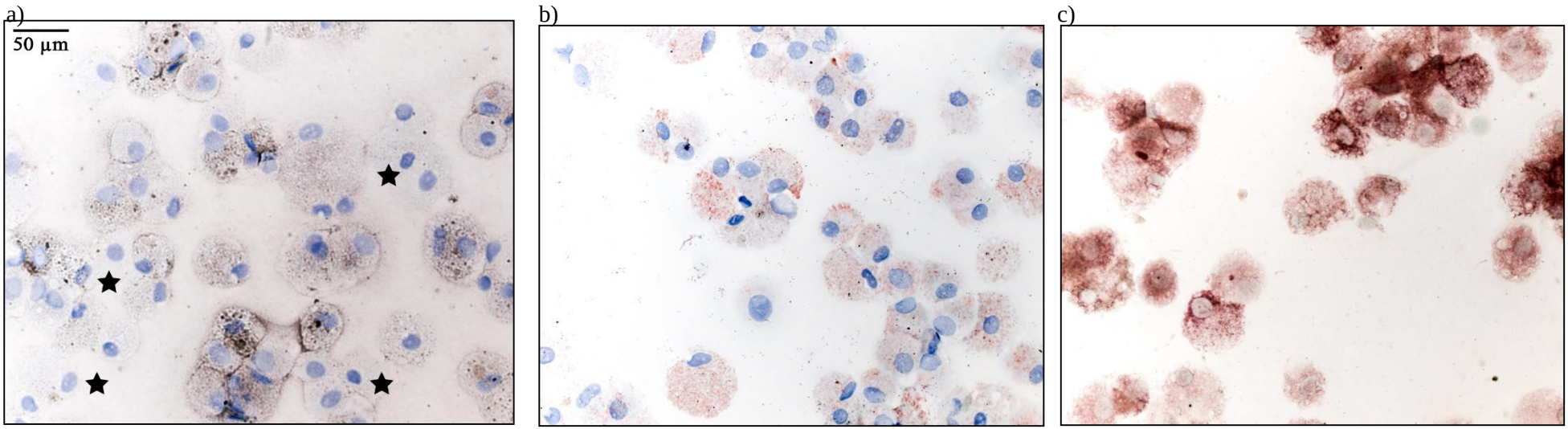


Figure 5.5 Fresh lung macrophages stained with ACE-2 and macrophage markers Lung macrophages from one subject isolated from fresh lung tissue and stained with a) ACE-2 b) CD68 c) non-specific esterase Stars indicate the macrophages that were not counted as positive for ACE-2.

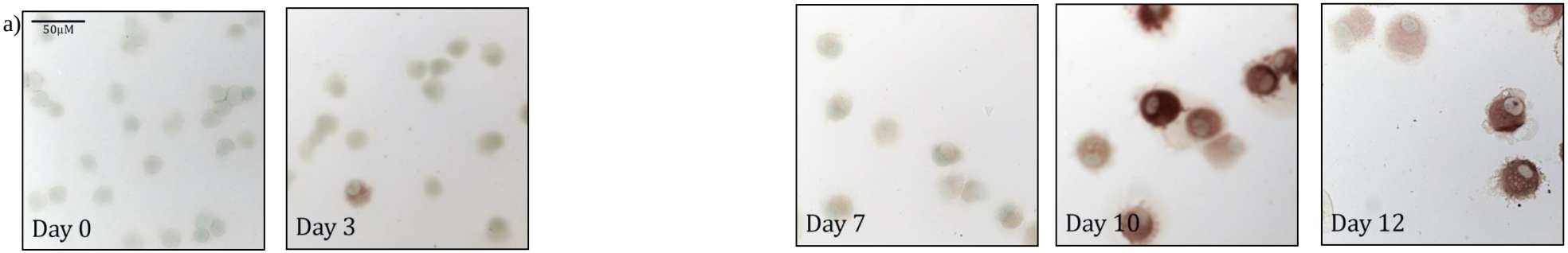
As seen in figure 5.5 lung tissue macrophages had dense staining of non-specific esterase (5.5c) as well as ACE-2 (5.5a) and CD68 (5.5b).

Non-specific esterase is a marker for mature macrophages and this enzyme was evaluated in the sample denoted by one subject (Mdm6). The reason for only staining one participant for non-specific esterase was because this was the last participant who donated their blood during this study. I was not able to secure any more volunteers willing to donate their blood.

The reason that the non-specific esterase staining technique was not used on participant numbers Mdm1, 2, 3, 4, and 5 was because analysis of these participant results revealed that the macrophage marker CD68 was not accurately identifying the macrophages at day 12. A more reliable technique to identify macrophages was required therefore the non-specific esterase was then implemented on the sixth volunteer (Mdm6) to demonstrate maturation from monocyte to macrophage.

This was demonstrated and quantified in figure 5.6.

a)



b)

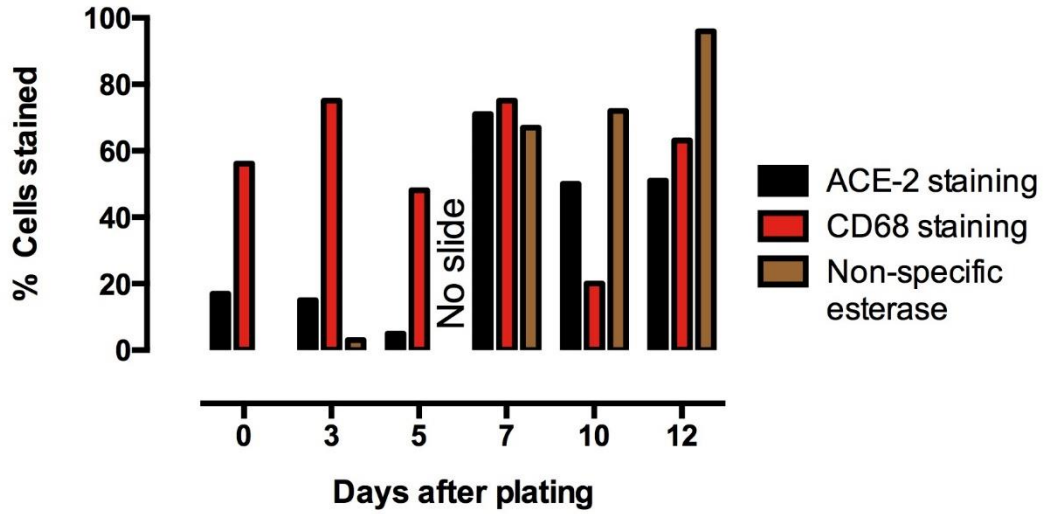


Figure 5.6 Non-specific esterase staining in Mdms throughout differentiation Cells were treated with GM-CSF to stimulate and maintain differentiation over a 12-day period cells. a) Images from subject Mdm6 of non-specific esterase staining (brown) b) Graph of percentage of total cells stained with ACE-2 (black bars) *CD68 (red bars) and non-specific esterase (brown bars) over time of MdMs isolated from one subject (Mdm6).* N.B no slide present day 7 for non-specific esterase

In figure 5.6b the percentage of cells stained positive for non-specific esterase were plotted alongside the percentage of cells stained positive for ACE-2 and CD68 for Mdm6 over 12 days.

At day 0 for Mdm6, there was no non-specific esterase staining on monocytes. Staining was, however, identified on day 3. At day 3, 3 % of total cells in the Mdm6 sample stained positively for non-specific esterase. This percentage continued to increase over 12 days until 96 % of cells were positive for non-specific esterase staining.

Numbers of ACE-2 positive and non-specific esterase positive cells had a similar pattern throughout the 12-day culture. For example, the percentage of ACE-2 positive cells was below 20 %, on days 0 and 3. This was matched by a low percentage of positively stained non-specific esterase cells. Culturing for longer led to an increase in ACE-2 positive cell numbers. For example at the later days of 7, 10 and 12 there were 70, 50 and 51 % of cells stained positively for ACE-2, respectively. This was mirrored with a high percentage of cells being positively stained for non-specific esterase, 67, 72 and 96 % at days 7, 10 and 12, respectively.

5.3.2.1 ACE-2 activity in monocyte-derived macrophages

ACE-2 expression in the Mdm6 sample was high at the end of the 12 days (figure 5.6b). The next experiment investigated whether this ACE-2 was functionally active. ACE-2 activity was measured in the Mdm cells at day 12 as shown in figure 5.7

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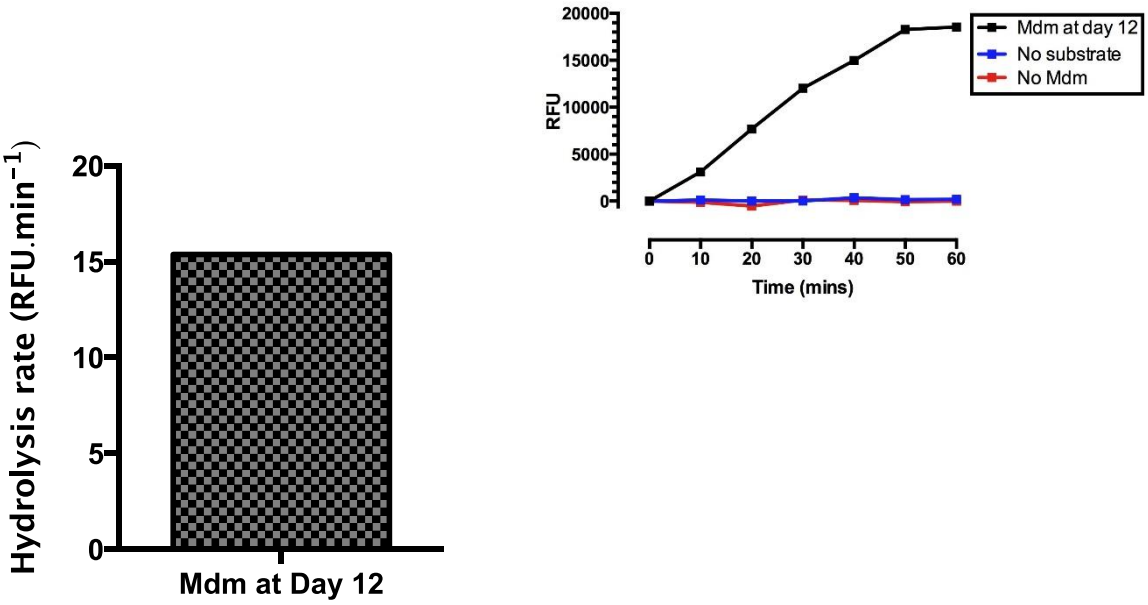

Figure 5.7 ACE-2 activity of Mdm at day 12 2.5 x 10^3 cells from subject Mdm6 were incubated with Mca-APK-OH (an ACE-2 specificsubstrate). The hydrolysis rate was calculated from the raw data over time (see section 5.2.2.3 for more detail). Insert: Raw data over time demonstrating no substrate and no Mdm controls. Results based on one experiment done in duplicate.

In figure 5.7 (insert), cells incubated without substrate did not have an increase in RFU over time and neither did the sample without any Mdms (blue and red lines). However, samples containing Mdms incubated with the substrate elicited a steady cumulative increase in RFU over 60 minutes (figure 5.7 insert). From this increase, a rate of 15 RFU.min^{-1} for Mdms per 2.5 x 10^3 cell sample after 12 days of culture.

5.3.2.2 Cigarette smoke extract exposure

The second factor that could influence ACE-2 expression in macrophage was cigarette smoke. CSE was used in this chapter. This was the mainstream smoke from the mouthpiece of a lit cigarette and the smoke was dissolved in HBSS. This CSE was applied to the Mdms in the middle

of maturation (day 7). At day 12 these exposed Mdms were evaluated for ACE-2 protein expression. Data are shown in figure 5.8.

Figure 5.8 shows that at day 0 the majority of freshly isolated monocytes were ACE-2 positive. By day 7, these cells were of similar morphology to mature macrophages and this was the time point at which CSE was applied. The monocytes were only isolated from one subject as this experiment was intended to be a preliminary experiment. More samples from other subjects were intended to be used however due to the constraints of time this was not possible.

Samples treated with acute exposure to 10 % CSE on day 7 appeared to contain more ACE-2 positive cells on day 12 than non-treated controls.

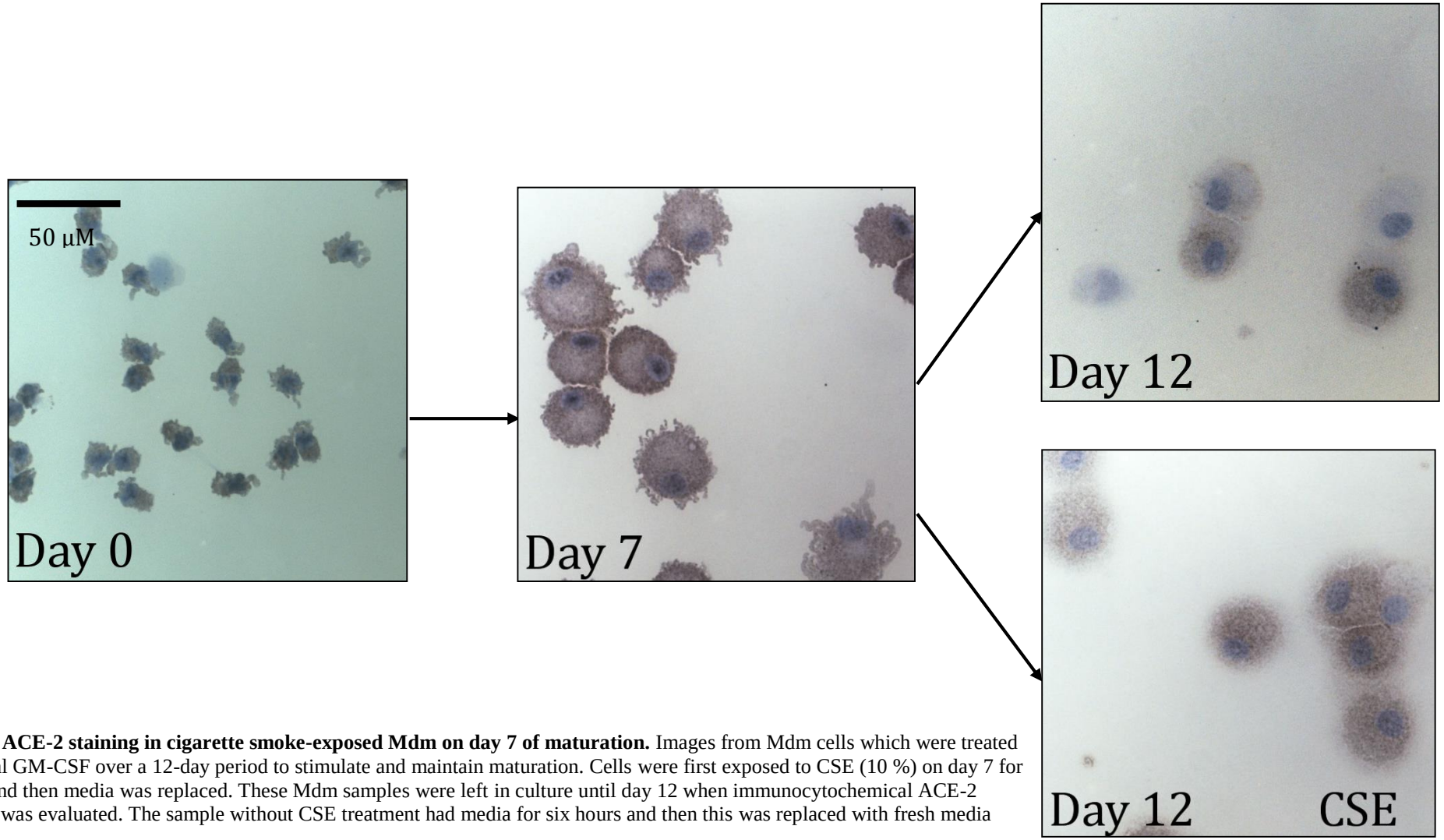

Figure 5.8 ACE-2 staining in cigarette smoke-exposed Mdm on day 7 of maturation. Images from Mdm cells which were treated with 2ng/ml GM-CSF over a 12-day period to stimulate and maintain maturation. Cells were first exposed to CSE (10 %) on day 7 for six hours and then media was replaced. These Mdm samples were left in culture until day 12 when immunocytochemical ACE-2 expression was evaluated. The sample without CSE treatment had media for six hours and then this was replaced with fresh media

5.3.3 ACE-2 Activity in Fresh Lung Fragments

ACE-2 activity after cigarette smoke treatment was evaluated in fragments of human lung tissue. ACE-2 activity was represented by relative fluorescent units (RFU). The change from analysing Mdms to analysing lung fragments was to enable the use of the same method of ACE-2 activity used in chapter 4 where fragments from 21 subjects were evaluated for their ACE-2 activity (figure 4.10). Before preparation of the lung sample for the ACE-2 activity assay (as described in methods 4.2.4), CSE was applied to the lung fragments for 30 minutes. The vehicle control for this experiment was lung fragments incubated in HBSS for 30 minutes. Data are shown in figure 5.9.

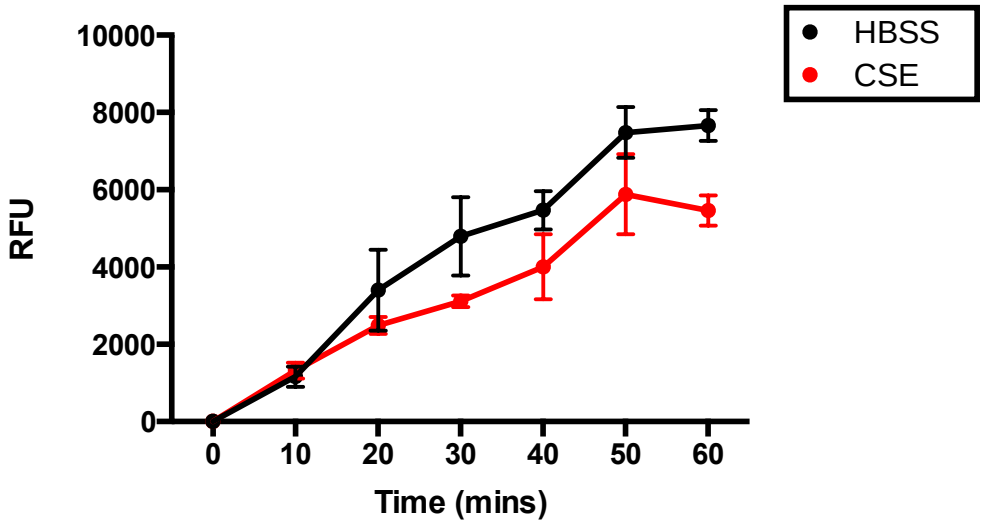

Figure 5.9 ACE-2 activity of lung fragments treated CSE Hydrolysis of fluoropeptide Mca-APK-DnP ACE-2 specific substrate normalised to blank by lung fragments either treated with CSE over time (red dots) or HBSS treatment (black dots). Data shown as mean ± SEM of three independent experiments of CSE exposure on lung macrophages isolated from the same subject. Two-way ANOVA with Sidak's multiple comparison test demonstrated an overall difference $p<0.01$ between two lines but not at a specific time point

In figure 5.9 the increase of ACE-2 activity observed in CSE treated lung fragments that were treated with CSE for 30 minutes was less rapid (a change of 4562 RFU between 10 and 50 minute time points) compared to the vehicle control, which remained in HBSS for 30 minutes (change of 6321 RFU between the same time points).

The ACE-2 activity in CSE treated lung fragments was significantly less than vehicle control over time, according to two-way ANOVA ($p < 0.01$, $F(1,28)=11.8$, $MSE=1.3 \times 10^7$).

The vehicle control sample that was incubated in HBSS for 30 minutes was also compared to a lung fragment sample from the same subject who was immediately processed for the measurement of ACE-2 activity (figure 5.10). This immediate processing was the same method used in chapter 4. This lung fragment that was immediately processed is referred to as no treatment because there was no treatment prior to processing.

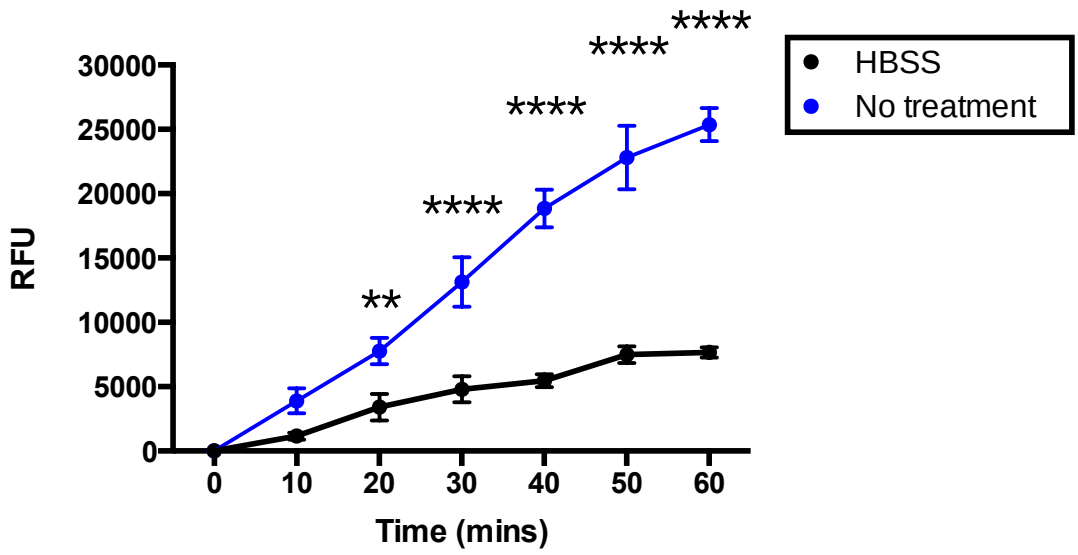

Figure 5.10 ACE-2 activity of lung fragments with or without treatment of HBSS Hydrolysis of fluoropeptide Mca-APK(Dnp) normalised to blank of lung fragments either treated with HBSS over time (black dots) or without treatment (blue dots). Data shown as mean ± SEM of three independent experiments. Two-way ANOVA with Sidak's multiple comparison test * $p<0.05$ ****$p<0.0001$.

In figure 5.10, the increase of ACE-2 activity was less rapid with a change of 6321 RFU between 10 and 50 minute time points compared to no treatment with a change of 18,914 RFU between the same time points.

The ACE-2 activity for the lung fragments incubated in HBSS was significantly less than the no treatment samples according to a two-way ANOVA ($p < 0.0001$, $F(1,21)=28.0$, $MSE=6.55 \times 10^8$).

5.4 Discussion

The aim of this chapter was to investigate what factors affect ACE-2 expression and activity within lung macrophages. In chapter 3, macrophage expression for ACE-2 was heterogeneous; some cells expressed strong ACE-2 staining whilst other neighbouring cells were negative. There was also a trend of more ACE-2 positive cells and activity in lung from current smokers compared to those of ex-smokers. In order to examine the cause of this heterogeneity, two factors were focussed on. The first was macrophage maturation as assessed in the Mdm model and the second was cigarette smoke, explored by exposing lung macrophages or lung fragments to CSE.

The main finding of this chapter is that, for the first time, catalytically active ACE-2 is present within lung macrophages isolated from fresh lung tissue. This chapter also reports that ACE-2 expression increases slightly at the beginning of macrophage maturation and decreases gently by from day 7 to the end of the experiment. Cigarette smoke intervention during maturation seemed to stop this natural decrease by the end of maturation. As this was an exploratory chapter, some of the experiments consisted of one subject only. It is therefore important to interpret these results with some degree of caution.

5.4.1 Method Rationale

To investigate maturation, the monocyte-derived macrophage (Mdm) technique was used as described by Tudhope & Finney-Hayward (2008). The reason for using this particular technique was because the mature macrophages produced were similar to human alveolar macrophages, characterised by their inflammatory cytokine profile, cluster of differentiation markers and co-receptors (Winkler *et al.*, 2008). Furthermore, this technique enabled the observation of ACE-2 expression by ICC at regular time points.

To investigate the effect of cigarette smoke, fresh CSE was applied to Mdms and lung fragments. CSE was chosen because it is a widely used and inexpensive *in vitro* model of cigarette smoke. The rationale is that the CSE bathes the Mdms as cigarette smoke would bathe macrophages in the human lung after it was inhaled and dissolve into the surrounding epithelial lining fluid.

5.4.2 ACE-2 in Isolated Lung Macrophages

ACE-2 expression in lung macrophages has not previously been investigated. Papers analysing ACE-2 expression by IHC and its colocalisation with SARS virus contain images that show apparent ACE-2 staining in lung macrophages. Despite this, the authors of these papers did not identify or discuss ACE-2 positive lung macrophages (He *et al*., 2006; Glowacka *et al*., 2011) because ACE-2 expression in macrophage was not the focus of their investigation.

Preliminary data from this chapter show that ACE-2 is present within macrophages isolated from human lung tissue and that this ACE-2 is functionally active. The activity of ACE-2 seemed to be a biphasic curve when plotted against the number of macrophages within each sample (figure 5.2). The more macrophages present in the sample, the higher the ACE-2 activity, until 50 x 10^3 cells, however, a higher number of macrophages than this value reduced the level of ACE-2 activity. This reduction in activity may be due to over-confluence of the macrophages in the assay plate leading to cell apoptosis and a lower number of cells exhibiting ACE-2 activity. Apoptosis could be measured by a viability assay such as trypan blue or tritium labelled thymidine.

Another suggestion for the reduction in ACE-2 activity with increasing concentrations of cells could be due to limited accessibility of synthetic substrate to ACE-2. To test this hypothesis, media incubated with this sample should then be transferred to a lower number of cells to see if there is a cleavage of synthetic substrate. If there is cleavage this could be a plausible suggestion.

One last suggestion could be that confluence was not a problem but the concentration of the synthetic substrate was not enough to accommodate the high number of cells. To check this, a number of concentrations of synthetic substrates should be trialled

Identifying that ACE-2 is enzymatically functional within macrophages could suggest that active ACE-2 could break-down Ang II within the surrounding area of the macrophage and lead to the production of the Ang-(1-7) peptide. Ang-(1-7) could then go on to have beneficial anti-inflammatory or antifibrotic effects within the macrophage or its surrounding area such as preventing an increase in inflammatory mediators during disease (Renno *et al.,* 2012) and collagen deposition in a model of lung fibrosis (Shenoy *et al.,* 2010). On the other hand, ACE-2 could also cleave Ang I and convert Ang I to Ang-(1-9).

Only in recent years has the function of Ang-(1-9) been delineated. The majority of work has focused on Ang-(1-9) in relation to high blood pressure and cardiovascular animal models. Some reports have written that Ang-(1-9) peptide in animal models of hypertension have increased blood pressure (Mogielnicki *et al*., 2014). Another group has demonstrated a beneficial effect of

lowered oxidative stress and reduced cardiac fibrosis (Ocaranza *et al.*, 2014). Furthermore, this paper also reported that the recombinant Ang-(1-9) has also reduced ACE activity, Ang II protein levels in the blood and also decreased oxidative stress. Mixed reports of Ang-(1-9) function at this very early stage means that commenting on the effects of Ang-(1-9) produced by ACE-2 in lung macrophages would be difficult but could mean that as a result Ang II protein levels and ACE activity will be reduced within the lung

5.4.3 Macrophage Maturation and ACE-2

Experiments of isolated monocytes matured into macrophages over 12 days were used to assess the level of ACE-2 protein expression during the 12-day period.

5.4.3.1 ACE-2 expression during maturation

ACE-2 was not only present in monocytes prior to maturation, but throughout the 12-day period of Mdm maturation for all subjects. The general pattern of ACE-2 expression seemed to decrease for the first five days, abruptly increase in the middle of maturation (day 7) and then gently return to baseline levels. This does not correspond to the literature reports of other zinc dependent metalloproteinases such as MMP. MMP expression was previously shown to increase from day 1 to day 7 of culture (Ghorpade *et al*., 2001). On the other hand, serine proteases such as human leukocyte elastase and cathepsin G were found to be significantly reduced during maturation (Campbell *et al*., 1991). The reason these studies may not corroborate with the findings in this present study is because the other studies in the literature did not measure at regular time intervals like this present study, but only at the beginning and the end of the maturation experiment. Furthermore, they are two different enzymes that have different mechanisms of action.

One thing to note is that Mdms from one particular subject had a consistently high percentage of ACE-2 positive cells prior to and throughout the 12 days of maturation. This contrasted with the other five subjects, whose results followed the general pattern described above. Personal details were not taken for subjects who donated blood for this work. As a result it is difficult to account for this discrepancy.

One possible explanation for this unusually high proportion of ACE-2 positive macrophages at the beginning of the experiment could be a mild infection of the subject. ACE-2 expression could be required at early stages of macrophage maturation for anti-inflammatory purposes. This suggestion was based on Thomas *et al*., (2010) who revealed that macrophages deficient in ACE-2 had a higher concentration of cytokines when stimulated compared to those isolated from wild-

type mice. This is one possibility. Further work on the effect of viruses and bacteria on ACE-2 expression in lung macrophages would help delineate this.

It is also important to highlight that ACE-2 identified at day 12 of the mature Mdms was functionally active (figure 5.7). ACE-2 activity in mature Mdms not only supports Mdms as a good model for isolated lung macrophages, but it also raises the question of whether ACE-2 is always active from monocyte to macrophage or whether ACE-2 activity is only optimally catalytic in mature macrophages only. This is something that could be investigated in further work.

5.4.3.2 CD68 positive macrophages during maturation

The macrophage marker CD68 is used widely for identifying macrophages using immunohistochemistry (Kunisch *et al.*, 2004; Lau *et al.*, 2004). An anti-CD68 antibody was used to identify mature macrophages within the Mdm samples. Percentages of CD68 positive cells in the Mdm samples were expected to increase during maturation.

Unexpectedly, the pattern of CD68 staining in the current chapter seemed to fluctuate erratically over the 12 days and showed no increase. This fluctuation raised the question of whether the Mdms had matured by day 12 of the experiment. This unexpected result may have arisen from the method rather than a question of whether the macrophages were mature or not.

The antibody used to identify CD68 in this experiment was the PG-M1 clone. This clone was first reported in the 1990s (Falini *et al*., 1993). It was, and still is, the best clone available because of its reliable identification of the CD68 epitope after the process of sample fixation in preparation for IHC. Many papers that have used this clone for fresh isolates of macrophages or freshly fixed tissue have shown that the PG-M1 clone is appropriate for IHC macrophage identification (Kunisch *et al.*, 2004; Berbic *et al.*, 2009).

The results in this thesis also support the notion that PG-M1 is a reliable clone for macrophage identification in freshly fixed tissue or fresh isolated macrophages. In chapter 3, macrophages isolated from human lung were stained with CD68 using the same PG-M1 clone (figure 3.10). Isolated lung macrophages were also positively stained for CD68 (figure 5.5). In contrast, Mdms cultured for 12 days did not demonstrate clear CD68 staining (figure 5.4).

From the evidence above, it seems that fresh tissue or cells isolated from human lung and processed shortly after, result in reliable CD68 positive staining by the PG-M1 antibody clone. However, cells that have been cultured for days do not. One explanation for this may be that the

epitope responsible for identifying CD68 may have been lost in culture or less of the epitope is exposed after a period of time in culture.

5.4.3.3 Non-specific esterase

Since the antibody against CD68 used for the IHC technique did not positively stain Mdms, an alternative technique was used to identify macrophages. Non-specific esterase detection was the chosen as an alternative technique as it has been used to identify macrophages in cell cultures as well as fresh tissue for over fifty years (Horwitz *et al.*, 1977).

Non-specific esterase was identified in macrophages isolated from fresh lung tissue (figure 5.5). In addition, increased staining was observed during the maturation of Mdms (figure 5.6). Both of these results suggest that Mdms are macrophage-like cells at day 12.

ACE-2 and non-specific esterase also had a similar staining pattern of cells during the maturation experiment. At the beginning of the experiment, some monocytes were stained with non-specific esterase. This was also the case with monocytes stained with ACE-2 especially for the subjects Mdm6 where there was a direct comparison of ACE and non-specific staining (figure 5.6). By day 12, however almost all the cells were stained with non-specific esterase or ACE-2 for Mdm6. This direct comparison of different stains on the same sample of Mdms is key. It could be interpreted that as the monocytes mature into macrophages, more cells are ACE-2 positive, although further experiments will need to confirm this.

Overall, it is difficult to draw conclusions about whether ACE-2 expression was affected by maturation. Only six subjects were sampled for this experiment and the ACE-2 positive percentages seemed to be variable across the period of maturation. The ACE-2 positive cell percentages of monocytes (day 0 of culture) compared with mature macrophage (day 12 of culture) did change but this change was not substantial.

5.4.4 Cigarette Smoke Treatment and ACE-2

Cigarette smoke is known to affect lung macrophage function and therefore the balance of protein expression associated with the lung macrophage (Karavitis *et al.*, 2011). Cigarette smoke inhalation can be modelled by the treatment of lung cells with CSE. In this current chapter, Mdms treated with CSE seemed to maintain ACE-2 protein expression that would have otherwise been diminished by day 12, as seen in the no-treatment controls. This particular CSE experiment was carried out only once. Caution must, therefore, be taken when interpreting these results. This maintenance of ACE-2 expression after CSE treatment aligns with the results in the previous

chapters where there was a trend for higher ACE-2 protein expression (figure 3.20) and catalytic activity (figure 4.12) in current smokers compared with ex-smokers. This evidence seems to suggest that there is a link between ACE-2 expression in macrophages and exposure to cigarette smoke. However, in the current chapter lung fragments treated with CSE had lower ACE-2 activity compared with vehicle control lung fragments incubated in HBSS for thirty minutes. This suggests that CSE lowers ACE-2 activity in lung tissue.

Importantly, the vehicle control where lung fragments were bathed in HBSS for thirty minutes was significantly lower in ACE-2 activity than lung fragments from the same subject that was immediately processed for the measurement of ACE-2 activity. The lung fragment that was immediately processed did not undergo any treatment prior to processing and this followed the same methods as in chapter 4 where lung tissue from 21 subjects were analysed for their ACE-2 activity (figure 4.2). Lower ACE-2 activity was unexpected as both lung fragment samples had the same solutions applied to them, the only variable being the length of time prior to processing. This suggests that the vehicle control bathed in HBSS for the effect of CSE on ACE-2 activity in lung fragments was not appropriate.

Reasons for the impact of the length of time that the lung fragments were bathed in HBSS media prior to processing may include the media not containing L-glutamine or sodium pyruvate as an energy source for the cells. This may lead to non-optimal conditions for the lung fragments, which may explain why ACE-2 activity was lowered due to reduced cell viability or metabolism. Suboptimal conditions could also lead to the degradation of the ACE-2 or lowered ACE-2 function. Lung fragments also have many other types of cells, which could influence or block the ACE-2 activity during that period of time which may not be able to happen when the lung tissue is processed immediately.

5.4.4.1 Comparison of ACE-2 activity in Mdms and lung fragments

ACE-2 activity in Mdms was different to ACE-2 activity in lung fragments. At the 50-minute time point of ACE-2 activity of mature Mdms (day 12) the value was double the number of ACE-2 activity for the lung fragments that were bathed in HBSS for thirty minutes but less than the ACE-2 activity of lung tissue fragments that were immediately processed with no treatment.

This variance among the different sample types could be due to the number of ACE-2 active cells within the sample. For example, if there are low numbers of ACE-2 positive cells within some samples this may lead to low ACE-2 activity in the sample.

5.4.4.2 Reasons for ACE-2 level change after cigarette smoke

Maintenance of ACE-2 expression after exposure to cigarette smoke has not been explored. One paper reportedly used a cigarette smoke rat model to investigate ACE-2 expression. They found that exposing rats to cigarette smoke twice a day for 6 months led to lower ACE-2 protein expression within lung homogenates compared to those exposed to fresh air (Han *et al*., 2010).

Also this paper described another separate *in vitro* experiment with rat airway smooth muscle cells isolated from normal rats. When they treated these cells with CSE, they found a lower ACE-2 expression compared to no treatment controls. The reason for this discrepancy could be due to many factors including species differences, the frequency of the smoke exposure, the smoking load and also the *in vitro or in vivo* model used.

As there are so many components in cigarette smoke the answer to what could be causing ACE-2 to change in human lungs after cigarette smoking exposure are endless. Evidence from primary cells indicated a change in inflammatory response after CSE application, which could induce a change in ACE-2 expression and activity. For example, human primary alveolar type II epithelial cells treated with CSE released fewer chemokines associated with the inflammatory response (MCP-1 and IL-8) compared to those without treatment (Witherden *et al*., 2004). In contrast, primary cells isolated from other areas of the human pulmonary system, such as the nasal epithelial cells, have higher IL-8 release after treatment with CSE compared to non-treated cells (Comer *et al*., 2014).

Another reason for ACE-2 change after cigarette smoke exposure could be due to the reduction of phagocytic function of the macrophage. For example CSE exposure reduces uptake of the *Haemophilus influenzae* virus by murine alveolar macrophages compared to non-treated controls (Martí-Lliteras *et al*., 2009), which is a reduction in function of the macrophage. Mdm treated with CSE released lower amounts of IL-8 and MMP-9 following stimulation (Koarai *et al.*, 2012).

5.4.4.3 Smoke components

To pinpoint which component from cigarette smoke influences the change in ACE-2 expression warrants a new PhD project. However, the literature does suggest that there are certain components that could influence the RAS.

Nicotine has reported to increase ACE activity of human umbilical vein endothelial cells (Ljungberg, 2009). Furthermore ROS have also had an effect on some parts of the RAS, mostly increasing the receptor that Ang II binds to, the AT_1 receptor. Banday *et* al., (2008) demonstrated

that oxidative stress increased AT_1 receptors in the kidney, and intracellular receptor NF-κB often induced by ROS also increase AT_1 receptors fibroblasts in culture (Cowling *et al.*, 2002).

Investigations into the effect of ROS on enzymes belonging to the RAS are non-existent. On the other hand, literature has been published demonstrating RAS and associated antagonists/inhibitors manipulating and affecting the generation of ROS. RAS could be useful as a potential treatment to dampen down elevated ROS in diseases. For example, application of recombinant ACE-2 or the antagonist of Ang-(1-7) receptor enhanced Ang II-mediated ROS production within cells of the sheep kidney (Gwathmey *et al*., 2010). Similar results were seen in human aortic endothelial cells (Sampaio *et al*., 2007). Treatment of these endothelial cells with Ang-(1-7) reduced the superoxide (O_2^-) induced by Ang II. More recently, Xia *et al.,* (2011) has demonstrated that ACE-2 KO mice have increased levels of ROS markers in the plasma and when ACE-2 is overexpressed in the brain region of the mice these markers are significantly reduced.

The evidence above demonstrates that ACE-2 expression and Ang-(1-7) reduces Ang II-induced production of ROS. This may be due to the break-down of Ang II itself by ACE-2 or Ang-(1-7) influencing the cell signalling of Ang II through the heterodimerisation of their receptors and biased agonism.

In summary, cigarette smoke seems to influence the expression and activity of ACE-2. After the treatment of CSE, ACE-2 protein expression is maintained in Mdms. It is difficult to conclude whether ACE-2 expression or activity is directly changed by cigarette smoke or whether it is secondary to the inflammatory or ROS processes that may be stimulated by cigarette smoke extract.

5.4.5 Limitations

One limitation was that the Mdm experiment was not fully optimised. Lung fragments that were incubated with HBSS for 30 minutes acting as the vehicle control for CSE treatment did not result in a similar activity to that of the no treatment controls (figure 5.10). This difference may suggest that ACE-2 activity would have been lost regardless if the cells were exposed to CSE or not.

Another limitation was that no toxicology experiments were done on the macrophages that were exposed to CSE. Reading through the literature, it is difficult to know what a physiologically relevant CSE concentration for lung macrophages could be due to a number of papers quoting different concentrations. For example Guzik *et al.,* (2011) used 100 % CSE smoke on peripheral blood cells for 30 minutes to measure apoptosis whereas Kent *et al,,* (2008) used 25 % CSE on

macrophages isolated from COPD patients for 6 hours and this was toxic to the macrophages. Not only are the concentrations are different in the literature but also the procedure for preparing the fresh CSE is different. In my study, I carried out the same preparation method as in Aosihiba *et al.*, (2001) who experimented on isolated human alveolar macrophages. They also demonstrated that 10 % CSE for 6 hours caused minimal cell death hence why I chose to follow this paper. In further experiments, measuring cell death by a trypan blue stain will be done as it is important to measure cell death so as not attribute an effect to live cells when a proportion of the cells are not living.

Time was also a limitation, therefore the experiment for ACE-2 activity within lung fragments was done as a preliminary experiment. It is, therefore, important that these results are interpreted with caution. With more time, the experiment would have been optimised and more lung fragment samples from different subjects would have been put through the experiment.

5.4.6 Summary

ACE-2 protein is expressed and has activity in lung macrophages and the reasons for this localisation is currently unknown. ACE-2 protein expressed in mature macrophages is also expressed throughout maturation therefore ACE-2 expression is not influenced by the age of the macrophages. The other condition of cigarette smoke as a factor seems to be inconclusive and more samples from different subjects are needed to understand whether cigarette smoke is an influential factor for the expression of ACE-2.

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Chapter 6 Discussion

COPD is the third leading cause of death in the world (Lozano *et al.,* 2013). This gradual destruction of alveolar tissue, fibrosis and build-up of inflammatory exudate in this disease leads to obstruction of the airway. No pharmaceutical treatments are available to slow down this decline in lung function.

An improvement in lung function however, has been seen in subjects prescribed ACEi compared to those without ACEi medication (Petersen *et al.,* 2014) and more COPD patients prescribed ACEi or ARBs were alive 90-days after being discharged from the hospital (Mortensen *et al.,* 2009). Mancini *et al.,* (2006) also described the benefit of prescribed ACEi demonstrating a reduction of the risk of death from COPD. Taken together, this suggests a possibly deleterious role for the RAS in COPD.

The work conducted for this thesis demonstrates that ACE, chymase and ACE-2 are present in the human lung. Renin was not identified in the lung sections sampled however a technique other than IHC is required to confirm this. ACE-2 is expressed in lung macrophages from parenchymal tissue. This was surprising since there is limited information about the colocalisation of ACE-2 with macrophages in the literature.

With regards to enzyme differences between COPD and no COPD controls, ACE activity in the lung is the only enzyme that is different. ACE activity is significantly lower in COPD subjects compared to the non-COPD controls. ACE activity is also lower in subjects who were prescribed ACEi compared to those subjects not prescribed ACEi. On the other hand, ACE-2 activity seems to be higher in COPD lung but this is not significant and there was no difference in subjects prescribed ACEi compared to no prescription.

As for smoking, the smoking status of the subject does not affect the protein or activity of the enzymes tested. ACE-2 protein expression and activity appears to be higher in current smokers however this is not statistically significant.

The research completed in this thesis contributes to the limited studies investigating RAS in COPD that have been published. It gives an insight into the enzymes, mainly ACE and ACE-2 in COPD lung that could be involved in the pathogenesis of COPD. If further research demonstrated these enzymes are a contributing factor to the cause of COPD then it could lead to these enzymes being a pharmacological target. The treatment that would target these enzymes could then stop

the decline in lung function observed in COPD and therefore ensure a better quality of life for COPD patients.

6.1.1 ACE Protein Expression and Activity

Even though there is evidence for an important role for RAS in COPD, ACE protein expression levels measured by the number of ACE positive vessels was similar in COPD compared to controls. Importantly though, despite the abundance of ACE being similar, ACE activity was lower in lung homogenates from COPD subjects compared to controls. This indicates that ACE is present within the COPD lung but is not working as efficiently or there have been post-translational modifications that occurred to ACE catalytic machinery. These modifications may have led to a proportion of ACE molecules not being able to cleave the substrate.

An example of a post-translational modification of ACE is glycosylation (Sadhukhan *et al.*, 1996). One particular *N*-glycosylation site of the ACE molecule out of five other sites is required to be glycosylated for ACE activity. Denaturation of this one glycosylation site of this ACE molecule in COPD subjects could be the reason for lower ACE activity. COPD subjects have been reported to have high numbers of defects in glycosylation of a molecule similar in structure to ACE called transferrin and this could extend to the ACE molecule too (Nihlen *et al.*, 2001).

6.1.2 ACE Inhibition

As mentioned above, in the literature subjects who were prescribed ACEi have an improvement in mortality rates in COPD patients. It seems counterintuitive for COPD patients who already have low ACE activity in the lung, as demonstrated by this current study, have their ACE activity reduced further by ACEi which then lead to these beneficial effects. This is because the original mechanism of action for ACEi was to lower the already heightened ACE activity in the serum of hypertensive patients in order to normalise their blood pressure.

Having said that, these data show that there is a potential window for further reduction of ACE activity by ACEi in COPD subjects. This is because the lowest ACE activity value in COPD subjects was higher than the negligible activity found in the group prescribed ACEi (figure 4.2). There is therefore potential for further reduction and this could be potent enough to influence the positive outcomes of a longer life and lower hospitalisations for COPD patients.

There may be some alternative suggestions that explain the beneficial effect of ACEi in COPD patients. COPD subjects are reported to have heightened ACE activity in the serum compared to

healthy controls (Brice *et al.,* 1995). ACEi prescribed to COPD patients could also target the heightened ACE activity in the serum of COPD patients to lower it.

A second mechanism could be that ACEi and ARB could be acting on alternative targets to ACE such as ROS within local and systemic areas, or even the receptors belonging to the peptides Ang II or Ang-(1-7). More recently, COPD is thought of as a disease with extrapulmonary systems (Rabe *et al.,* 2007) therefore ACEi/ARB could be treating the extrapulmonary comorbidities of COPD such as cardiovascular and musculoskeletal pathologies in COPD patients (Agustí *et al.,* 2003). This work has therefore opened up the possibility that lung and serum ACE activity are targets of ACEi and this may be the case in COPD patients.

6.1.3 ACE-2

ACE-2 is known to be protective for inflammatory diseases. Recombinant human ACE-2 (rhACE-2) administered to an animal model of severe acute lung injury rescued the symptoms of the disease, suggesting a therapeutic role for ACE-2 in disease (Imai *et al.* 2005).

ACE-2 has been implicated mostly in the lung disease SARS. This enzyme is identified as the protein that receives the coronavirus responsible for SARS (Wong et al. 2004). The virus binds to ACE-2 located on bronchial epithelial cells.

A finding of this study is that ACE-2 is present and active in macrophages and this may be the starting point for developing pharmacological treatments for lung diseases. Not much has been reported within the literature regarding ACE-2 and macrophages however ACE-2 activity has been measured in cultured macrophages that were derived from blood monocytes (Keidar *et al.,* 2005; 2007). This author showed that ACE-2 activity was elevated in the Mdms taken from heart failure patients who were receiving mineralcorticoid therapy compared to no therapy (Keidar *et al.,* 2005) as well as patients that were on the cusp of becoming hypertensive compared to hypertensive patients (Keidar *et al.,* 2007).

This finding of ACE-2 activity within macrophages has not been found before and it opens up new questions: what is the function of ACE-2 within the macrophage and could ACE-2 as a target in macrophages be a beneficial therapy for inflammatory diseases? The work of Keidar *et al*. (2005, 2007), seems to suggest that ACE-2 activity is elevated during treatment or just before presentation of a disease, which shows that ACE-2 could be employed in a defence mechanism against disease. This elevation of ACE-2 activity was also observed in COPD subjects.

During treatment or just before the presentation of disease symptoms, ACE-2 could be cleaving Ang I or Ang II and aiding homeostasis in its own microenvironment. This suggestion was based on my finding from preliminary experiments that ACE-2 was active in both lung macrophages isolated from lung tissue as well as Mdms. The location of ACE-2 could mean that ACE-2 may dampen down the inflammatory cytokines during first onset of disease symptoms as demonstrated when recombinant ACE-2 was given to animals at the early stage of demonstrating symptoms of ARDS (Imai *et al.*, 2005). If this were so, it suggests that there may be a protective effect of the raised ACE-2 activity.

The protein expression levels of ACE-2 and its activity seemed also to be higher in smokers compared to ex-smokers. Again, the same viewpoint of ACE-2 increasing to combat disease could still stand. ACE-2 could be triggered by inhalation of cigarette smoke in current smokers which would combat the inflammation and pathogenesis in the lung that is caused by cigarette smoke. In contrast, it could be argued that smoking raises ACE-2 and therefore regular smoking could be beneficial for lung function and inflammatory status in the lung. Perhaps a product formed by inhaling smoke could increase ACE-2 activity but it is highly unlikely that smoking could be beneficial to lung disease.

6.1.4 ACE and ACE-2 Imbalance

The current work also challenges the suggested balance between ACE and ACE-2 and its dysregulation in diseased tissue. It is hypothesised that this imbalance causes the raised Ang II peptide levels observed in diseases, which leads to fibrosis, inflammation, oxidative stress, and ultimately damage to lung tissue. For example, Imai et *al*. (2005) published that ACE-2 KO mice have an increase in Ang II protein levels in tissue and this is linked to pathogenesis within the lung. Resetting the balance by injecting rhACE-2 into these mice lowers this heightened Ang II. Furthermore knocking out ACE altogether in a different cohort of ACE-2 KO mice led to fewer symptoms of induced severe lung failure. From this, Imai *et al*., concluded that there is a fine balance between ACE and ACE-2 in tissue and if this is optimally maintained it can act as a prophylactic for lung damage in disease.

In contrast to the study explained above the results of this thesis demonstrated that ACE-2 protein and activity seems to be higher in COPD compared to non COPD lung, although this is not significant and ACE activity is lower in COPD subjects compared to controls. There are no other studies that have explored ACE-2 in COPD lung, although in IPF there does seem to be a disruption in the ACE and ACE-2 balance (Li *et al.*, 2008).

It is possible that raised ACE-2 leads to the increased break-down of its pro-inflammatory substrate, Ang II. This break-down not only removes the pro-inflammatory effects of Ang II but, in so doing, leads to the formation of Ang-(1-7) with its anti-inflammatory effects. On the other hand ACE activity is also lowered so there seems to be a homeostatic mechanism in the lung to stop further injury. Lower ACE activity leads to less Ang II formation from the break-down of Ang I.

There are not enough studies in the literature that focus on enzyme activity. The vast majority of work only seems to address mRNA and protein expression. It is true that these ways of measuring do give indications of how much of a target there is, via transcription and translation, but these do not take into account activity or efficiency nor the role of cellular environment or post-translational modifications. Even when ACE-2 activity is addressed it is not discussed in its relation to ACE. For example, Li *et al.* (2008) only investigated ACE-2 activity in the human lung and the protein expression was done in animal models of the disease. This particular paper demonstrated a decrease of ACE-2 activity within the human lung, but no mention of ACE activity, Ang II or Ang-(1-7) peptides.

The results of this study should provoke the research field into investigating the functional activity of the enzymes of RAS rather than the protein expression or mRNA. The reason being is that protein expression does not always correlate with activity as shown by the ACE activity and IHC protein expression in chapter 4. A more detailed analysis of a range of RAS components is required when analysing tissue by carrying out measurements of peptides such as Ang II and Ang-(1-7) and the enzymes of the RAS in one sample. These measurements are required especially within a human lung sample if we are to better our knowledge of the RAS in the lung.

6.1.5 Strengths and Limitations

This thesis has done well in localising enzymes within the lung tissue using a technique to find whether enzymes were present in particular areas of human lung. The design of this research study has allowed the reader to observe an association with personal characteristic information of subjects and RAS enzymes within the lung. This information was able to explain why lung ACE activity was so low in some subjects; because they were prescribed ACEi medication. Again it was also able to explain the trend that ACE-2 activity was seemingly higher in some subjects because they were current smokers.

On the other side, there were some limitations that were recognised. As mentioned previously, this research has not been able to measure the peptides of the RAS. Due to this limitation, I was

unable to observe whether the lowering of ACE activity leads to a lowering of Ang II also within COPD lung. On many occasions the size of tissue collected was only sufficient for the activity assay and this is the reason why peptide protein expression was not measured. Alternatively another cohort of patient samples could have been used only for investigating peptide levels however there was only so much that could be done within the laboratory period of three years.

For some experiments completed in this study, there was a low number of subjects. For example, ACE-2 activity measured in lung macrophages were only isolated from one subject's lung tissue. Similarly, blood monocytes from one volunteer was sampled to mature into Mdms for the measurement of ACE-2 protein expression after CSE application. The reason for this was because the experiment carried out intended to be a preliminary experiment that was to be followed up with sampling from a number of subjects however time did not permit this. Nonetheless, these preliminary experiments have opened up new possibilities with regards to ACE-2. For example the ACE-2 present in macrophages could be active within lung and cigarette smoke inhalation could have a direct effect on the expression of this ACE-2. More subjects samples would be needed to conclude this.

This research has not identified whether the lower ACE activity in COPD subjects was due to having the disease or whether it was causing the disease. This is because it was not in the remit of this study. The design of this study aimed to investigate the natural levels of RAS enzymes in COPD subjects. Manipulating ACE or ACE-2 expression in mouse models of COPD would be a more appropriate way of investigating cause and effect.

The subjects who donated their lung sample in this research study were patients in surgery for lung cancer. There could have been a possibility that cancerous tumour formation could have interfered with the RAS, however, the lung samples were taken farthest away from the site of the tumour. Human lung samples were required for this and taking samples from subjects who were already scheduled to have surgery was a better approach to obtain lung tissue.

6.1.6 Further Work

As a result of my study, further work should be conducted on the peptides of the RAS namely Ang II, Ang-(1-7), Ang-(1-9) and Ang-(1-5) within the human lung. This work should further the understanding of the RAS mechanisms in COPD lung as well as lung without COPD. The aforementioned peptides will be measured by radioimmunoassay or ELISA in human lung homogenate donated by COPD subjects and compared to non-COPD subjects. There will be a drive to sample lung tissue from COPD subjects of a wide range of severities instead of the

mild/moderate COPD severity (GOLD I and II) that was sampled in this thesis. This work will help annotate the network of the RAS peptides in relation to each other as well as the enzymes already observed in this thesis and give more information as to whether RAS is associated with COPD pathogenesis.

As ACE-2 expression in macrophages is novel, the function of ACE-2 when expressed in the macrophages should be explored in more detail. To explore, confocal microscopy will be used to observe any effects that cytokines have on the macrophage with regards to the flagged ACE-2 protein. This will help to find out whether ACE-2 in macrophages function as a receptor and also whether the localisation of ACE-2 to structures of the macrophage change with cytokine treatment.

ACE-2 could also function as a catalyst. To investigate this, Ang II will be placed into a culture of lung macrophages isolated from normal lung tissue. Both Ang II and Ang-(1-7) will be measured before and after treatment. Depletion of Ang II peptide in the culture would suggest that ACE-2 is functioning as a catalyst in the macrophage on its surrounding peptides.

For a more reliable way to investigate the effect of cigarette smoke on ACE-2 expression, instead of using Mdms, freshly isolated macrophages from BAL fluid of smokers will be used and directly compared to controls. These macrophages will undergo ACE-2 activity analysis, as done before in this thesis.

6.1.7 Conclusion

The results from this thesis have led me to partially accept my hypothesis: COPD patients have lower protein expression and activity levels of RAS enzymes compared to subjects without COPD. Indeed, the RAS enzyme ACE in the lung was significantly lower in activity within subjects classified with COPD compared to controls however the ACE protein expression or abundance measured by IHC did not differ. Other RAS enzymes analysed in this study were not different, with the exception of ACE-2 activity that seemed higher in COPD however this needs to be clarified with more samples.

A RAS enzyme activity imbalance within COPD lung seems to be occurring with regards to ACE and ACE-2. This imbalance has never been explored before in the human lung of COPD patients. Furthermore the experiments in this thesis have demonstrated ACE-2 localisation to the lung macrophage. This ACE-2 is also catalytically active. This could potentially be important in the treatment of COPD because of its cellular location within the lung.

The implications of this new knowledge from the results of this thesis warrant further work by academics and research scientists to gain a greater understanding of the feedback loops and interplay in the components of the RAS within human lung in COPD. Once explored, the extensive knowledge of RAS may determine when pathogenesis of COPD occurs and may lead to effective strategies to stop the decline of lung function in COPD in the future.

List of References

Agustı, A. G. N., Noguera, A., Sauleda, J., Sala, E., Pons, J., Busquets, X. (2003). Systemic effects of chronic obstructive pulmonary disease. *Eur Respir J*, *21*(2), 347-60.

Akhtar, T., Ullah, Z., Khan, M. H., Nazli, R. (2007). Chronic bronchitis in women using solid biomass fuel in rural Peshawar, Pakistan. *Chest, 132*(5), 1472-1475.

Andersson, C K, Mori, M., Bjermer, L., Löfdahl, C.G., Erjefält, J. S. (2009). Novel site-specific mast cell subpopulations in the human lung. *Thorax*, *64*(4), 297–305

Andersson, C K, Mori, M., Bjermer, L., Löfdahl, C. G., Erjefält, J. S. (2010). Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease. *Am J Resp Crit Care*, *181*(3), 206–17

Andreas, S., Herrmann-Lingen, C., Raupach, T., Lüthje, L., Fabricius, J. A., Hruska, N., Korber, W., Buchner, B., Criee, CP., Hasenfuss, G., Calverley, P. (2006). Angiotensin II blockers in obstructive pulmonary disease: a randomised controlled trial. *Eur Respir Journal*, *27*(5), 972-79.

Aoshiba, K., Tamaoki, J., Nagai, A. (2001). Acute cigarette smoke exposure induces apoptosis of alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol*, *281*(6),1392-1401

Austin, M. A., Wills, K. E., Blizzard, L., Walters, E. H., Wood-Baker, R. (2010). Effect of high flow oxygen on mortality in chronic obstructive pulmonary disease patients in prehospital setting: randomised controlled trial. *Brit Med J 341*, c5462

Baarsma, H. A., Engelbertink, L. H., Hees, L. J., Menzen, M. H., Meurs, H., Timens, W., Postma D.S., Kerstjens, H.A., Gosens, R. (2013). Glycogen synthase kinase-3 (GSK-3) regulates TGF-β1-induced differentiation of pulmonary fibroblasts. *Br J Pharmacol*, *169*(3), 590-603

Balzar, S., Chu, H. W., Strand, M., Wenzel, S. (2005). Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *Am J Resp Crit Care*, *171*(5), 431-9.

Banday, A. A., Lokhandwala, M. F. (2008). Oxidative stress-induced renal angiotensin AT1 receptor upregulation causes increased stimulation of sodium transporters and hypertension*, Am J Physiol-Renal, 295*(3), 698–706

Barnes, P. J. (2006). Theophylline for COPD. *Thorax*, *61*(9), 742-3.

Belz, G. G., Butzer, R., Kober, S., Mang, C., Mutschler, E. (1999). Time course and extent of angiotensin II antagonism after irbesartan, losartan, and valsartan in humans assessed by angiotensin II dose response and radioligand receptor assay. *Clin Pharmacol Ther 66*(4), 367-73

Benigni, A., Cassis, P., Remuzzi, G. (2010). Angiotensin II revisited: new roles in inflammation, immunology and aging. *EMBO molecular medicine*, *2*(7), 247-57

Berbic, M., Schulke, L., Markham, R., Tokushige, N., Russell, P., Fraser I. S. (2009). Macrophage expression in endometrium of women with and without endometriosis. *Hum Reprod, 24(*2), 325-32

Berger, P., Laurent, F., Begueret, H., Perot, V. (2003). Structure and Function of Small Airways in Smokers : Relationship between Air Trapping at CT and Airway Inflammation. *Radiology*, *228*(1), 85–94

Bernstein, K. E., Shen, X. Z., Gonzalez-Villalobos, R. A., Billet, S., Okwan-Duodu, D., Ong, F. S. Fuchs, S. (2011). Different in vivo functions of the two catalytic domains of angiotensin-converting enzyme (ACE). *Curr Opin Pharmacol*, *11*(2), 105-11

Bernstein, K. E., Ong, F. S., Blackwell, W. L. B., Shah, K. H., Giani, J. F., Gonzalez-Villalobos, R. A., Shen X.Z., Fuchs, S., Touyz, RM. (2013). A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacol Rev, 65*(1), 1-46.

Bradding, P., Feather, P. H., Wilson, S., Bardin, P. C., Heuser, C. H., Howarth, P. H. (1993). lmmunolocalization of Cytokines in the Nasal Mucosa of Normal and Perennial Rhinitic Subjects. *J Immunol*, *151*(7), 3853–65

Brice, E. A., Friedlander, W., Bateman, E. D., Kirsch, R. E. (1995). Serum angiotensin-converting enzyme activity, concentration, and specific activity in granulomatous interstitial lung disease, tuberculosis, and COPD. *Chest 107*(3), 706-10.

Brown, N. J.,Vaughan, D. E. (1998). Angiotensin-Converting Enzyme Inhibitors. *Circulation*, *97*(14),1411–20

Buhl, R., Maltais, F., Abrahams, R., Bjermer, L., Derom, E., Ferguson, G., Flezar, M., Hebert, J., McGarvet, L., Oizzichini, E., Reid, J., Veale, A., Gronke, L., Hamilton, A., Korducki, L., Tetzlaff, K., Waitere-Wijkerm S., Watz, H., Bateman, E. (2015). Tiotropium and olodaterol fixed-dose combination versus mono-components in COPD (GOLD 2–4). *Eur Respir J*, *45*(4), 969-79

Burge, S. Wedzicha, J. A. (2003). COPD exacerbations: definitions and classifications. *Eur Respir J*, *21*(4), 46-53

Busquets, X. (2007). Angiotensin-converting-enzyme gene polymorphisms, smoking and chronic obstructive pulmonary disease. *Int J Chrin Obstuct Pulmon Dis*, *2*(3), 329–34

Booker J., Bent A., The Consesus Reference Group (2004). Chronic obstructive pulmonary disease: national clinical guideline on management of chronic obstructive pulmonary disease in adults in primary and secondary care. *Thorax*, *59*(1), 1-232

Camargo, S. M., Singer, D., Makrides, V., Huggel, K., Pos, K. M., Wagner, C. A., Kuba, K., Danilczyk, U., Skovby, F., Kleta, R., Penninger, J. M. (2009). Tissue-specific amino acid transporter partners ACE2 and collectrin differentially interact with hartnup mutations. *Gastroenterology*, *136*(3), 872-82

Campbell, E. J., Cury, J.D., Shapiro, S.D., Godlbery,G.I., Weigus, H.G. (1991). Neutral Proteinases of Human Mononuclear Phagocytes Cellular differentiation markedly alters cell phenotype for serine proteinases, metalloproteinases, and tissue inhibitor of metalloproteinases. *J Immunol*, (4), 1286-93

Caughey, G. H. (2007). Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev*, *217*(1), 141-54.

Chana, K. K., Fenwick, P. S., Nicholson, A. G., Barnes, P. J., Donnelly, L. E. (2014). Identification of a distinct glucocorticosteroid-insensitive pulmonary macrophage phenotype in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol*, *133*(1), 207-16

Chen, F., Gon, L., Zhang, L., Wnag, H., Qi, X., Wu, X., Xia, Y., Cai, Y., Liu,L., Li, X., Ren, J. (2007) Early lung injury contributes to lung fibrosis via AT1 receptor in rats. *Acta Pharmacol Sin, 28*(2), 222- 37

Churg, A., Zhou, S., Wright, J. L. (2012). Matrix metalloproteinases in COPD. *Eur Respir J*, *39*(1), 197-209

CKS (Clinical Knoweldge Summaries) Updated September 2015 *http://cks.nice.org.uk/chronic-obstructive-pulmonary-disease#!scenario* Accessed on 4th January 2015

Clarke, N. E., Turner, A. J. (2012). Angiotensin-converting enzyme 2: the first decade. *Int J Hypertens, 2012*(1), 307315

Comer, D. M., Elborn, J. S., Ennis, M. (2014). Inflammatory and cytotoxic effects of acrolein, nicotine, acetylaldehyde and cigarette smoke extract on human nasal epithelial cells. *BMC Pulm Med*, *14*(1), 32

Cope, G. F., Wu, H. H., O'Donovan, G. V., Milburn, H. J. (2012). A new point of care cotinine test for saliva to identify and monitor smoking habit. *European Respiratory Journal*, *40*(2), 496-7.

Cowling, R. T., Gurantz, D., Peng, J., Dillmann, W. H., Greenberg, B. H. (2002). Transcription factor NF-kappa B is necessary for up-regulation of type 1 angiotensin II receptor mRNA in rat cardiac fibroblasts treated with tumor necrosis factor-alpha or interleukin-1 beta. *J Biol Chem, 277*(8), 5719–24

Culpitt, S. V., Rogers, D. F., Fenwick, P. S., Shah, P., De Matos, C., Russell, R. E., Barnes, P. J., Donnelly, L. E. (2003). Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. *Thorax*, *58*(11), 942-6

Dai, Q., Xu, M., Yao, M., Sun, B. (2007). Angiotensin AT1 receptor antagonists exert anti-inflammatory effects in spontaneously hypertensive rats. *Br J Pharmacol*, *152*(7), 1042-8.

Dandona, P., Dhindsa, S., Ghanim, H., Chaudhuri, A. (2007). Angiotensin II and inflammation: the effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockade. *J Hum Hypertens*, *21*(1), 20-7

De Marco, R., Pesce, G., Marcon, A., Accordini, S., Antonicelli, L., Bugiani, M Casali L., Ferrari M., Nicolini G., Panico M.G., Pirina P, Zanolin M, E., Cerveri I., Verlato, G. (2013). The coexistence of asthma and chronic obstructive pulmonary disease (COPD): prevalence and risk factors in young, middle-aged and elderly people from the general population. *PLoS One*, *8*(5), e62985

Demedts, I. K., Morel-Montero, A., Lebecque, S., Pacheco, Y., Cataldo, D., Joos, G. F., Pauwels R. A., Brusselle, G. G. (2006). Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax*, *61*(3), 196-201

Department of Health (2011) An outcomes strategy for chronic obstructive pulmonary disease (COPD) and asthma in England *Downloaded from http://www.dh.gov.uk/publications* Accessed on 11th July 2015

Di Napoli, M., Papa, F. (2003). Angiotensin-converting enzyme inhibitor use is associated with reduced plasma concentration of C-reactive protein in patients with first-ever ischemic stroke. *Stroke*, *34*(12), 2922-9.

Donnelly, L. E., Tudhope, S. J., Fenwick, P. S.,Barnes, P. J. (2010). Effects of formoterol and salmeterol on cytokine release from monocyte-derived macrophages. *Eur Respir J*, *36*(1), 178–86

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K., Jeyaseelan, R., Breitbart, R. E., Acton, S. (2000). A novel angiotensin-converting enzyme–related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res, 87*(5), e1-e9.

Dubar, V., Gosset, P., Aerts, C., Voisin, C., Wallaert, B., Tonnel, A. B. (1993). In vitro acute effects of tobacco smoke on tumor necrosis factor a and interleukin-6 production by alveolar macrophages. *Exp Lung Res*, *19*(3), 345-9

El-Hashim, A. Z., Renno, W. M., Raghupathy, R., Abduo, H. T., Akhtar, S., Benter, I. F. (2012). Angiotensin-(1–7) inhibits allergic inflammation, via the MAS1 receptor, through suppression of ERK1/2-and NF-κB-dependent pathways. *Br J Pharmacol, 166*(6), 1964-76.

EMC, 2015 Product characteristics for perindoprilat (8mg tablet) Accord Healthcare Ltd *Downloaded from https://www.medicines.org.uk/emc/medicine/25988* on 7th September 2015

English, W. R., Corvol, P., Murphy, G. (2012). LPS activates ADAM9 dependent shedding of ACE from endothelial cells. *Biochem Biophys Res Commun*, *421*(1), 70-5

Erdös, E. G. (2006). The ACE and I: how ACE inhibitors came to be. *The FASEB Journal*, *20*(8), 1034-8

Evnin, L. B.,Vásquez, J. R., Craik, C. S. (1990). Substrate specificity of trypsin investigated by using a genetic selection. *Proc Natl Acad Sci U S A*, *87*(17), 6659-63

Falini, B., Flenghi, L., Pileri, S., Gambacorta, M., Bigerna, B., Durkop, H., Eitelbach, F., Thiele, J., Pacini, R., Cavaliere, A., Martelli, M., Cardarelli, N., Sabattini, E., Poggi, S., Stein, H. (1993). PG-M1 : A New Monoclonal Antibody Directed against a Fixative-Resistant Epitope on the Macrophage-Restricted Form of the CD68 Molecule, *Am J Pathol, 142*(5), 1359–72

Fallica, J., Boyer, L., Kim, B., Serebreni, L., Varela, L., Hamdan, O., Wang, L., Simms, T., Damarla, M., Kolb, T., Bucala, R., Mitzner, W., Hassoun, P, Damico, R. (2014). Macrophage Migration Inhibitory Factor Is a Novel Determinant of Cigarette Smoke–Induced Lung Damage. *Am J Respir Cell Mol Biol*, *51*(1), 94-103

Faure, P., Oziol, L., Le Bihan, M.-L., Chomard, P. (2004). Cell culture media are potent antioxidants that interfere during LDL oxidation experiments. *Biochimie*, *86*(6), 373–8

Ferrario, C. M., Jessup, J., Chappell, M. C., Averill, D. B., Brosnihan, K. B., Tallant, E. A., Diz, D, I ., Gallagher, P. E. (2005). Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation*, *111*(20), 2605-10

Fleming, I. (2006). Signaling by the angiotensin-converting enzyme. *Circ Res*, *98*(7), 887-96.

Friedland, J., Silverstein, E., Drooker, M. (1981). Human Lung Angiotensin Converting Enzyme. *J.Clin. Invest.*, *67*, 1151–60

Fuchs, S., Xiao, H. D., Cole, J. M., Adams, J. W., Frenzel, K., Michaud, A., Zhao, H., Keshelava, G., Capecchi, M. R., Corvol, P., Bernstein, K. E. (2004). Role of the N-terminal catalytic domain of angiotensin-converting enzyme investigated by targeted inactivation in mice. *J Biol Chem*, *279*(16), 15946-53

Fujimoto, S., Satoh, M., Horike, H., Hatta, H., Haruna, Y., Kobayashi, S., Namikoshi, T., Arakawa, S., Tomita, N., Kashihara, N. (2008). Olmesartan ameliorates progressive glomerular injury in subtotal nephrectomized rats through suppression of superoxide production. *Hypertens Res*, *31*(2), 305

Gartlehner, G., Hansen, R.A., Carson, S.S., Lohr, K.N. (2006). Efficacy and safety of inhaled corticosteroids in patients with COPD: a systematic review and meta-analysis of health outcomes. *Ann Fam Med*, *4*(3), 253-62

Ghazi-Khansari, M., Mohammadi-Karakani, A., Sotoudeh, M., Mokhtary, P., Pour-Esmaeil, E., Maghsoud, S. (2007). Antifibrotic effect of captopril and enalapril on paraquat-induced lung fibrosis in rats. *J App Toxicol, 27*(4), 342-9.

Ghorpade, A., Persidskaia, R., Suryadevara, R., Che, M., Liu, X. J., Persidsky, Y., Gendelman, E. (2001). Mononuclear Phagocyte Differentiation , Activation , and Viral Infection Regulate Matrix Metalloproteinase Expression : Implications for Human Immunodeficiency Virus Type 1-Associated *J Virol*, *75*(14), 6572- 83

Ghosh, S. N., Zhang, R., Fish, B. L., Semenenko, V. A., Li, X. A., Moulder, J. E., Jacobs E. R., Medhora, M. (2009). Renin-angiotensin system suppression mitigates experimental radiation pneumonitis. *Int J Radiat Oncol Biol Phys, 75*(5), 1528-36.

Glowacka, I., Bertram, S., Müller, M. A., Allen, P., Soilleux, E., Pfefferle, S., Steffen, I. Tsegave, T.S., He, Y., Gnirss, K., Niemeyer, D., Schneider, H., Drosten, C., Pöhlmann, S. (2011). Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. *J Virol*, *85*(9), 122–34

GOLD (2015) Global Initiative for Chronic Obstructive Lung Disease Pocket Guide to COPD Diagnosis, Management and Prevention, A guide for Health Care Professionals. Updated 2015. *Downloaded from http://www.goldcopd.org on 11th July 2015*

Gosman, M. M., Postma, D. S., Vonk, J. M., Rutgers, B., Lodewijk, M., Smith, M., Luinge, M. A., Ten Hacken, N. H., Timens, W. (2008). Association of mast cells with lung function in chronic obstructive pulmonary disease. *Respir Res*, *9*, 64

Grobe, N., Weir, N. M., Leiva, O., Ong, F. S., Bernstein, K. E., Schmaier, A. H., Morris,M., Elased, K. M. (2013). Identification of prolyl carboxypeptidase as an alternative enzyme for processing of renal angiotensin II using mass spectrometry. *Am J Physiol Lung Cell Mol Physiol 304*(10), 945-53

Gross, J., Lapiere, C. M. (1962). Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U.S.A*, *48*(6), 1014-22

Gross, J. L., De Azevedo, M. J., Silveiro, S. P., Canani, L. H., Caramori, M. L., Zelmanovitz, T. (2005). Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes care*, *28*(1), 164-176.

Guo, Y., Zhao, G., Tanaka, S., Yamaguchi, T. (2009). Differential responses between monocytes and monocyte-derived macrophages for lipopolysaccharide stimulation of calves. *Cell Moll Immunol, 6*(3), 223.

Guzik, K., Skret, J., Smagur, J., Bzowska, M., Gajkowska, B., Scott, D. A., Potempa, J. S. (2011). Cigarette smoke-exposed neutrophils die unconventionally but are rapidly phagocytosed by macrophages. *Cell Dear Dis*, *2*(3), 131.

Gwathmey, T. M., Pendergrass, K. D., Reid, S. D., Rose, J. C., Diz, D. I., Chappell, M. C. (2010). Angiotensin-(1-7)-angiotensin-converting enzyme 2 attenuates reactive oxygen species formation to angiotensin II within the cell nucleus. *Hypertension*, *55*(1), 166–71

Hamming, I., Timens, W., Bulthuis, M. L., Lely, A. T., Navis, G. J., van Goor, H. (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol*, *203*(2), 631–7

Han, S.X., He, G.M., Wang, T., Chen, L., Ning, Y.Y., Luo, F., Yang J., Dong, J., Liao, Z., Xu, D., Wen, F.Q. (2010). Losartan attenuates chronic cigarette smoke exposure-induced pulmonary arterial hypertension in rats: possible involvement of angiotensin-converting enzyme-2. *Toxicol Appl Pharmacol*, *245*(1), 100–7

Haschke, M., Schuster, M., Poglitsch, M., Loibner, H., Salzberg, M., Bruggisser, M, Penninger, J., Krähenbühl, S. (2013). Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. *Clin Pharmacokin*, *52*(9), 783-92.

Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M. B., Leboeuf, M., Becker, C.D., See. P., Price, J., Lucas, D., Greter, M., Mortha, A., Boyer, S., Forsberg, C., Tanaka, M., Rooijen, N., Garcia-Sastre, A., Stanley, R E., Ginhoux, F., Frenette , P., Merad, M. (2013). Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*, *38*(4), 792–804

Hautamaki, R. D., Kobayashi, D. K., Senior, R. M., Shapiro, S. D. (1997). Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science*, *277*(5334), 2002-4

He, L., Ding, Y., Zhang, Q., Che, X., He, Y., Shen, H., Wang, H., Li, Z., Zhao, L., Geng, J., Deng, Y., Yang, L., Li, J., Cai, J., Qiu, L., Wen, K., Xu, X., Jiang, S. (2006). Expression of elevated levels of pro-inflammatory cytokines in SARS-CoV-infected ACE2+ cells in SARS patients: relation to the acute lung injury and pathogenesis. *J Pathol*, *210*(3), 288–97

Heard, B. E. (1958). A pathological study of emphysema of the lungs with chronic bronchitis. *Thorax*, *13*(2), 136-49

Heran, B. S., Wong, M. M., Heran, I. K., Wright, J. M. (2008). Blood pressure lowering efficacy of angiotensin converting enzyme (ACE) inhibitors for primary hypertension. *Cochrane Database Syst Rev*, *4*. CD003823

Herath, C. B., Warner, F. J., Lubel, J. S., Dean, R. G., Jia, Z., Lew, R. A., Smith, I., Burrell, L.M., Angus, P. W. (2007). Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1–7) levels in experimental biliary fibrosis. *J Hepatol, 47*(3), 387-95

Hermanns, M. I., Müller, A. M., Tsokos, M., Kirkpatrick, C. J. (2014). LPS-induced effects on angiotensin I-converting enzyme expression and shedding in human pulmonary microvascular endothelial cells. *In Vitro Cell Dev Biol Anim*, *50*(4), 287-95

HSCIC Statistics on Smoking, England (2015) *Health and Social Care Information Centre.* ISBN 978-1-78386-327-3

Hodge, S., Holmes, M., Banerjee, B., Musk, M., Kicic, A., Waterer, G., Reynolds, P.N., Hodge, G., Chambers, D. C. (2009). Posttransplant bronchiolitis obliterans syndrome is associated with bronchial epithelial to mesenchymal transition. *Am J Transplant, 9*(4), 727-33.

Hogg, J. C. (2004). Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet*, *364*(9435), 709-21

Hogg, J. C., Timens, W. (2009). The pathology of chronic obstructive pulmonary disease. *Annu Rev Pathol, 4*, 435-59

Hollenberg, N. K., Fisher, N. D., Price, D. A. (1998). Pathways for angiotensin II generation in intact human tissue evidence from comparative pharmacological interruption of the renin system. *Hypertension*, *32*(3), 387-92

Holtzman, M. J., Tyner, J. W., Kim, E. Y., Lo, M. S., Patel, A. C., Shornick, L. P., Agapov, E., Zhang, Y. (2005). Acute and Chronic Airway Responses to Viral Infection: Implications for Asthma and Chronic Obstructive Pulmonary Disease. *Proc Am Thorac Soc,* , *2*(2), 132–40.

Horwitz, D. A., Allison, A. C., Ward, P., Kight, N. (1977). Identification of human mononuclear leucocyte populations by esterase staining. *Clin Exp immunol*, *30*(2), 289-98

Huang, M., Li, X., Meng, Y., Xiao, B., Ma, Q., Ying, S., Wu, P.S., Zhang, Z. (2010). Upregulation of angiotensin-converting enzyme (ACE) 2 in hepatic fibrosis by ACE inhibitors. *Clin Exp Pharamcol Physiol 37*(1), e1–6

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., Yan, P., Sarao, R., Wada, T., Leong-Poi, H., Crackower, M.A., Fukamizu, A., Hui, C.C., Hein, L., Uhlig, S., Slutsky, A.S., Jiang, C., Penninger, J. M. (2005). Angiotensin-converting enzyme 2 protects from severe acute lung failure. *Nature*, *436*(7047), 112–6

Jia, H. P., Look, D. C., Tan, P., Shi, L., Hickey, M., Gakhar, L., McCray, P. B. (2009). Ectodomain shedding of angiotensin converting enzyme 2 in human airway epithelia. *Am J Physiol Lung Cell Mol Physiol*, *297*(1), L84-L96.

Jurewicz, M., McDermott, D. H., Sechler, J. M., Tinckam, K., Takakura, A., Carpenter, C. B., Milford, E., Abdi, R. (2007). Human T and natural killer cells possess a functional renin-angiotensin system: further mechanisms of angiotensin II-induced inflammation. *J Am Soc Nephrol, 18*(4), 1093–102

Kanazawa, H., Hirata, K., Yoshikawa, J. (2003). Effects of captopril administration on pulmonary haemodynamics and tissue oxygenation during exercise in ACE gene subtypes in patients with COPD: a preliminary study. *Thorax, 58*(7), 629-31

Karavitis, J., Kovacs, E. J. (2011). Macrophage phagocytosis: effects of environmental pollutants, alcohol, cigarette smoke, and other external factors. *J Leukoc Biol.*, *90*(6), 1065-78

Keidar, S., Gamliel-Lazarovich, A., Kaplan, M., Pavlotzky, E., Hamoud, S., Hayek, T., Abassi, Z. (2005). Mineralocorticoid receptor blocker increases angiotensin-converting enzyme 2 activity in congestive heart failure patients. *Circ Res*, *97*(9), 946-53.

Keidar, S., Kaplan, M., Gamliel-Lazarovich, A. (2007). ACE2 of the heart: from angiotensin I to angiotensin (1–7). *Cardiovasc Res*, *73*(3), 463-9.

Kent, L., Smyth, L., Clayton, C., Scott, L., Cook, T., Stephens, R., Singh, D. (2008). Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages. *Cytokine*, *42*(2), 205-16.

Kim, V., Criner, G. J. (2013). Chronic bronchitis and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med, 187*(3), 228-37

Kleinstreuer, C., Feng, Y. (2013). Lung deposition analyses of inhaled toxic aerosols in conventional and less harmful cigarette smoke: a review. *Int J Environ Res Public Health*, *10*(9), 4454-85

Koarai, A., Traves, S. L., Fenwick, P. S., Brown, S. M., Chana, K. K., Russell, R. E., Donnelly, L. E. (2012). Expression of muscarinic receptors by human macrophages. *European Respiratory Journal*, *39*(3), 698-704.Kohlstedt, K., Brandes, R. P., Müller-Esterl, W., Busse, R., Fleming, I. (2004). Angiotensin-converting enzyme is involved in outside-in signaling in endothelial cells. *Circ Res 94*(1), 60-67.

Kohlstedt, K., Brandes, R. P., Müller-Esterl, W., Busse, R., Fleming, I. (2004). Angiotensin-converting enzyme is involved in outside-in signaling in endothelial cells. *Circ Res*, *94*(2), 50-64.

Kunisch, E., Fuhrmann, R., Roth, A., Winter, R., Lungershausen, W., Kinne, R. W. (2004). Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry. *Annals of the rheumatic diseases*, *63*(7), 774-84.

Kuschner, W. G., D'Alessandro, A., Wong, H., Blanc, P. D. (1996). Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. *European Respiratory Journal*, *9*(10), 1989-1994.Lahzami, S., Bridevaux, P. O., Soccal, P. M., Wellinger, J., Robert, J. H., Ris, H. B., Aubert, J. D. (2010). Survival impact of lung transplantation for COPD. *Eur Respir J*, *36*(1), 74-80

Lahzami, S., Bridevaux, P. O., Soccal, P. M., Wellinger, J., Robert, J. H., Ris, H. B., Aubert, J. D. (2010). Survival impact of lung transplantation for COPD. *Eur Respir J*, *36*(1), 74-80.

Lambert, D. W., Yarski, M., Warner, F. J., Thornhill, P., Parkin, E. T., Smith, a I., Hooper, N.M., Turner, A. J. (2005). Tumor necrosis factor-alpha convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2). *J Biol Chem*, *280*(34), 30113–9

Lau, S. K., Chu, P. G., Weiss, L. M. (2004). CD163: a specific marker of macrophages in paraffin-embedded tisse samples . *Am J Clin Pathol*, *122*(5), 794-801

Lee, J., Nordestgaard, B. G., Dahl, M. (2009). Elevated ACE activity is not associated with asthma, COPD, and COPD co-morbidity. *Respir Med*, *103*(9), 1286–92

Leopold, J. G., Gough, J. (1957). The centrilobular form of hypertrophic emphysema and its relation to chronic bronchitis. *Thorax*, *12*(3), 219-35

Li, X., Molina-Molina, M., Abdul-Hafez, A., Uhal, V., Xaubet, A., Uhal, B. D. (2008). Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. *Am J Physiol Lung Cell Mol Physiol, 295*(1), 178–85

Lieberman, J. (1975). Elevation of serum angiotension-converting-enzyme (ACE) level in sarcoidosis. *Am J Med*, *59*(3), 365-72.

Lieberman, J., Winter, B., Sastre, A. (1986). Alpha 1-antitrypsin Pi-types in 965 COPD patients. *Chest*, *89*(3), 370-3.

Lieschke, G. J., Burgess, A. W. (1992). Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. *NEJM*, *327*(1), 28-35.

Lijnen, P., Papparella, I., Petrov, V., Semplicini, A., Fagard, R. (2006). Angiotensin II-stimulated collagen production in cardiac fibroblasts is mediated by reactive oxygen species. *J Hypertens*, *24*(4), 757-66.

Liu, M., Proud, D., Lichtenstein, L., MacGlashan, D.W., Schieimer, R.P., Adkinson, N.F., Kagey-Sobotka,A., Schulman, E.S., Plaut, M., (1986). Human lung macrophage-derived histamine-releasing activity is due to IgE-dependent factors. *J Immunol*, *136*(7), 2588–95

Ljungberg, L., Persson, K. (2009). Effect of nicotine and nicotine metabolited on angiotensin convering enzyme in humn endothelial cells. *Endothelium,* 15, 239-45

Løkke, A., Lange, P., Scharling, H., Fabricius, P., Vestbo, J. (2006). Developing COPD: a 25 year follow up study of the general population. *Thorax*, *61*(11), 935-9

Lozano R., Naghavi M., Foreman K., Lim S., Shibuya, K., Aboyans, V., Abraham J., Adair T., Aggarwal R., Ahn S. AlMazroa M., Alvarado M., Anderson R.H., Anderson L.M., Andrews K.G., Atkinson C., Andrews K.G., Atkinson C., Baddour L.M., Barker- Collo S., Bartels D.H., Bell M.L Cross M. (2013). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, *380*(9859), 2095-128

Makinde, T., Murphy, R. F., Agrawal, D. K. (2007). The regulatory role of TGF-β in airway remodeling in asthma. *Immunol Cell Biol*, *85*(5), 348-56.

Malerba, M., Ponticiello, A., Radaeli, A., Bensi, G., Grassi, V. (2004). Effect of twelve-months therapy with oral ambroxol in preventing exacerbations in patients with COPD. Double-

blind, randomized, multicenter, placebo-controlled study (the AMETHIST Trial). *Pulm Pharmacol Ther*, *17*(1), 27-34

Mancini, G. J., Etminan, M., Zhang, B., Levesque, L. E., FitzGerald, J. M., Brophy, J. M. (2006). Reduction of morbidity and mortality by statins, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers in patients with chronic obstructive pulmonary disease. *J Am Coll Cardiol, 47*(12), 2554-60.

Marco van der Toorn, Rezayat, D., Kauffman, H. F., Bakker, S. J., Gans, R. O., Koëter, G. H., Slebos, D. J. (2009). Lipid-soluble components in cigarette smoke induce mitochondrial production of reactive oxygen species in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* (1), 109-14.

Marks, L. S., Maxwell, M. H. (1979). Tigerstedt and the discovery of renin. An historical note. *Hypertension*, *1*(4), 384-8

Marshall, R. P., McAnulty, R. J., Laurent, G. J. (2000). Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor. *Am J Respir Crit Care Med, 161*(6), 1999-2004.

Martí-Lliteras, P., Regueiro, V., Morey, P., Hood, D. W., Saus, C., Sauleda, J.,Agusti, A., Bengoechea, J., Garmendia, J. (2009). Nontypeable Haemophilus influenzae clearance by alveolar macrophages is impaired by exposure to cigarette smoke. *Infect Immun*, *77*(10), 4232–42

Matsuoka, S., Washko, G. R., Dransfield, M. T., Yamashiro, T., Estepar, R. S. J., Diaz, A., Hatabu, H. (2010). Quantitative CT measurement of cross-sectional area of small pulmonary vessel in COPD: correlations with emphysema and airflow limitation. *Acad Radiol*, *17*(1), 93-9.

McCollum, L. T., Gallagher, P. E., Tallant, E. A. (2012). Angiotensin-(1–7) abrogates mitogen-stimulated proliferation of cardiac fibroblasts. *Peptides*, *34*(2), 380-8

Mercer, P. F., Shute, J. K., Bhowmik, A., Donaldson, G. C., Wedzicha, J. A., Warner, J. A. (2005). MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res*, *6*(1-9), 151.

Metzger, R., Bohle, R. M., Chumachenko, P., Danilov, S. M., Franke, F. E. (2000). CD143 in the development of atherosclerosis. *Atherosclerosis*, *150*(1), 21-31.

Metzger, R., Franke, F. E., Bohle, R. M., Alhenc-Gelas, F., Danilov, S. M. (2011). Heterogeneous distribution of angiotensin I-converting enzyme (CD143) in the human and rat vascular systems: vessel, organ and species specificity. *Microvasc Res*, *81*(2), 206-15.

Miura, S., Sadashiva S. Karnik, K. K. (2011). Review: angiotensin II type 1 receptor blockers: class effects versus molecular effects. *J Renin Angiotensin Aldosterone Syst,* 12.1 (2011): 1-7.

Mogielnicki, A, Kramkowski, K., Hermanowicz, J. M., Leszczynska, A, Przyborowski, K., Buczko, W. (2014). Angiotensin-(1-9) enhances stasis-induced venous thrombosis in the rat because of the impairment of fibrinolysis. *J Renin Angiotensin Aldosterone Syst*, *15*(1), 13–21

Molet, S., Belleguic, C., Lena, H., Germain, N., Bertrand, C. P., Shapiro, S. D., Lagente, V. (2005). Increase in macrophage elastase (MMP-12) in lungs from patients with chronic obstructive pulmonary disease. *Inflamm Res*, *54*(1), 31-36.

Montes, E., Ruiz, V., Checa, M., Maldonado, V., Melendez-Zajgla, J., Montaño, M., Selman, M. (2012). Renin is an angiotensin-independent profibrotic mediator: role in pulmonary fibrosis. *Eur Respir J*, *39*(1), 141-8.

Morrell, N. W., Atochina, E. N., Morris, K. G., Danilov, S. M., Stenmark, K. R. (1995). Angiotensin converting enzyme expression is increased in small pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *J Clin Invest*, *96*(4), 1823-33

Mortensen, E. M., Copeland, L. A, Pugh, M. J. V, Restrepo, M. I., de Molina, R. M., Nakashima, B., Anzueto, A. (2009). Impact of statins and ACE inhibitors on mortality after COPD exacerbations. *Respir Res*, *10*(1), 45–54

Natesh, R., Schwager, S. L., Sturrock, E. D., Acharya, K. R. (2003). Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. *Nature*, *421*(6922), 551-4.

NICE guidelines [CG101] 2010 Chronic obstructive Pulmonary Disease in over 16s: diagnosis and management. *Downloaded from https://www.nice.org.uk/guidance/CG101/chapter/1-*Guidance Accessed on 10th March 2016

Nihlen, U., Montnemery, P., Lindholm, L., Lofdahl, C. (2001). Increase serum levels in carbohydrate-deficient transferring in pateints with chronic obstructive pulmonary disease *Scan J clin Lab Inv, 31* (5), 341-7

Ocaranza, M. P., Moya, J., Barrientos, V., Alzamora, R., Hevia, D., Morales, C., Pinto, M., Escudero, N., Garcia, L., Novoa, U., Ayala, P., Diaz-Araya. G., Godoy, I., Chiong, M., Lavandero, S., Jalil, J.E., Michea, L. (2014). Angiotensin-(1-9) reverses experimental hypertension and cardiovascular damage by inhibition of the angiotensin converting enzyme/Ang II axis. *J Hypertension*, *32*(4), 771–83

Office for National Statistics Adult Smoking Habits in Great Britain (2013) *Downloaded from http://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/healthandlifeexpectancies/compendium/opinionsandlifestylesurvey/2015-03-19/adultsmokinghabitsingreatbritain2013* Accessed on 10th March 2016

Orfanos, S. E., Armaganidis, A., Glynos, C., Psevdi, E., Kaltsas, P., Sarafidou, P., Catravas, J.D., Dafni, U.G., Langleben, D., Roussos, C. (2000). Pulmonary capillary endothelium-bound angiotensin-converting enzyme activity in acute lung injury. *Circulation*, *102*(16), 2011-8

Orte, C., Polak, J. M., Haworth, S. G., Yacoub, M. H., Morrell, N. W. (2000). Expression of pulmonary vascular angiotensin-converting enzyme in primary and secondary plexiform pulmonary hypertension. *J Pathol*, *192*(3), 379-84.

Pabst, S., Theis, B., Gillissen, A., Lennarz, M., Tuleta, I., Nickenig, G., Skowasch, D., Groohe, C. (2009). Angiotensin-converting enzyme I/D polymorphism in chronic obstructive pulmonary disease. *Eur J Med Res*, *14*,177–81

Passos-Silva, D. G., Verano-Braga, T., Santos, R. A. (2013). Angiotensin-(1–7): beyond the cardio-renal actions. *Clinical Science*, *124*(7), 443-56

Pedersen, K. B., Sriramula, S., Chhabra, K. H., Xia, H., Lazartigues, E. (2011). Species-specific inhibitor sensitivity of angiotensin-converting enzyme 2 (ACE2) and its implication for ACE2 activity assays. *Am J Physiol Regul Integr Comp Physiol 301*(5), R1293–9

Pejler, G., Rönnberg, E., Waern, I., Wernersson, S. (2010). Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood*, *115*(24), 4981-90.

Perrin-Fayolle, M., Pacheco, Y., Harf, R., Montagnon, B., Biot, N. (1981). Angiotensin converting enzyme in bronchoalveolar lavage fluid in pulmonary sarcoidosis. *Thorax*, *36*(10), 790-2.

Pesci, A., Balbi, B., Majori, M., Cacciani, G., Bertacco, S., Alciato, P., Donner, C. F. (1998). Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J*, *12*(2), 380-6

Petersen, H., Sood, A., Meek, P. M., Shen, X., Cheng, Y., Belinsky, S. A., Owen, C.A., Washko, G., Pinto-Plata, V., Kelly, E., Celli, B., Tesfaigzi, Y. (2014). Rapid lung function decline in smokers is a risk factor for COPD and is attenuated by angiotensin-converting enzyme inhibitor use. *Chest*, *145*(4), 695–703

Pinto-Plata, V. M., Müllerova, H., Toso, J. F., Feudjo-Tepie, M., Soriano, J. B., Vessey, R. S., Celli, B. R. (2006). C-reactive protein in patients with COPD, control smokers and non-smokers. *Thorax*, *61*(1), 23-8

Podowski, M., Calvi, C., Metzger, S., Misono, K., Poonyagariyagorn, H., Lopez-Mercado, A., Cheadle, C. (2012). Angiotensin receptor blockade attenuates cigarette smoke–induced lung injury and rescues lung architecture in mice. *J Clin Invest*, *122*(1), 229-40.

Pons, A. R., Sauleda, J., Noguera, A., Pons, J., Barcelo, B., Fuster, A., Agusti, A. G. N. (2005). Decreased macrophage release of TGF-β and TIMP-1 in chronic obstructive pulmonary disease. *Eur Respir J, 26*(1), 60-66.

Rabe, K. F., Hurd, S., Anzueto, A., Barnes, P. J., Buist, S. A., Calverley, P., Zielinski, J. (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Resp Crit Care*, *176*(6), 532-55.

Regalado, J., Pérez-Padilla, R., Sansores, R., Paramo Ramirez, J. I., Brauer, M., Paré, P., Vedal, S. (2006). The effect of biomass burning on respiratory symptoms and lung function in rural Mexican women. *Am J Resp Crit Care*, *174*(8), 901-5.

Ren, X., Glende, J., Al-Falah, M., de Vries, V., Schwegmann-Wessels, C., Qu, X., Tan, L., Tschernig, T., Deng, H., Naim, H. Y., Herrier, G (2006). Analysis of ACE2 in polarized epithelial cells: surface expression and function as receptor for severe acute respiratory syndrome-associated coronavirus. *J Gen Virol*, *87*(6), 1691–5

Renno, W. M., Al-Banaw, A. G., George, P., Abu-Ghefreh, A. A., Akhtar, S., Benter, I. F. (2012). Angiotensin-(1–7) via the Mas receptor alleviates the diabetes-induced decrease in GFAP and GAP-43 immunoreactivity with concomitant reduction in the COX-2 in hippocampal formation: an immunohistochemical study. *Cell Mol Neurobiol*, *32*(8), 1323-36.

Ribeiro-Oliveira Jr, A., Nogueira, A. I., Pereira, R. M., Boas, W. W. V., dos Santos, R. A. S., Silva, A. C. S. (2008). The renin–angiotensin system and diabetes: an update. *Vasc Health Risk Manag*, *4*(4), 787.

Rice, G., Thomas, D.A., Grant, P.J., Turner, A.J., Hooper, N.M. (2004). Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *Biochem J*, *383*(1), 45-51.

Richard, V., Hurel-Merle, S., Scalbert, E., Ferry, G., Lallemand, F., Bessou, J. P., Thuillez, C. (2001). Functional evidence for a role of vascular chymase in the production of angiotensin II in isolated human arteries. *Circulation*, *104*(7), 750-2.

Rigat, B., Hubert, C., Alhenc-Gelas, F., Cambien, F., Corvol, P., Soubrier, F. (1990). An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the varince of serum enzyme levels. *J Clin Inves,*89(4), 1343-6

Rodrigues-Machado, M. G., Magalhães, G. S., Cardoso, J. A., Kangussu, L. M., Murari, A., Caliari, M. V., Oliveira, M.L., Cara, D.C., Noviello,M.L., Marques, F.D., Pereira, J.M., Lautner, R.Q., Santos, R.A., Campagnole-Santos, M. J. (2013). AVE 0991, a non-peptide mimic of angiotensin-(1–7) effects, attenuates pulmonary remodelling in a model of chronic asthma. *Br J Pharmacol*, *170*(4), 835-46

Rodríguez-Vita, J., Sánchez-López, E., Esteban, V., Rupérez, M., Egido, J., Ruiz-Ortega, M. (2005). Angiotensin II activates the Smad pathway in vascular smooth muscle cells by a transforming growth factor-β–independent mechanism. *Circulation*, *111*(19), 2509-17.

Rovina, N., Koutsoukou, A., Koulouris, N. G. (2013). Inflammation and immune response in COPD: where do we stand?. *Mediators Inflamm*, *2013*.

Rueckschloss, U., Quinn, M.T., Holtz, J., Morawietz, H. (2002). Dose-Dependent Regulation of NAD(P)H Oxidase Expression by Angiotensin II in Human Endothelial Cells: Protective Effect of Angiotensin II Type 1 Receptor Blockade in Patients With Coronary Artery Disease. *Arterioscler Thromb Vasc Biol, 22*(11), 1845–51

Russell, R. E., Culpitt, S. V., DeMatos, C., Donnelly, L., Smith, M., Wiggins, J., Barnes, P. J. (2002). Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol*, *26*(5), 602-9.

Sadhukhan, R., Sen, I. (1996). Different glycosylation requirements for the synthesis of enzymatically active angiotensin-converting enzyme in mammalian cells and yeast. *J Biol Chem,*, *271*(11), 6429-34.

Sakaguchi, K., Chai, S. Y., Jackson, B., Johnston, C. I., Mendelsohn, F. A. (1988). Inhibition of tissue angiotensin converting enzyme. Quantitation by autoradiography. *Hypertension*, *11*(3), 230-8

Salvi, S. S., Barnes, P. J. (2009). Chronic obstructive pulmonary disease in non-smokers. *The lancet*, *374*(9691), 733-743.Sampaio, W. O., Henrique de Castro, C., Santos, R. a. S., Schiffrin, E. L., Touyz, R. M. (2007). Angiotensin-(1-7) Counterregulates Angiotensin II Signaling in Human Endothelial Cells. *Hypertension*, *50*(6), 1093–8

Sampaio, W. O., de Castro, C. H., Santos, R. A., Schiffrin, E. L., Touyz, R. M. (2007). Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension*, *50*(6), 1093-8.

Santos, R. A., e Silva, A. C. S., Maric, C., Silva, D. M., Machado, R. P., de Buhr, I., Mendes, E. P. (2003). Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *PNAS*, *100*(14), 8258-63.

Santos, R. A., Ferreira, A. J., Verano-Braga, T., Bader, M. (2013). Angiotensin-converting enzyme 2, angiotensin-(1–7) and Mas: new players of the renin–angiotensin system. *J Endocrinol*, *216*(2), 1-17.

Segura-Valdez, L., Pardo, A., Gaxiola, M., Uhal, B. D., Becerril, C., Selman, M. (2000). Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest*, *117*(3), 684-94

Shabihkhani, M., Lucey, G. M., Wei, B., Mareninov, S., Lou, J. J., Vinters, H. V., Yong, W. H. (2014). The procurement, storage, and quality assurance of frozen blood and tissue biospecimens in pathology,biorepository, and biobank settings. *Clin Biochem*, *47*(4), 258-66.

Shah, A., Oh, Y. B., Lee, S. H., Lim, J. M., Kim, S. H. (2012). Angiotensin-(1–7) attenuates hypertension in exercise-trained renal hypertensive rats. *Am J Heart Circ Physiol, 302*(11), 2372-80.

Shahab, L., Jarvis, M. J., Britton, J., West, R. (2006). Prevalence, diagnosis and relation to tobacco dependence of chronic obstructive pulmonary disease in a nationally representative population sample. *Thorax*, *61*(12), 1043-47

Shaykhiev, R., Krause, A., Salit, J., Strulovici-Barel, Y., Harvey, B. G., O'Connor, T. P., Crystal, R. G. (2009). Smoking-dependent reprogramming of alveolar macrophage polarization: implication for pathogenesis of chronic obstructive pulmonary disease. *J Immunol*, *183*(4), 2867-83.

Shenoy, V., Ferreira, A. J., Qi, Y., Fraga-Silva, R. A., Díez-Freire, C., Dooies, A., Jun, J.Y, Sriramula, S., Mariappan, N., Pourang, D., Venugopal, C.S., Francis, J., Reduelhuber, T., Santos, R.A., Patenl, J.P., Raizada, M K., Katovich, M. J. (2010). The angiotensin-converting enzyme 2/angiogenesis-(1–7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. *Am H Resp Crit Care*, *182*(8), 1065-72

Shieh, W. J., Hsiao, C. H., Paddock, C. D., Guarner, J., Goldsmith, C. S., Tatti, K., Packard, M., Mueller, L., Wu, M., Rollin, P., Su, I., Zaki, S. R. (2005). Immunohistochemical, in situ hybridization, and ultrastructural localization of SARS-associated coronavirus in lung of a fatal case of severe acute respiratory syndrome in Taiwan. *Hum Pathol*, *36*(3), 303-9

Silver, R. B., Reid, A. C., Mackins, C. J., Askwith, T., Schaefer, U., Herzlinger, D., Levi, R. (2004). Mast cells: a unique source of renin. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(37), 13607-13612. Silverstein, E., Friedland, Q. J., Lyons, H. A., Gourin, A. (1975). Enzyme in granulatmous lymph nodes and serym in sarcoidosis: clinical and possible pathogenic significance. *Ann N Y Acad Sci,* 278, 498-51

Silverstein, E., Friedland, J., Lyons, H. A., Gourin, A. (1976). Elevation of angiotensin-converting enzyme in granulomatous lymph nodes and serum iin sarcoidosis: clinical and possible pathogenic signficiance. *Ann N Y Acad Sci*, *278*(1), 498-513.

Sims, A. C., Baric, R. S., Yount, B., Burkett, S. E., Collins, P. L., Pickles, R. J. (2005). Severe Acute Respiratory Syndrome Coronavirus Infection of Human Ciliated Airway Epithelia : Role of Ciliated Cells in Viral Spread in the Conducting Airways of the Lungs. *J Virol*, *79*(24), 15511–24

Simsek, S., Tekes, S., Oral, D., Turkyilmaz, A. (2013). The insertion / deletion polymorphism in the ACE gene and chronic obstructive pulmonary disease. *Genet Mol Res*, *12*(2), 1392–8

Skeggs, L. T., Kahn, J. R., Shumway, N. P. (1956). The preparation and function of the hypertensin-converting enzyme. *J Exp Med*, *103*(3), 295-99.

Sopel, M. J., Rosin, N. L., Lee, T. D., Légaré, J. F. (2011). Myocardial fibrosis in response to Angiotensin II is preceded by the recruitment of mesenchymal progenitor cells. *Lab Invest*, *91*(4), 565-78.

Specks, U., Martin, W. J., Rohrbach, M. S. (1990). Bronchoalveolar lavage fluid angiotensin-converting enzyme in interstitial lung diseases. *Am Rev Respir Dis*, *141*(1), 117-23.

Spira, A., Beane, J., Shah, V., Liu, G., Schembri, F., Yang, X., Palma. J., Brody, J. S. (2004). Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc Natl Acad Sci U S A, 101*(27), 10143-48

Staples, K. J., Nicholas, B., McKendry, R. T., Spalluto, C. M., Wallington, J. C., Bragg, C. W., Robinson, E., Martin, K., Djukanovic, R., Wilkinson, T. M. (2015). Viral Infection of Human Lung Macrophages Increases PDL1 Expression via IFNβ. *PloS one*, *10*(3), e0121527

Suissa, S., Patenaude, V., Lapi, F., Ernst, P. (2013). Inhaled corticosteroids in COPD and the risk of serious pneumonia. *Thorax*, *68*(11), 1029-36

Takeuchi, K., Shimizu, T., Ohishi, N., Seyama, Y., Takaky, F., Yotsumoto, H. (1989). Purification of Human Lung Angiotensin-Converting Enzyme by High-Performance Liquid Chromatography: Properties and N-Terminal Amino Acid Sequence. *J. Biochem.*, *45*, 442–5

Talhout, R., Schulz, T., Florek, E., Van Benthem, J., Wester, P., Opperhuizen, A. (2011). Hazardous compounds in tobacco smoke. *Int J Environ Res Public Health*, *8*(2), 613-28

Tardif, J., Borgeat, P., Laviolette, M. (1990). Inhibition of human alveolar macrophage production of leukotriene B4 by acute in vitro and in vivo exposure to tobacco smoke. *Am J Respir Cell Mol Biol*, *2*(2), 155-161.

Taylor, A. E., Finney-Hayward, T. K., Quint, J. K., Thomas, C. M. R., Tudhope, S. J., Wedzicha, J. A., Barnes P.J., Donnelly, L. E. (2010). Defective macrophage phagocytosis of bacteria in COPD. *Eur Respir J*, *35*(5), 1039-47

Taylor, A. A., Siragy, H., Nesbitt, S. (2011). Angiotensin receptor blockers: pharmacology, efficacy, and safety. *J Clin Hypertens*, *13*(9), 677-86.

Thomas, M. C., Pickering, R. J., Tsorotes, D., Koitka, A., Sheehy, K., Bernardi, S., Toffoli, B., Nguyen-Huu, T., Head, G., Fu, Y., Chin-Dusting, J., Cooper, M., Tikellis, C. (2010). Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. *Circ Res*, *107*(7), 888–97

Thorley, A. J., Tetley, T. D. (2007). Pulmonary epithelium, cigarette smoke, and chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis, 2*(4), 409-28

Tikellis, C., Bialkowski, K., Pete, J., Sheehy, K., Su, Q., Johnston, C., Thomas, M. C. (2008). ACE2 deficiency modifies renoprotection afforded by ACE inhibition in experimental diabetes. *Diabetes*, *57*(4), 1018-25

Tipnis, S. R., Hooper, N. M., Hyde, R., Karran, E., Christie, G., Turner, A. J. (2000). A human homolog of angiotensin-converting enzyme cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem*, *275*(43), 33238-43.

Tudhope, S., Finney-Hayward, T., Nicholson, A.G., Mayer, RJ., Barnette, MS., Barnes, P.J., Donnelly, L.E. (2008). Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage. *J Pharmacol Exp Ther, 324* (1), 306–12

Uçar, G., Yildirim, Z., Ataol, E., Erdoğan, Y., Biber, Ç. (1997). Serum angiotensin converting enzyme activity in pulmonary diseases: correlation with lung function parameters. *Life Sci*, *61*(11), 1075-82.

Uhal, B. D., Kyong Kim, J., Li, X., Molina-Molina, M. (2007). Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. *Current pharmaceutical design*, *13*(12), 1247-56.

Uhal, B. D., Dang, M., Dang, V., Llatos, R., Cano, E., Abdul-Hafez, A., Markey, J., Piasecki, C.C., Molina-Molina, M. (2013). Cell cycle dependence of ACE-2 explains downregulation in idiopathic pulmonary fibrosis. *Eur Respir J*, *42*(1), 198–210

Urata, H., Kinoshita, A., Misono, K. S., Bumpus, F. M., Husain, A. (1990). Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem, 265*(36), 22348-57.

van der Toorn, M., Rezayat, D., Kauffman, H. F., Bakker, S. J., Gans, R. O., Koëter, G. H., Choi, A,M., van Oosterhout A.J., Slebos, D. J. (2009). Lipid-soluble components in cigarette smoke induce mitochondrial production of reactive oxygen species in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol, 297*(1), 109-14

van Diemen, C. C., Postma, D. S., Vonk, J. M., Bruinenberg, M., Nolte, I. M., Boezen, H. M. (2006). Decorin and TGF-β1 polymorphisms and development of COPD in a general population. *Resp Res*, *7*(1), 89.

Veerappan, A., Reid, A. C., Estephan, R., O'Connor, N., Thadani-Mulero, M., Salazar-Rodriguez, M., Levi, R., Silver, R. B. (2008). Mast cell renin and a local renin-angiotensin system in the airway: role in bronchoconstriction. *Proc Natl Acad Sci I S A 105*(4), 1315–20

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Acton, S. (2002). Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem*, *277*(17), 14838-43.

Wallace, A. M., Loy, L. B., Abboud, R. T., D'Armiento, J. M., Coxson, H. O., Muller, N. L., Finley, R. J. (2014). Expression of Matrix Metalloproteinase-1 in Alveolar Macrophages, Type II Pneumocytes, and Airways in Smokers: Relationship to Lung Function and Emphysema. *Lung*, *192*(4), 467-72.

Wang, T., Han, S.-X., Zhang, S.-F., Ning, Y. Y., Chen, L., Chen, Y. J., He, G. M., Xu, D., An, J., Yang, T., Zhang, X. H.,Wen, F.Q. (2010). Role of chymase in cigarette smoke-induced pulmonary artery remodeling and pulmonary hypertension in hamsters. *Respir Res*, *11*(1) 36

Wang, J., Chen, L., Chen, B., Meliton, A., Liu, S. Q., Shi, Y., Li, Y. C. (2015). Chronic Activation of the Renin-Angiotensin System Induces Lung Fibrosis. *Sci Rep*, *23*(5)

Watkins, A. J., Wilkins, A., Cunningham, C., Perry, V. H., Seet, M. J., Osmond, C., Eckert, J.J., Torrens, C., Cagampang, F.R., Cleal, J., Gray, W.P., Hanson, M.A., Fleming, T. P. (2008). Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. *J Physiol, 586*(8), 2231–44

Wei, C., Hase, N., Inoue, Y., Bradley,E.W., Yahiro, E., Li, M., Naqvi, N., Powell, P.C., Shi, K., Takahashi, Y., Saku, K., Urata, H., Dell'italia, L.J., Husain, A. (2010). Mast cell chymase limits the cardiac efficacy of Ang I–converting enzyme inhibitor therapy in rodents. *J Clin Invest 120*(4), 1229–39

Weidner, N., Austen, K. F. (1993). Heterogeneity of mast cells at multiple body sites: fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase, and carboxypeptidase content. *Pathol Res Pract,189*(2), 156-62

Willemse, B. M., Ten-Hacken, N. H. T., Rutgers, B., Lesman-Leegte, I. G.T., Postma, D. S., Timens, W. (2005). Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J, 26*(5), 835-45

Winkler, A. R., Nocka, K. H., Sulahian, T. H., Kobzik, L., Williams, C. M. (2008). In vitro modeling of human alveolar macrophage smoke exposure: enhanced inflammation and impaired function. *Exp Lung Res*, *34*(9), 599–629

Witherden, I. R., Vanden Bon, E. J., Goldstraw, P., Ratcliffe, C., Pastorino, U., Tetley, T. D. (2004). Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. *Am J Respir Cell Mol Biol, 30*(4), 500–9

Wong, S. K., Li, W., Moore, M. J., Choe, H., Farzan, M. (2004). A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J Bio Chem*, *279*(5), 3197-201.

Wösten-van Asperen, R. M., Lutter, R., Specht, P. A., Moll, G. N., van Woensel, J. B., van der Loos, C. M., Bos, A. P. (2011). Acute respiratory distress syndrome leads to reduced ratio of ACE/ACE2 activities and is prevented by angiotensin-(1–7) or an angiotensin II receptor antagonist. *J Pathol*, *225*(4), 618-627.

Wynn, T. A. (2011). Integrating mechanisms of pulmonary fibrosis. *J Exp Med*, *208*(7), 1339-50.

Wysocki, J., Ye, M., Soler, M. J., Gurley, S. B., Xiao, H. D., Bernstein, K. E., Batlle, D. (2006). ACE and ACE2 activity in diabetic mice. *Diabetes*, *55*(7), 2132-39.

Xia, H., Suda, S., Bindom, S., Feng, Y., Gurley, S. B., Seth, D., Navar, L.G., Lazartigues, E. (2011). ACE2-mediated reduction of oxidative stress in the central nervous system is associated with improvement of autonomic function. *PloS one*, *6*(7), e22682

Xue, T., Wei, N., Xin, Z., Qingyu, X. (2014). Angiotensin-converting enzyme-2 overexpression attenuates inflammation in rat model of chronic obstructive pulmonary disease. *Inhal Toxicol*, *26*(1), 14–22

Yang, Z., Yu, X., Cheng, L., Miao, L., Li, H., Han, L., Jiang, W. (2013). Effects of enalapril on the expression of cardiac angiotensin-converting enzyme and angiotensin-converting enzyme 2 in spontaneously hypertensive rats. *Arch Cardiovasc Dis, 106*(4), 196–201

Yohannes, A.M. (2007). Palliative care provision for patients with chronic obstructive pulmonary disease. *Health Qual Life Outcomes*, *5*(1), 17-22

Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M., Strauss-Ayall, D., Viukov, S., Guilliams, M., Misharin, A., Hume, D.A., Periman, H., Malissen, G., Zelzer, E., Jung, S. (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*, *38*(1), 79–91

Zahradka, P., Werner, J. P., Buhay, S., Litchie, B., Helwer, G., Thomas, S. (2002). NF-κB activation is essential for angiotensin II-dependent proliferation and migration of vascular smooth muscle cells. *J Mol Cell Cardiol, 34*(12), 1609-21.

Zandvoort, A., Postma, D. S., Jonker, M. R., Noordhoek, J. A., Vos, J. T., Timens, W. (2008). Smad gene expression in pulmonary fibroblasts: indications for defective ECM repair in COPD. *Respir Res*, *9*(83),

Zeng, M., Li, Y., Jiang, Y., Lu, G., Huang, X., Guan, K. (2013). Local and systemic oxidative stress and glucocorticoid receptor levels in chronic obstructive pulmonary disease patients. *Can Respir J*, *20*(1), 35-41