

Effects of vitamin D on inflammation and oxidative stress in airway epithelial cells

Rosemary Norton

A Thesis Presented for the Degree of Doctor of Philosophy at
the University of East Anglia, Norwich, UK

August 2012

©This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognise that its copyright rests with the author and that use of any information derived there from must be in accordance with current UK Copyright Law. In addition, any quotation or extract must include full attribution.

Dedicated to Brian

Abstract

Chronic obstructive pulmonary disease (COPD) is a multifactorial and systemic disease driven by inflammation and oxidative stress, predominantly caused by smoking, but exacerbated by infection and pollution. Current therapies aim to treat specific aspects of the disease and/or symptoms rather than the disease as a whole.

Vitamin D, more commonly known for its importance in calcium and phosphorus homeostasis, has now been established as an important immunomodulatory agent. Recent studies have shown vitamin D deficiency to be associated with reduced lung function and COPD disease severity. Equally vitamin D has been shown to modulate a number of inflammatory mediators which are important in COPD pathology. Together, these suggest that vitamin D may be beneficial in COPD. Airway epithelial cells play a central role in COPD pathogenesis and have been shown to constitutively activate vitamin D. This study investigated the effects of vitamin D on inflammation and oxidative stress in human airway epithelial cells, crucially on mediators important in COPD pathology and also investigated the potential mechanisms involved in the action of vitamin D.

Three different human airway epithelial cell lines A549, NCI-H292, 16HBE14o- and primary human small airway epithelial cells were shown to express similar inflammatory mediators, with the 16HBE14o- cells shown to be a useful model of primary cells. Vitamin D significantly modulated IL-6 mRNA and protein expression in the primary cells and reduced MUC5AC mRNA and protein expression in the mucous producing NCI-H292 cells. Interestingly, vitamin D treatment gave a biphasic effect on induction of inflammatory mediators by TNF α in the NCI-H292 cells. Vitamin D also modulated the induction of oxidative stress, antioxidant gene expression and Nrf2 protein expression in the NCI-H292 cells.

This data emphasises the complexity of vitamin D modulation of inflammatory mediators and oxidative stress in airway epithelial cells and provides new novel avenues of future study.

Publications and Presentations arising from thesis

Publications:

- **'Vitamin D: Potential in the Prevention and Treatment of Lung Cancer.'** Norton R and O'Connell M A. *Anticancer Research* 32:211-222 (2012).
- **'Vitamin D and Respiratory Health'** Hughes DA and Norton R, *Clinical and Experimental Immunology* 158: 20–25 (2009).

Abstracts/Presentation:

- **'Response of lung epithelial cells to inflammatory stimuli following exposure to the active form of vitamin D.'** Norton R, Sexton DW, Clark IM, Wilson AM, Hughes DA and O'Connell MA. *Proceedings of the Nutrition Society* 70(OCE3), E87 (2011).

Poster presentation: Nutrition Society Conference on Vitamins in early development and healthy ageing: impact on infectious and chronic disease, Cork, June 2011.

- **'The potential benefits of vitamin D on lung inflammation'** *Oral presentation:* BSI Early Career Inflammation and Immunology symposium, Norwich, June 2011.

- **'Immunomodulatory effects of 1,25(OH)₂D₃ in normal and transformed respiratory epithelium.'** R Norton, DW Sexton, IM Clark, AM Wilson, DA Hughes and MA O'Connell. *Anticancer Research* 31: 1499 (2011).

Oral presentation: 4th Symposium on Vitamin D and Analogs in Cancer Prevention and Therapy, Homburg, May 2011.

- **'Finding a 2D in vitro model to study lung inflammation'** *Oral presentation:* Abcam conference on Injury and Repair Mechanisms in Chronic Lung Disease, London, April 2011.

- **'Immunomodulatory effects of vitamin D in respiratory epithelium'** *Poster presentation:* British Society of Immunology Annual Congress, Liverpool, December 2010.

➤ **'A breath of sunshine, vitamin D in respiratory disease' *Invited Talk***: British Society of Immunology East Anglian Group Seminar Series, Norfolk and Norwich Hospital, Norwich, June 2010.

➤ **'Vitamin D deficiency' Poster and Oral Pechu Kucha Presentation**: June 2011, University of East Anglia Postgraduate showcase event at the forum in Norwich disseminating research to members of the public.

*** Winner, Vice Chancellors Award for 'Best Overall Poster' ***

*** Winner, 'Most Creative/Original Poster' ***

*** Highly Commended 'Public Engagement Award' ***

Acknowledgements

Firstly, I would like to thank the **Norwich Research Park** for funding my studentship and the **Nutricia Research Foundation** who funded the research carried out in this thesis.

Supervisors

I would like to thank all my supervisors for their support in this really interesting project. Dr Maria O'Connell, Dr David Hughes, Professor Ian Clark, Dr Darren Sexton, Dr Roy Bongaerts and Dr Andrew Wilson. Thank you to Maria for supporting me throughout my lab research, encouraging me to get involved in different activities and helping me to attend many conferences and get experience of presenting my research. I am truly grateful for all your help, support and friendship both then and now. To David, for continuing to support the project and for his kindness and encouragement throughout, but especially for his guidance and thoughtfulness during a difficult time writing up. To Ian without whom I would not be doing a PhD, his inspirational leadership and support during my undergraduate research projects had a great influence on me. To Darren whose enthusiasm for research is infectious and constant ideas, support and discussion have been invaluable.

I would also like to thank members of our lab, past and present for their help, support and friendship. Lydia Wherry, Jenna Bradley, Jonathan Cowan, Richard Jacques, Clara Mayano-Cabenero, Jason Kerr and Adrian Churchman. Project students; Alfisal Taymoor, Jade Robinson and Ben Farrington. Thanks also to Dr Chris Morris for his help with cells.

I have to thank all the past and present members of the Clark lab who have been a constant help with guidance and supplies and were a huge part of me choosing to do a PhD. Tracey Swingler, Ursula Rogers, Rosemary Davidson, Janine Wilkinson and Alison Surridge. A special thanks to Eleanor Jones for her help with gelatin zymography and Kirsty Culley who was a big influence on my future choice to do a PhD.

I am grateful to Dr Andrew Goldson for his help with the flow cytometry and for obtaining funding for us to do some of the flow cytometric bead arrays.

I would also like to thank my parents for their constant support and guidance in helping me to become who I am and get where I am today, and for encouraging me through the toughest parts of my PhD.

Last but not least, I would like to thank Brian for his continued support throughout my PhD, who was there in times of despair and frustration in good times and bad and encouraged me on to the end, and for being patient and helping me to do what I needed to do to get my thesis finished.

1.1.7	<i>Current therapies</i>	36
1.1.8	<i>Future therapies</i>	37
1.2	Vitamin D	40
1.2.1	<i>Sources and Metabolism</i>	41
1.2.2	<i>Immunomodulation</i>	43
1.3	Vitamin D in lung infection and disease	45
1.3.1	<i>Tuberculosis</i>	46
1.3.2	<i>Influenza and the common cold</i>	47
1.3.3	<i>Cystic fibrosis</i>	48
1.3.4	<i>Asthma</i>	49
1.3.5	<i>Lung cancer</i>	50
1.3.6	<i>COPD</i>	51
1.4	Aims	54
Chapter 2: Materials and Methods		55
2.1	Materials	56
2.1.1	<i>General Materials</i>	56
2.1.2	<i>Cell Culture</i>	56
2.1.2.1	<i>Consumables</i>	56
2.1.2.2	<i>Cell culture reagents</i>	56
2.1.3	<i>Nucleic acid isolation</i>	56
2.1.4	<i>qRT-PCR</i>	57
2.1.5	<i>Protein detection</i>	57
2.1.5.1	<i>Western blotting</i>	57
2.1.5.2	<i>Flow cytometric bead array</i>	57
2.1.5.3	<i>Gelatin zymography</i>	58
2.1.6	<i>Reactive oxygen species assay</i>	58
2.1.7	<i>Cytotoxicity / Proliferation assay</i>	58
2.1.8	<i>ELISA</i>	58
2.1.9	<i>Immunohistochemistry for MUC5AC expression</i>	58
2.2	Methods	59
2.2.1	<i>Cell culture</i>	59
2.2.1.1	<i>Heat inactivation of FCS</i>	59

2.2.1.2 Preparation of cell culture media	59
2.2.1.3 Cells	59
2.2.1.3.1 A549	59
2.2.1.3.2 NCI-H292	59
2.2.1.3.3 16HBE14o-	60
2.2.1.3.4 SAEC	60
2.2.1.4 Cell enumeration and viability	61
2.2.1.5 Cryopreservation	61
2.2.1.5.1 A549 and NCI-H292	61
2.2.1.5.2 16HBE14o-	62
2.2.1.5.3 SAEC	62
2.2.1.6 Cell stimulations	62
2.2.1.6.1 LPS	62
2.2.1.6.2 TNF α	62
2.2.1.6.3 Vitamin D	62
2.2.1.6.4 Inhibitors	62
2.2.1.6.5 tBHP and H ₂ O ₂	63
2.2.2 Nucleic acid isolation	63
2.2.2.1 RNA extraction using tri-reagent	63
2.2.2.2 RNA quantification	63
2.2.3 qRT-PCR	64
2.2.3.1 cDNA synthesis from RNA extracts	64
2.2.3.2 cDNA synthesis directly from cell cultures	64
2.2.3.3 qRT-PCR	65
2.2.3.4 Primer design	65
2.2.3.5 Analysis	67
2.2.4 ROS assay	67
2.2.4.1 Method optimisation	67
2.2.4.2 Optimised assay procedure	68
2.2.5 Cytotoxicity/Proliferation assay	68
2.2.6 Protein detection	68
2.2.6.1 Western blotting	68
2.2.6.1.1 Extract preparation	68

2.2.6.1.2 SDS-PAGE	68
2.2.6.1.3 Gel transfer	69
2.2.6.1.4 Gel staining	69
2.2.6.1.5 Immunoblotting	69
2.2.6.1.6 Chemiluminescence	69
2.2.6.1.7 Reprobing	70
2.2.6.2 Flow cytometric bead array	70
2.2.6.3 Gelatin zymography	70
2.2.6.3.1 Gel preparation	71
2.2.6.3.2 Extract preparation	71
2.2.6.4 ELISA	71
2.2.6.5 Immunohistochemistry for MUC5AC expression	72
2.2.7 Statistical analysis	72
 Chapter 3: Characterisation of airway epithelial cells	 74
3.1 Introduction	75
3.2 Aims	77
3.3 Results	78
3.3.1 Morphology of the cell lines used in this study	78
3.3.2 Expression of inflammatory mediators in airway epithelial cells	79
3.3.3 Induction of inflammatory mediators by <i>Pseudomonas Aeruginosa</i> LPS	81
3.3.4 Induction of mRNA expression for inflammatory mediators by TNF α	84
3.3.5 Induction of inflammatory cytokine protein expression by TNF α	86
3.3.6 Induction of MMPs and MUC5AC mRNA expression by TNF α	87
3.3.7 MMP9 functional activity	89
3.3.8 MUC5AC protein expression in A549 and NCI-H292 cells	90
3.4 Discussion	92

3.5 Conclusion	99
-----------------------	-----------

Chapter 4: Effects of vitamin D on inflammatory mediators in airway epithelial cells	101
4.1 Introduction	102
4.2 Aims	103
4.3 Results – Part one – Effects of vitamin D on basal expression of inflammatory mediators in human airway epithelial cells	104
<i>4.3.1 Effects of vitamin D treatment on cell proliferation in lung cancer cell lines A549 and NCI-H292</i>	<i>104</i>
<i>4.3.2 Sensitivity of airway epithelial cells studied to vitamin D treatment</i>	<i>104</i>
<i>4.3.3 Effects of vitamin D on basal expression of IL-6 and IL-8 in cells sensitive to vitamin D treatment</i>	<i>106</i>
<i>4.3.4 Mechanism of vitamin D modulating basal IL-6 mRNA expression</i>	<i>107</i>
<i>4.3.5 Effects of vitamin D on basal expression of mRNA for other inflammatory mediators</i>	<i>109</i>
<i>4.3.6 Effects of vitamin D on MMP9 enzyme activity in airway epithelial cells</i>	<i>111</i>
<i>4.3.7 Mechanism of action of vitamin D on induction of TNFα mRNA in NCI-H292 cells</i>	<i>112</i>
<i>4.3.8 Effect of Vitamin D on the basal expression of MUC5AC in the different cell types</i>	<i>114</i>
Results – Part two – Effects of vitamin D on inflammatory responses induced by TNFα or LPS	114
<i>4.3.9 Effects of vitamin D on TNFα induction of inflammatory mediators</i>	<i>114</i>
<i>4.3.10 Dose dependent effects of vitamin D in SAEC and NCI-H292 cells</i>	<i>115</i>
<i>4.3.11 Vitamin D does not affect IL-6 and IL-8 expression in the CYP24 over-expressing A549 cells</i>	<i>116</i>
<i>4.3.12 Effects of vitamin D on LPS induced IL-6</i>	

<i>and IL-8 mRNA expression</i>	117
4.3.13 <i>Effects of vitamin D on TNFα/LPS induction of mRNA for other inflammatory mediators in NCI-H292 cells</i>	118
4.3.14 <i>Effects of vitamin D pretreatment in MMP9 mRNA expression and enzyme activity</i>	119
4.3.15 <i>Effects of vitamin D pre-treatment on MUC5AC induction by known inducers</i>	121
4.4 Discussion	123
4.5 Conclusion	130
Chapter 5 – Effects of vitamin D on oxidative stress in airway epithelial cells	131
5.1 Introduction	132
5.2 Aims	133
5.3 Results	134
5.3.1 <i>Optimisation of methodology for the ROS assay</i>	136
5.3.2 <i>Induction of ROS by positive controls H₂O₂ and tBHP</i>	139
5.3.3 <i>Effect of vitamin D on ROS production in airway epithelial cells</i>	140
5.3.4 <i>Effect of vitamin D on the induction of ROS by H₂O₂ and tBHP</i>	141
5.3.5 <i>Effect of vitamin D on genes controlled by the antioxidant response element</i>	145
5.3.6 <i>Effects of molecular inhibitors on induction of antioxidant genes in NCI-H292 cells by vitamin D treatment</i>	147
5.4 Discussion	150
5.5 Conclusion	153
Chapter 6 – General discussion and future studies	154
6.1 Airway epithelial cells and lung inflammation	155
6.2 Vitamin D and lung inflammation	158
6.3 Vitamin D and oxidative stress	159
6.4 New hypotheses from this study to be investigated	160
6.5 Human studies	161

6.6 Conclusion	162
Appendix I Cytotoxicity of reagents and inhibitors	163
Appendix II Buffers and solutions	168
Appendix III Dissociation curves and standard curves for SYBR green qRT-PCR	173
Appendix IV Standard curves for flow cytometric bead array	181
References	182

List of Figures

Figure 1.1 COPD Pathology

1.2 Key signalling pathways activated by inflammation

1.3 Vitamin D citations by year

1.4 Simplified version of vitamin D metabolism

3.1 Morphology of cell lines used in study

3.2 Induction kinetics of pro-inflammatory mediators by LPS

3.3 Induction kinetics of pro-inflammatory mediators by TNF α

3.4 TNF α induction of IL-6 and IL-8 protein expression in airway epithelial cells

3.5 Induction kinetics of MMPs and MUC5AC by TNF α

3.6 MMP9 enzyme activity in SAEC and 16HBE14o- cells

3.7 MUC5AC protein expression in NCI-H292 and A549 cells

3.8 LPS and PMA significantly induce MUC5AC protein expression in NCI-H292 cells

4.1 Effects of vitamin D on lung cancer cell proliferation

4.2 Sensitivity of lung epithelial cells to vitamin D treatment

4.3 Effects of vitamin D on the basal IL-6 and IL-8 mRNA and protein expression

4.4 Effects of signalling pathway inhibitors on vitamin D mediated inhibition of basal IL-6 mRNA in 16HBE14o- cells

4.5 Effects of vitamin D on the basal mRNA expression of other inflammatory mediators

4.6 Effect of vitamin D on MMP9 enzyme activity

4.7 Effect of signalling pathway inhibitors on vitamin D mediated activation of basal TNF α mRNA in NCI-H292 cells

4.8 Vitamin D reduces basal MUC5AC mRNA expression and protein secretion

4.9 Effects of vitamin D on TNF α induced IL-6 and IL-8 mRNA and protein expression

- 4.10 Dose dependent effects of vitamin D on TNF α induced IL-6 mRNA expression**
- 4.11 Vitamin D does not affect TNF α induced IL-6 and IL-8 mRNA expression in A549**
- 4.12 Effects of vitamin D on LPS induced IL-6 and IL-8 mRNA expression**
- 4.13 Biphasic effects of vitamin D on TNF α and LPS induction of IL-1 β and TNF α in NCI-H292 cells**
- 4.14 Effect of vitamin D on MMP9 expression and enzyme activity in SAEC and 16HBE14o- cells**
- 4.15 Vitamin D pretreatment blocks induction of MUC5AC mRNA expression and protein secretion by inflammatory stimuli.**
- 5.1 Optimisation of loading agents for ROS assay**
- 5.2 Dose dependent induction of ROS by H₂O₂ and tBHP in airway epithelial cells**
- 5.3 Vitamin D induced ROS production in airway epithelial cells**
- 5.4 Effects of vitamin D on H₂O₂ induced oxidative stress in A549 and NCI-H292 cells**
- 5.5 Effects of vitamin D on tBHP induced oxidative stress in A549 and NCI-H292 cells**
- 5.6 Vitamin D synergistically induces ROS in 16HBE14o- cells**
- 5.7 Effect of vitamin D on antioxidant gene expression in airway epithelial cells**
- 5.8 Vitamin D treatment increases Nrf2 protein expression**
- 5.9 Effect of signalling pathway inhibitors on vitamin D mediated activation of basal HO-1 mRNA in NCI-H292 cells**
- 5.10 Effect of signalling pathway inhibitors on vitamin D mediated activation of basal Ferritin H mRNA in NCI-H292 cells**

List of Tables

Table 2.1 Primers for qRT-PCR (100 μ M)

3.1 Summary of mRNA expression for inflammatory mediators

3.2 Summary of protein expression for inflammatory mediators

5.1 Summary of literature on H₂DCFDA and their methodology

6.1 Summary of the similarities and differences between the cell types used in the study and conclusions

Abbreviations

1,25(OH) ₂ D ₃	1,25 dihydroxyvitamin D / calcitriol
25(OH)D ₃	25-hydroxyvitamin D (Calcidiol)
AP-1	Activator Protein 1
APC	Antigen Presenting Cells
ARE	Antioxidant Response Element
BAL	Bronchoalveolar lavage fluid
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
COPD	Chronic Obstructive Pulmonary Disease
CXCL-1	CC(CXC-motif) ligand 1
CYP 24	Mitochondrial Cytochrome P450 24-Hydroxylase
CYP27B1	25-hydroxyvitamin D ₃ 1-alpha Hydroxylase
DBP	Vitamin D Binding Protein
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EPC	Endothelial Progenitor Cell
ERK	Extracellular Signal Regulated Kinase
FBS	Foetal Bovine Serum
FEV1	Forced Expiratory Volume in 1 second
FITC	Fluorescein isothiocyanate
GEF's	Guanosine Nucleotide Exchange Factors
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GPCR	G-protein Coupled Receptor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HBSS	Hanks Buffered Salt Solution
HCl	Hydrochloric acid

HEPES	<i>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</i>
ICAM	Intercellular adhesion molecule
IKK	Inhibitor of κ B kinase
IL-	Interleukin
IFN- γ	Interferon gamma
I κ B	Inhibitor of κ B
JNK	c-jun N-terminal kinase
Keap1	kelch-like ECH-associated protein 1
LDS	Lithium Dodecyl Sulphate
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAPK	Mitogen activated protein kinase
MEM	Minimum Eagles Medium
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MHC	major histocompatibility complex
MMLV	Mouse moloney murine leukaemia virus
MMP	Matrix Metalloproteinase
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
MUC5AC	Mucin subtype 5AC
NaCl	Sodium chloride
NF κ B	Nuclear Factor kappa B
NO	Nitric oxide
NQO1	NADP(H), quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2 p45-related factor 2
<i>P. aeruginosa</i>	<i>Pseudomonas Aeruginosa</i>
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3 Kinase
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RNA	Ribonucleic acid
RNS	Reactive nitrogen species

ROCK	Rho kinase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
S. Enteritidis	Salmonella Enteritidis
SABM	Small airway epithelial cell basal medium
SAEC	Small airway epithelial cells
SAGM	Small airway epithelial cell growth factors
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SLPI	Secretory leukocyte protease inhibitor
TE	Trypsin EDTA
TGF	Transforming growth factor
THXR1	Thioredoxin reductase 1
TIMPS	Tissue inhibitors of metalloproteinases
TLR	Toll like receptor
TNF α	Tumor necrosis factor alpha
URTI	Upper respiratory tract infection
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Introduction

1.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD is defined as a progressive airflow obstruction which is not fully reversible, with an abnormal inflammatory response ⁽¹⁾. It is estimated that 210 million people have COPD worldwide and it is predicted to be the third leading cause of death by 2020 ⁽²⁾. In the UK, the prevalence is 1% increasing to 10% in males over the age of 75 (World Health Organisation (WHO)). 90% of COPD related deaths occur in low-middle income countries due to the reduced accessibility to treatments and preventative measures.

1.1.1 Aetiology

More than 90% of all COPD cases are associated with smoking ⁽²⁾. However, only 15% of smokers develop COPD, suggesting that there are differences in an individual's susceptibility to the disease. Although an inflammatory response to cigarette smoke is present in all smokers, in some the inflammation persists on smoking cessation ⁽³⁾. The differences in an individual's susceptibility to COPD and ability to modulate responses to cigarette smoke is key to understanding the vast spectrum of airway disease present in COPD. Air pollution is another contributor to COPD development including occupational dust, chemicals and the indoor use of solid fuel for cooking and heating in low-income countries (WHO). Genetic factors can also influence COPD, including variability in genes involved in protease production, response to cigarette smoke and mucociliary clearance ⁽⁴⁾.

1.1.2 Signs and Symptoms

The main symptoms of patients with COPD are breathlessness, a chronic cough and an abnormal sputum / excessive mucous production (WHO). Clinically, the forced expiratory volume in one second (FEV1) measured by spirometry, is an indicator of the degree of airway obstruction and a decline in FEV1 is correlated with disease severity.

1.1.3 Pathology

COPD encompasses a range of different disease pathologies. The terms emphysema and chronic bronchitis are no longer used but are included in the diagnosis of COPD (WHO)⁽¹⁾. Emphysema, chronic bronchitis with airway obstruction, and small airway

disease are the distinct phenotypes of COPD but most patients show overlapping pathologies ⁽⁵⁾. COPD is characterised by airflow obstruction with excessive mucous secretion, and destruction of airspaces with loss of lung elastic recoil, chronic inflammation and tissue remodelling without fibrosis ^(1, 3, 6). Remodelling causes the airway wall to thicken reducing the elasticity and the alveolar attachments are disrupted from the membrane leading to alveolar collapse. These pathologies collectively lead to a reduction in FEV1.

1.1.4 Systemic effects

The complexity of COPD is not restricted to the respiratory system; COPD is a systemic disease involving skeletal muscle and the cardiovascular system with weight loss and muscle weakness all associated with an increased risk of mortality ^(6, 7). Malnutrition is common in patients with COPD, affecting 50% of patients with advanced stage disease ⁽⁷⁾. The cause of weight loss is a matter of debate with a high metabolic rate thought to play a major role. There are several proposed mechanisms contributing to an increased metabolic rate. These include; increased oxygen consumption by respiratory muscles to overcome obstruction, the medications given to patients, and inflammatory mediators. Depletion of muscle mass in COPD is associated with mitochondrial abnormalities and loss of contractile proteins with malfunction of any remaining muscle ⁽⁷⁾. Alongside COPD, cigarette smoke is associated with a 50% increased risk of heart failure so it is not surprising that heart disease is a co-morbidity of COPD and vice versa. With COPD characterised by a low grade systemic inflammation, this can contribute to the progression of atherosclerosis and cardiovascular events ⁽⁸⁾.

1.1.5 Exacerbations

An exacerbation is defined as an acute and sustained worsening of a patient's symptoms from their usual state beyond the normal day to day variation ⁽⁹⁾. Exacerbations of COPD play a major role in the morbidity, mortality and severity of the disease. Acute exacerbations are the most frequent cause of hospital admission and death amongst patients with chronic lung disease and are a major burden on the health service ⁽¹⁰⁾. Although infection is the major cause of exacerbations, air pollution can also contribute. The prominent bacteria found in COPD exacerbations

are *Haemophilus influenza*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* which is the most frequent bacterial pathogen in severe COPD and elicits the greatest inflammation giving accelerated lung function decline⁽¹⁰⁻¹⁴⁾. The rhinovirus is the dominant viral pathogen in acute exacerbations⁽¹¹⁾. Both viral and bacterial exacerbations increase inflammation with cell infiltration and cytokine production, leading to progression of the disease.

1.1.6 Pathogenesis

The pathogenesis of COPD involves many different processes, including cell infiltration, alveolar cell apoptosis, extracellular matrix proteolysis, inflammation, oxidative stress and epigenetic modulation⁽⁶⁾, all of which will be discussed in the next sections.

1.1.6.1 Cell infiltration

COPD is characterised by a neutrophil and macrophage-induced inflammation that develops over many years⁽²⁾. There is a chronic inflammatory cell infiltration of the bronchial wall, goblet cell hyperplasia and metaplasia and a decrease in the number and length of the cilia, reducing mucocilliary clearance causing airway obstruction. A recent theory is that bronchiole vascular remodelling is one of the first steps leading to COPD pathology and is thought to involve the mobilisation of endothelial progenitor cells (EPCs) into the lung from the bone marrow to produce new blood vessels⁽¹⁵⁾. These new vessels are the means by which cytokines and chemokines interact with the lung epithelium and immune cells. Recent work suggests that cigarette smoke-induced angiogenesis and leukocyte trafficking may be important for the recruitment of inflammatory cells to the airways⁽¹⁶⁾, with smokers lungs having increased numbers of neutrophils, lymphocytes and macrophages⁽⁶⁾.

Cigarette smoke induces increased levels of circulating neutrophils in the lungs which are sequestered in the lung capillaries⁽³⁾ through the rolling of neutrophils on endothelial cells. Neutrophils are an abundant cell type found in both the bronchoalveolar lavage fluid (BAL) and sputum samples from patients with COPD⁽³⁾. They are a prominent source of inflammatory mediators⁽³⁾.

The healthy lung contains resident macrophages although in COPD it is the infiltrating inflammatory macrophages that are the main source of cytokines. Macrophages are activated by cigarette smoke and they are also increased in BAL fluid of COPD patients. The number of macrophages in the alveolar wall correlates with destruction, and in the airways correlates with the severity of obstruction providing evidence of the major role of macrophages in COPD pathophysiology⁽¹⁷⁾.

CD8 positive cells are the most abundant T cell subset in COPD. They are increased in the epithelium and their numbers correlate with reduced lung function. They also release a number of different inflammatory mediators⁽¹⁷⁾.

The airway epithelium is now being recognised as an important contributor of a wide range of cytokines and chemokines that recruit both neutrophils and macrophages, which are important in the pathogenesis of COPD^(17, 18). Primary small airway epithelial cells (SAEC) and normal human bronchial epithelial cells (NHBE) are the cell lines which are the closest models to *in vivo* lung however they are expensive and have a relatively short lifespan which means experimentally they are used infrequently shown by the small amount of literature with these cell lines studying lung inflammation. There are numerous lung epithelial cell lines used as model cell systems to study lung inflammation, most of which are derived from cancerous tissue. Although they may show different expression patterns than the primary cells, they provide an alternative in which to optimise experiments and give indications of what inflammatory mediators are produced by the airway epithelium. The A549 cell line is derived from a lung adenocarcinoma however they are used as a model of the alveolar epithelial type II cells and there is extensive literature on all aspects of this cell line including production of cytokines, mucins, matrix metalloproteinases and reactive oxygen species in response to different stimuli^(19, 20). NCI-H292 are a mucoepidermoid lung cancer cell lines which have been used amongst other things to study the effect of lipopolysaccharide on mucin production in airway epithelial cells⁽²¹⁾. 16HBE14o- are a SV-40 transformed bronchial epithelial cell line which has been used widely to study epithelial barrier function and ion transport and shows similar characteristics to primary bronchial cells⁽²²⁾.

1.1.6.2 Cell death

COPD patients have a greater number of apoptotic cells within their lung tissue including alveolar and bronchial epithelial cells and endothelial cells in the parenchyma ⁽²³⁾. Cigarette smoke can directly induce necrosis in cells via caspase inhibition ⁽²⁴⁾. Cells undergoing programmed cell death can be quickly removed by the surrounding milieu of cells, not only by professional phagocytes, but also fibroblasts, endothelial, epithelial, stromal and smooth muscle cells ⁽²³⁾. However, in COPD this clearance of apoptotic cells is reduced. Surfactant proteins SF-A and D are also thought to be important in aiding the clearance of apoptotic cells and these are decreased in the lungs of smokers ⁽²³⁾.

1.1.6.3 Inflammation

There is a cellular homeostatic balance between the production of pro and anti-inflammatory mediators. In the lungs, cigarette smoke, oxidative stress and infections contribute to produce a pro-inflammatory profile. In lungs of smokers there is an increased production of interleukins (IL-): IL-1 β , IL-6, IL-8, IL-10, IL12p70 and tumour necrosis factor alpha (TNF α) in breath condensates ⁽⁶⁾, some of which correspond to those that are increased in the sputum during exacerbations of COPD; IL-6, IL-1 β , TNF α , chemokine ligand 1 (CXCL1), monocyte chemoattractant protein 1 (MCP-1), and IL-8. Clinical markers of the severity of COPD include; IL-6, IL-8, IL-10 and TNF α with bronchial biopsies of COPD patients having an increased expression of IL-10 ⁽³⁾. Inflammation may also be responsible for systemic manifestations and co-morbidities of COPD such as muscle dysfunction, cardiovascular disease and osteoporosis ⁽²⁵⁾ with elevated levels of systemic pro-inflammatory cytokines such as TNF α and IL-6 observed in COPD patients ⁽⁷⁾.

IL-6 is a multifunctional cytokine which plays a central role in host defence due to its range of immune activities and ability to induce the acute phase response. It is induced in infection, trauma or other stress when it contributes to inflammation by leukocytosis, thrombocytosis, lymphocyte activation and acute phase protein synthesis ⁽²⁶⁾. IL-6 can also activate pulmonary epithelial cells ⁽³⁾.

TNF α is elevated in bronchial biopsies, induced sputum and BAL fluid of COPD patients and is also significantly increased in the sputum during acute exacerbations⁽²⁷⁾. It is produced by a range of cell types including alveolar macrophages, neutrophils and epithelial cells after contact with cigarette smoke⁽²⁷⁾. TNF α stimulation of the pulmonary epithelium is a major contributor of acute lung injury⁽²⁸⁾. Using TNF α receptor knock out mice Churg et al (2004) showed that 60-70% of airway enlargement in COPD is dependent on TNF α . TNF α is also important for the production of matrix metalloproteinases (MMPs) although this process isn't completely TNF α dependent⁽²⁹⁾. TNF α may also increase fibroblast proliferation, differentiation and extracellular matrix deposition as well as promoting MMP induction⁽³⁰⁾. TNF α can inhibit macrophage apoptotic cell uptake and therefore clearance of apoptotic cells from the lung⁽²³⁾, leading to further airway obstruction.

IL-8 (also known as CXCL8) is a key contributor to COPD pathology as a neutrophil chemoattractant and is also an activator of immune responses. It is found in high concentrations in sputum and BAL fluid of patients with COPD⁽²⁴⁾ and can be produced by activated bronchial epithelial cells, macrophages and neutrophils in a mechanism of positive feedback⁽³⁾.

Leukotriene B4 (LTB4) is also a neutrophil chemoattractant and is secreted by alveolar macrophages and neutrophils⁽²⁴⁾. Leukocyte recruitment is an important step in tissue inflammation. Firm adhesion of neutrophils to endothelial cells is important for them to migrate to sites of inflammation and occurs via binding of neutrophil integrins to intercellular adhesion molecule -1 (ICAM-1)⁽³¹⁾. In tracheal epithelial cells ICAM-1 can be induced by TNF α and IL-1 β . In alveolar type 1 or 2 cells it is also induced by hyperoxia or lipopolysaccharide (LPS)⁽³²⁾. LPS is a structural component of the outer membrane of gram negative bacteria and can activate nuclear factor kappa B (NF κ B) via signalling through toll like receptor 4 (TLR4) on the cell surface of numerous cell types, which is important in COPD exacerbations⁽³³⁾. The antimicrobial peptide cathelicidin LL-37 is produced by neutrophils, macrophages and respiratory epithelial cells on TLR4 activation, is increased in the sputum of patients with COPD and is suggested to be a biomarker of COPD progression⁽³⁴⁾.

In relation to airway remodelling, transforming growth factor beta 1 (TGFβ1) is increased in the small airways and alveolar epithelial cells in patients with COPD and participates in fibrotic processes in the smaller airways⁽²⁴⁾. Endothelin -1 is increased in COPD sputum and is important in pulmonary vascular remodelling. Genetic deletion of vascular endothelial growth factor (VEGF) causes airspace enlargement, and leads to alveolar apoptosis and COPD development in rats, suggesting that VEGF is an important contributor to the destructive processes^(24, 35).

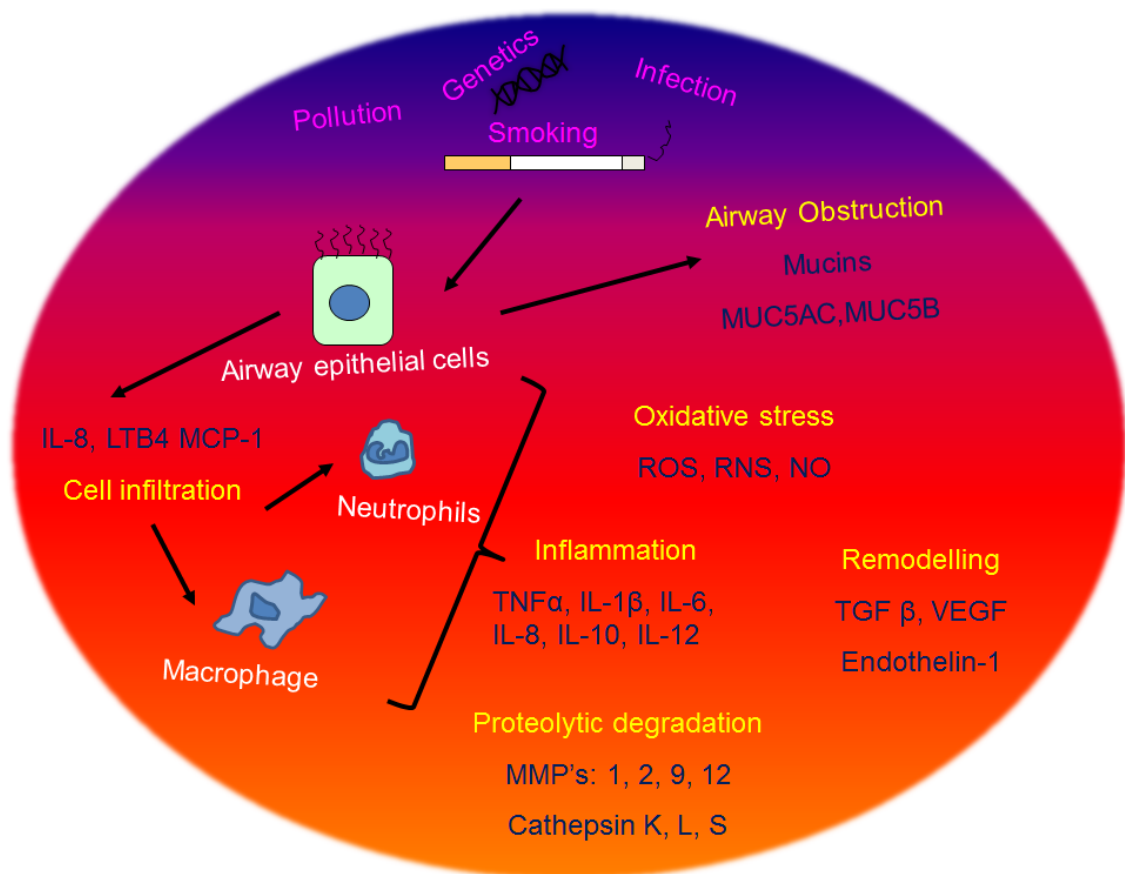


Figure 1.1 – COPD Pathology
Simplified diagram showing the main factors important in COPD pathology. The contributors, cell types, processes and molecules associated with COPD.

1.1.6.3.1 – Signalling pathways activated by inflammation (Figure 1.2)

1.1.6.3.1.1 NFκB

Nuclear Factor kappa B (NFκB) is an ubiquitous transcription factor regulating a large number of genes involved in inflammation, growth, apoptosis and carcinogenesis⁽³⁶⁾. Patients with COPD have increased NFκB activation in macrophage and epithelial cells, and it is further activated during exacerbations⁽³⁷⁾. NFκB consists of a family of transcription factors, classically as a heterodimeric complex of p50 and p65/RelA subunits. In an unstimulated cell, NFκB is in the

cytoplasm bound to inhibitor of κ B (I κ B) which masks the nuclear localisation signal holding NF κ B in the cytoplasm. Upon cell stimulation including cytokines, pathogens and oxidative stress, I κ B is rapidly phosphorylated by inhibitor of κ B kinase (IKK) targeting it for ubiquitination and subsequent degradation by the 26S proteasome, releasing NF κ B to translocate to the nucleus and activate target genes⁽³⁸⁾. NF κ B is a key transcription factor involved in the transcription of a wide number of inflammatory mediators including TNF α , interleukins, adhesion molecules, matrix metalloproteinases and oxidative stress such as hydrogen peroxide, all of which are important in COPD pathogenesis^(1, 37). I κ B α levels are significantly decreased whilst NF κ B DNA binding significantly increased in healthy smokers and current smokers with moderate COPD compared to healthy non smokers⁽⁶⁾.

1.1.6.3.1.2 MAPK pathway

The Mitogen Activated Protein Kinase (MAP kinase) signal transduction pathways are activated in COPD and play a key role in its pathogenesis⁽³⁷⁾. These pathways are activated by different stimuli and can activate the same or different phosphorylation cascades regulating a diverse range of cellular events.

1.1.6.3.1.2.1 ERK

The classical MAP kinase pathway is the Ras / extracellular signal regulated kinase (ERK) pathway. This is typically activated by mitogenic stimuli such as growth factors. Binding leads to tyrosine kinase receptor dimerisation and autophosphorylation activating Guanosine nucleotide Exchange Factors (GEF's) activating the GTPase Ras recruiting and activating MAPKKK leading to phosphorylation of dual specificity MAPKK (MKKs or MEKs) which activate ERK1/2 activating transcription factors implicated MMP-1 release, MUC5AC production and growth factors eg epidermal growth factor (EGF) release^(39, 40). ERK1/2 is significantly elevated in airway and alveolar epithelial cells in patients with emphysema compared to controls⁽³⁹⁾.

1.1.6.3.1.2.2 p38 MAPK

The p38 MAPK family consists of four different isoforms expressed in different tissues, regulating and activating different kinases and phosphorylation cascades

causing diverse and opposing effects ⁽³⁷⁾. The α isoform is expressed in airway smooth muscle, epithelial cells and immune cells and studies on COPD have focused on this isoform ⁽³⁷⁾. p38 MAPK is activated in response to many inflammatory signals including inflammatory cytokines, smoking, infection and oxidative stress. p38 MAPK activation is key in COPD and is correlated with the degree of lung function impairment and inflammation ⁽³⁷⁾.

1.1.6.3.1.2.3 JNK

JNK is a stress-activated protein kinase that is activated by a wide range of cellular insults including ROS and inflammatory stimuli ⁽⁴¹⁾. JNK was first identified as the enzyme responsible for phosphorylation the N-terminus of c-Jun a component of the Activator Protein 1 (AP-1) transcription factor regulating expression of many physiological processes including cytokine and inflammatory gene expression ⁽⁴¹⁾. JNKs are protein kinases that phosphorylate target proteins such as transcription factors, adaptor proteins and cytoskeletal proteins. JNK1 and 2 are the relevant isoforms for respiratory disease ⁽⁴¹⁾. In a mouse model of chronic lung inflammation inhibition of JNK significantly inhibited TNF α , IL-4, IL-13 and CCL5 expression. The role of JNK in cell types central to respiratory disease is poorly understood ⁽⁴¹⁾. Activation of JNK has been shown to be involved in TNF α induced MMP-9 expression in lung epithelial cells ⁽⁴²⁾.

There is also crosstalk between MAPK pathways for example ERK1/2 and JNK have been shown to activate NF κ B signalling in airway epithelial cells ⁽³⁹⁾. As multiple kinases and signalling pathways can activate airway inflammation, blocking one kinase may just lead to increased activity of another ⁽³⁷⁾.

1.1.6.3.1.3 PI3K

PI3K Phosphatidylinositol 3-kinase (PI3K) is a family of proteins that catalyse the phosphorylation of phosphoinositides and generate lipids that control a wide variety of intracellular signalling pathways. They play a crucial role in the expression and activation of inflammatory mediators, inflammatory cell recruitment, airway remodelling and corticosteroid insensitivity ^(43, 44). PI3K is important for macrophage and neutrophil activation and is also involved in leukocyte migration ⁽⁴⁵⁾. It also

regulates MMP-9 expression ⁽⁴³⁾. Abnormal PI3K activation in bronchial epithelium of smokers is an early event in the development of lung cancer ⁽⁴⁴⁾.

1.1.6.3.1.4 Rho Kinase

Rho/Rho kinase signalling has emerged as a key regulatory pathway in several processes underlying airway inflammation in COPD including the trafficking of inflammatory cells and remodelling of the airway epithelium ^(46, 47). The Rho family of small GTPases are involved in regulating actin remodelling and adhesion important in inflammatory cell and airway epithelial cell migration ⁽⁴⁶⁾. The G protein RhoA is the main upstream activator of Rho kinases (ROCK) and is highly expressed in airway smooth muscle and can be activated by a variety of G protein coupled receptors (GPCR) through interaction with GEFs. In addition, cytokines and ECM proteins have emerged as inducers of the Rho/Rho kinase signalling pathway ⁽⁴⁸⁾.

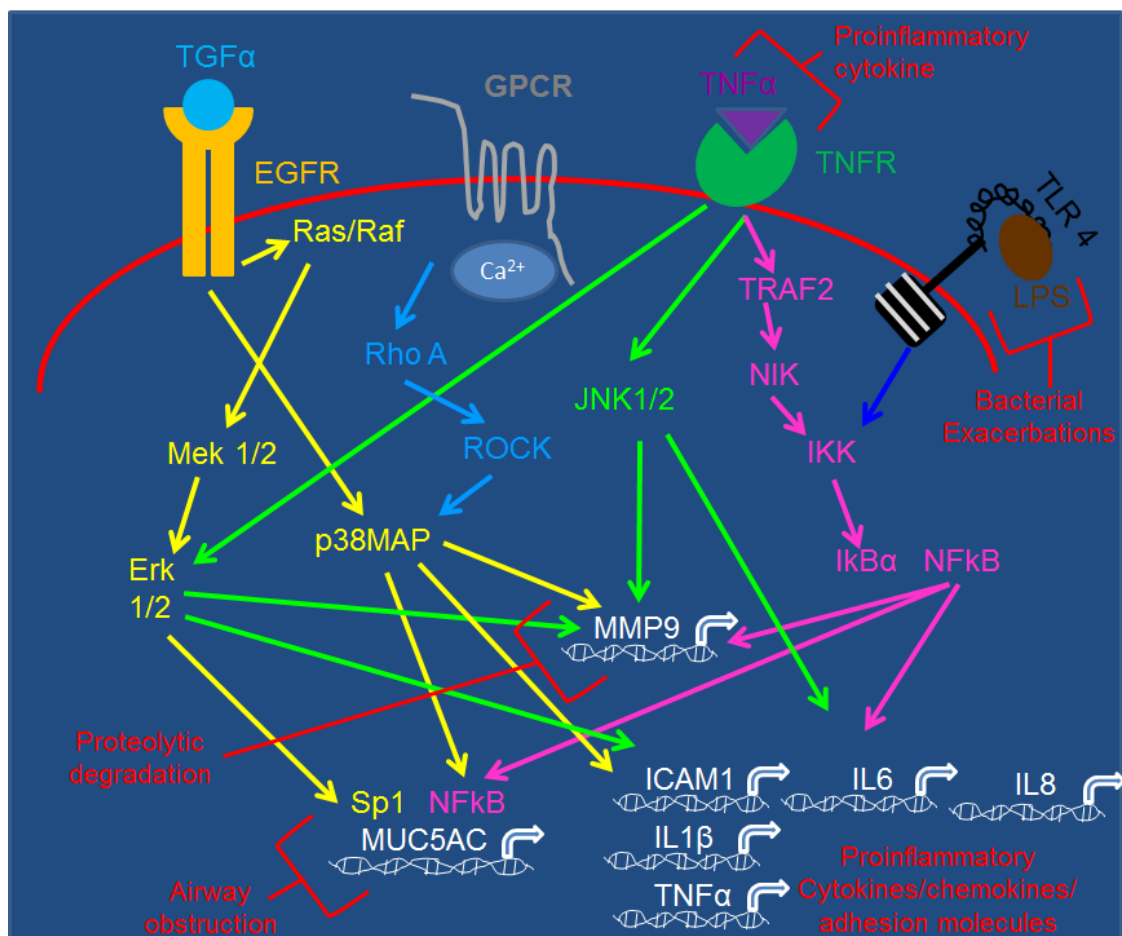


Figure 1.2 – Key signalling pathways activated by inflammation
Simplified diagram showing the main inflammatory signalling pathways activating expression of key mediators in COPD pathology.

1.1.6.4 Proteases

In healthy individuals there is a balance between proteases and their inhibitors important for airway remodelling. However, in COPD proteases are increased and their inhibitors are decreased potentiating the destructive processes within the lungs⁽⁴⁹⁾.

Anti alpha trypsin is an important anti-protease enzyme found in the serum which inhibits neutrophil elastase⁽⁴⁾. α 1 anti-trypsin deficiency is one of the genetic causes of COPD particularly in smokers, but only accounts for <1% of cases⁽²⁴⁾. Elastase breaks down extracellular matrix leading to lung damage and alveolar collapse and mice lacking this enzyme are protected from alveolar destruction induced from chronic cigarette smoke exposure⁽⁵⁰⁾. Proteinase 3 and Cathepsin G are other serine proteases with elastolytic activity found in neutrophils. Cathepsins B, K, L and S are cysteine proteases also with elastolytic activity and are elevated in lung macrophages in patients with COPD^(3, 24). Secretory leukocyte protease inhibitor (SLPI) is secreted by airway epithelial cells and is an important inhibitor of neutrophil elastase^(24, 51). Low concentrations of SLPI are associated with an increased risk of COPD exacerbations⁽⁵¹⁾.

Matrix metalloproteinases (MMPs) are a family of enzymes involved in the breakdown of a wide range of extracellular matrix components important for remodelling. This family of enzymes is produced by neutrophils, alveolar macrophages and airway epithelial cells^(3, 24). MMP-12, also termed macrophage metalloelastase, can degrade elastin and therefore has implications in alveolar destruction. Neutrophil elastase and MMP-12 can cleave receptors on macrophages that are important for the recognition and clearance of apoptotic cells⁽²³⁾. MMP-12 activity in patients with COPD has been directly associated with the extent of emphysema⁽⁵²⁾. Polymorphisms in MMP1 and MMP12 have been shown to reduce FEV1⁽⁴⁾ and patients with COPD have increased levels of neutrophil collagenase MMP-8 and gelatinase B MMP-9⁽⁴⁾. Patients with COPD also have increased levels of MMP-1 and MMP-9 in BAL fluid and BAL macrophages express more MMP-9 and MMP-1 than cells from control subjects⁽²⁴⁾. After smoking cessation for 3 months patients still had the presence of collagenase MMP-1 in the lungs suggesting that

cigarette smoke provides more long term changes in protease expression remaining long after smoking cessation ⁽⁵³⁾. Higher levels of MMP-9 are found in the serum of patients with COPD than control patients and it is associated with the stage of COPD and may play a role in systemic inflammatory responses ⁽⁵⁴⁾. MMP-9 and -12 are current research targets for inhibitors to use for COPD therapy ^(55, 56).

Natural inhibitors of MMPs within the body are tissue inhibitors of metalloproteinases (TIMPs). Polymorphisms in the TIMP2 gene are associated with COPD development ⁽⁴⁾. Both airway macrophages alone and those subjected to IL-1 β and LPS from smokers release more MMP-9 and TIMP-1 than age matched controls from non smokers ⁽⁵⁷⁾. During exacerbations of COPD, there is increased MMP-9 causing an imbalance between MMP-9/TIMP-1 in favour of destruction ⁽⁵⁸⁾.

1.1.6.5 Mucous hypersecretion

Mucous hypersecretion is important in COPD pathology and can be caused from smoking, infection and inflammatory cells activating mucin transcription ⁽³⁾. The mucous layer normally plays a protective role in the airways against inhaled pathogens, toxins and other foreign particles by mucociliary clearance ⁽⁵⁹⁾. However, abnormal mucous production and clearance contribute to COPD pathology with goblet cell hyperplasia and metaplasia. The major mucins of airway mucous secretions are MUC5AC and MUC5B, with MUC5AC being the secretion from the goblet cells on the epithelium and MUC5B from the submucosal glands ^(59, 60).

1.1.6.6 Oxidative Stress

Oxidative stress is thought to be one of the main driving forces behind COPD inflammation ⁽¹⁾. The lung epithelium is constantly exposed to oxidants generated endogenously during respiration and also from activated infiltrating inflammatory cells and air pollution ⁽⁵⁾. In addition, inhaled cigarette smoke contains reactive oxygen species (ROS), reactive nitrogen species (RNS), and nitric oxide (NO) ⁽⁶¹⁾. Oxidative stress occurs when the oxidant burden is unable to be neutralised by resident antioxidants. Generation of ROS are directly associated with oxidative modification of proteins, lipids, carbohydrates and DNA. Oxidative stress can cause cell damage, cell necrosis, apoptosis, autophagy, remodelling of ECM and blood

vessels, inactivation of anti-proteinases, premature senescence and elevated mucous secretion and therefore is pivotal in COPD pathology ⁽⁶²⁾. Both environmental or inflammatory cell derived ROS can influence inflammatory responses through activation and phosphorylation of the NFκB and the MAPK family, including ERK, JNK and p38 MAPK as well as PI3K ^(5, 63).

Both oxidative and nitrate stress can activate macrophages and macrophages from smokers produce more H₂O₂ than those from non smokers. Equally, smokers and COPD patients have higher H₂O₂ levels in exhaled breath condensates than control patients, which is even higher during COPD exacerbations ^(64, 65). H₂O₂ concentration in exhaled air is a marker of oxidative stress and can be used to monitor COPD exacerbations with it increasing with disease and decreasing with treatment ⁽⁶⁶⁾

There are two main classes of antioxidant systems employed by the respiratory tract to counteract oxidative stress; non enzymatic and enzymatic antioxidants. Non enzymatic antioxidants include small molecules such as vitamin C, vitamin E, β-carotene and thiol-containing compounds such as glutathione and thioredoxin. Enzymatic antioxidants include; glutathione peroxidase, superoxide dismutase, catalase, phase II detoxifying enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) which contribute to xenobiotic detoxification and stress response proteins such as haem-oxygenase-1 (HO-1) ^(64, 65). Phase II detoxifying enzymes contribute to recycling of thiols or facilitate excretion of reactive metabolites during xenobiotic detoxification ⁽⁶⁷⁾. Stress response proteins such as HO-1 and ferritin heavy and light chains are cytoprotective against various oxidant or pro-oxidant insults ⁽⁶⁷⁾. HO-1 contributes to the degradation of pro-oxidant haem molecule which also generates antioxidant products carbon monoxide and bilirubin with release of iron ⁽⁶⁷⁾. Ferritin is inducible by iron and is antioxidant by sequestering iron from participation in free radical formation ⁽⁶⁷⁾.

Most of the antioxidants and phase II cytoprotective genes are regulated by Nuclear factor erythroid 2 p45-related factor 2 (Nrf2) ^(5, 35). Nrf2 is a basic-leucine zipper transcription factor which plays a pivotal role in cellular defence against oxidative

stress⁽⁵⁾ and is mainly expressed in alveolar macrophages and epithelial cells within the lungs⁽⁶⁵⁾. In response to various cellular stressors, Nrf2 detaches from its cytosolic inhibitor kelch-like ECH-associated protein 1 (Keap1), and translocates into the nucleus binding with cofactors to the anti-oxidant response element (ARE) of genes leading to the induction of stress response genes⁽⁵⁾ including glutathione S-transferases, NQO1 and HO-1⁽⁶⁵⁾.

Deletion of Nrf2 in airway epithelium has been shown to exacerbate acute lung injury and impairs inflammation resolution⁽⁶⁸⁾. Nrf2 also plays a role in protection against elastase induced emphysema and pulmonary inflammation⁽⁶⁹⁾. Impaired Nrf2 signalling causes decline in proteasomal activity and increases endoplasmic reticulum stress response in lungs of COPD patients⁽⁷⁰⁾. In one study, Nrf-2 deficient mice exposed for 6 months to cigarette smoke developed airway destruction^(35, 71, 72). Levels of Nrf2 are decreased in lungs of patients with COPD and this results in enhanced susceptibility to cigarette smoke induced damaged and more pronounced oxidative stress in the lung⁽⁵⁾. However, in lung cancer uncontrolled Nrf2 activation provides a growth advantage and leads to chemoresistance of neoplastic cells⁽⁶⁷⁾.

Overall data indicates that Nrf2 exerts its protective effects on COPD phenotypes through transcriptional activation of anti-proteases as well as antioxidants in alveolar macrophages⁽⁶⁷⁾.

1.1.6.7 Epigenetics

Epigenetic changes are also important in COPD. DNA is wrapped around histones and these histones can undergo post translational modifications, most commonly via acetylation. Histone acetyl transferases catalyse the addition of acetyl groups to specific lysine residues on the tails of histones 3 and 4. The positive charge of the acetyl groups reduces the affinity of the DNA to the histones allowing the transcriptional machinery to access the DNA enabling transcriptional activation. Histone deacetylase (HDACs) catalyse the opposite reaction therefore promoting transcriptional repression. Cigarette smoke inhibits HDACs enabling transcriptional activation of inflammatory genes such as TNF α and IL-8. HDAC2 expression has

been found to be decreased in lung macrophages from patients with COPD ⁽¹⁾. Histone 4 acetylation at the NFκB binding site of IL-8 was increased in lung tissue from patients with COPD ⁽⁷³⁾ suggesting that epigenetic changes could be contributing to the increases in cytokine expression in COPD. They showed a positive correlation between HDAC activity and disease severity, and the clinical stage of COPD could be related to reduced HDAC activity therefore increased transcription of inflammatory genes ⁽⁷³⁾.

1.1.7 Current Therapies

Managing COPD is often complex due to its many systemic effects and co-morbidities. Treatment aims to manage symptoms and reduce the frequency of exacerbations which worsen the disease and often result in hospital admission, presenting a huge cost for healthcare providers. Smoking cessation is the only therapeutic intervention shown to reduce disease progression ⁽⁷⁴⁾. Pharmacological treatments include inhaled corticosteroids, bronchodilators and long term oxygen therapy and non pharmacological interventions include nutrition, exercise and lung volume reduction surgery ⁽⁷⁵⁾.

Bronchodilators are important for the treatment of COPD symptoms and are usually the initial choice of treatment ⁽⁷⁶⁾. They have been shown to increase the FEV1 in patients ⁽⁷⁵⁾ and to reduce airway inflammation *in vitro* ⁽⁷⁴⁾.

With inflammation being one of the major processes in COPD, many anti-inflammatory therapies have clinical benefit in controlling the disease symptoms, progression, and preventing exacerbations. Inhaled corticosteroids are extensively used as anti-inflammatory treatment alone or in combination with long-acting bronchodilator drugs ⁽⁷⁶⁾. Corticosteroids were a controversial treatment due to side effects and steroid resistance ⁽⁷⁷⁾, although are now a more accepted treatment shown to reduce the frequency of exacerbations in COPD and hospital admissions, which therefore helps to slow the progression of the disease ⁽⁷⁵⁾.

Long term oxygen therapy is the only treatment so far shown to improve decline in lung function associated with COPD ⁽⁷⁵⁾, although patient compliance is a problem.

COPD exacerbations are usually treated with a short course of oral corticosteroids or antibiotics ⁽⁷⁶⁾. Antibiotics are used for their anti-infective properties however; there is evidence to suggest they have little effect on airway and systemic inflammatory markers so other treatment is required to manage symptoms and inflammation ⁽⁷⁶⁾. Further studies are required to assess the effects of long-term antibiotics on exacerbation frequency with special attention to antibiotic resistance ⁽⁷⁶⁾ and new safer anti-inflammatory agents need to be developed for treatment of acute exacerbations ⁽⁷⁶⁾. A recent study has highlighted the heterogeneity of COPD exacerbations and defined independent subtypes of exacerbation with specific biomarkers, giving the potential for a more targeted and phenotype-specific management of COPD exacerbations in the future ⁽⁷⁸⁾.

1.1.8 Future therapies

New anti-inflammatory therapies aim to target specific inflammatory mediators. Phosphodiesterase 4 (PDE4) is expressed by neutrophils, CD8+ cells and macrophages and its inhibition suppresses inflammatory cell function ⁽⁷⁵⁾. However, inhibitors are associated with gastrointestinal side effects. There are a number of PDE4 inhibitors in clinical development for COPD reviewed in ⁽⁴³⁾. LTB4 is also a target for inhibition and antagonists are in early stage clinical trials however, the efficacy of such drugs is as yet unknown ⁽⁷⁵⁾.

Other potential therapeutics for COPD is the molecular targeting of specific inflammatory signalling pathways. p38 MAPK activity is increased in alveolar macrophages from patients with COPD. Selective inhibitors of p38 MAPK have been shown *in vitro* to inhibit IL-8 production and increase the suppressive effect of dexamethasone ⁽⁷⁹⁾. A study in COPD patients demonstrated p38 MAPK inhibitor SB-681323 decreased LPS stimulated TNF α in serum, and sputum neutrophils. However, there are multiple side effects reported ^(25, 74) and so p38 MAPK inhibitors that can be administered by inhalation are now being explored ^(37, 43, 74).

PI3K activity is significantly increased in peripheral blood monocytes from patients with COPD compared with control subjects and is associated with a decreased

sensitivity to corticosteroids ⁽⁷⁹⁾. Addition of a PI3K inhibitor restores sensitivity and a number of these inhibitors are under development which may be effective in steroid insensitive COPD ^(43, 79).

The NFκB pathway is a key target for anti-inflammatories and targeting this pathway for COPD treatment has been reviewed extensively ⁽⁷⁷⁾. Numerous IKK2 inhibitors are being evaluated as potential anti-inflammatories however, as yet there have been no clinical trials and animal data suggests that inhibition of this pathway may have multiple side effects ^(37, 74). HDACs also play a role in the transcriptional regulation of NFκB and represent another therapeutic target ⁽¹⁾.

Levels of TNFα in induced sputum correlate directly with pack-years of smoking and inversely with FEV1. Etanercept, infliximab and adalimumab antibodies have been developed clinically to inhibit TNFα in inflammatory diseases. Infliximab has been found to have little effect on inflammatory mediators or spirometry measurements in COPD patients. In addition, side effects of TNFα blockage include increased risk of infection ^(37, 74). Further studies are required to determine if TNFα antagonists are beneficial to all COPD patients or specific phenotypes of COPD patients ⁽⁴³⁾. Many other different inflammatory mediators have been targeted as potential therapies reviewed in ⁽⁸⁰⁾.

Proteases are also a therapeutic target. Inhibitors of neutrophil elastase have been studied now for many years but compounds failed in clinical development because of poor pharmacokinetics and low therapeutic index ⁽⁴³⁾ although this area is still being pursued. Equally α1 antitrypsin replacement therapy in patients with α1 antitrypsin deficiency related emphysema has not yet been proven effective ⁽⁴³⁾. MMP inhibitors are of interest in COPD therapy however, these inhibitors tend to have adverse side effects and further development is required for highly selective, oral MMP inhibitors with acceptable pharmacokinetics ⁽⁸¹⁾.

Many of the pathogenic mechanisms in COPD involve oxidative stress and hence this is a target for treatment by decreasing oxidant generation or enhancing antioxidants. Antioxidant therapy for COPD has been extensively reviewed recently

^(5, 62). Current agents are used symptomatically, mainly for mucolytic activity. However, other antioxidant therapies may be of therapeutic benefit either alone or in combination with other therapeutic agents and this requires further investigation with carefully designed clinical trials defining COPD phenotypes ⁽⁶²⁾.

Resistance to glucocorticoids is a common problem in the treatment of COPD and therefore restoring steroid sensitivity is a potential target in COPD ⁽⁷⁹⁾. Glucocorticoid suppression of inflammation requires recruitment of HDAC2 resulting in deacetylation of histones and decrease in inflammatory gene expression ⁽⁴³⁾. Oxidative stress reduces nuclear HDAC2 expression thereby contributing to the amplification of disease severity and corticosteroid resistance ⁽⁶⁵⁾. Activation of Nrf2 has been shown to reverse corticosteroid insensitivity in alveolar macrophages from COPD patients by dinitrosylating HDAC2 ⁽⁸²⁾. Nrf2 is also a novel therapeutic target in COPD with its ability to mitigate inflammation, improve antibacterial defence and restore corticosteroid responses ⁽⁸³⁾.

Many of these therapies are too toxic to be used systemically and therefore inhaled preparations are being investigated to reduce lung inflammation with low systemic exposure ⁽⁸⁴⁾. However, systemic inflammation is also important in COPD pathology and its co-morbidities and requires treatment but inhaled preparations may have less effect on systemic inflammation ⁽⁸⁴⁾. Investigation is required to look at if successful treatment of co-morbidities positively influences the course of the lung disease ⁽⁸⁴⁾.

All of these treatments only seem to target one aspect of COPD either specific molecules, symptoms or exacerbations even though COPD is a multifactorial disease. New therapies are required that are beneficial in many different aspects of the disease without having multiple side effects which outweigh their benefits. Vitamin D has this potential.

1.2 Vitamin D

Vitamin D is a steroid hormone thought to have first been produced over 200 million years ago in phytoplankton exposed to sunlight ⁽⁸⁵⁾. Conventionally vitamin D is known for its actions in bone mineralisation and calcium homeostasis although the role of vitamin D is a field which is expanding rapidly with deficiencies of vitamin D being a worldwide problem. Vitamin D deficiency is defined as being below 30 ng/ml of serum 25(OH)D (25-hydroxyvitamin D) the inactive form of the vitamin ⁽⁸⁶⁾. Deficiencies of vitamin D in children are associated with growth retardation and symptoms of rickets. In adults it can exacerbate osteoporosis increasing the risk of fractures ⁽⁸⁵⁾. There is now mounting evidence supporting the action of vitamin D in immunity and inflammation with deficiencies now also being associated with an increased risk of some cancers, autoimmune and infectious diseases ⁽⁸⁵⁾. The discovery of the vitamin D receptor expressed in many different cell types and organs around the body ignited further interest in this hormone ⁽⁸⁷⁾. Over the last 5-10 years there has been an exponential increase in the research on vitamin D for many diseases including different cancers, multiple sclerosis, diabetes, tuberculosis, asthma and heart disease (Figure 1.3).

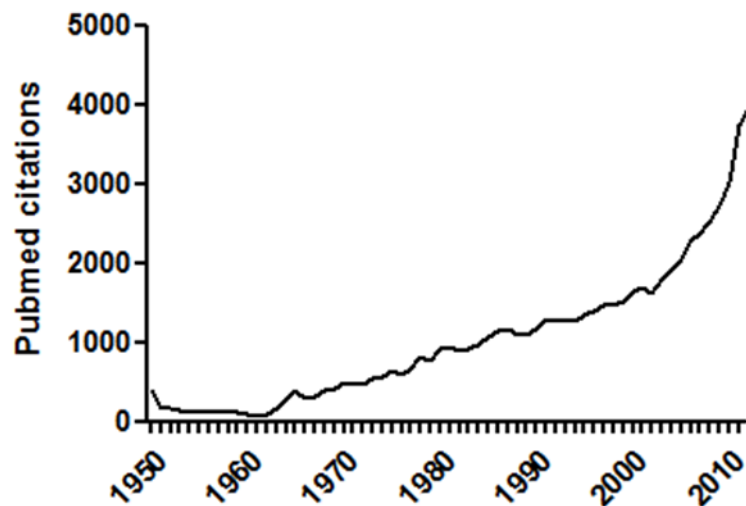


Figure 1.3 – Vitamin D citations by year
Graph showing the number of new publications for vitamin D on pubmed per year.

1.2.1 Sources and Metabolism

1, 25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) is the active form of vitamin D, which is mainly sourced from endogenous production within the skin but is also found in some dietary products. In the skin, UVB radiation on 7-dehydrocholesterol leads to the production of vitamin D metabolites ⁽⁸⁸⁾. Melanin impairs the ability of the solar radiation to produce vitamin D within the skin of those of Afro-Caribbean ethnicity so they have much lower circulating levels of 25 hydroxyvitamin D (25(OH)D) than those of white ethnicity ⁽⁸⁹⁾. Dietary sources of vitamin D metabolites include fortified dairy products, fatty fish and fish liver oils ⁽⁸⁸⁾.

The precursors of vitamin D from the skin and the gut are hydroxylated in the liver to form 25(OH)D through the action of mitochondrial cytochrome p450 enzymes CYP3A4 and CYP2R1 ⁽⁹⁰⁾. The active form of vitamin D is formed by further hydroxylation of 25(OH)D₃ to 1,25(OH)₂D₃ catalysed by 25-hydroxyvitamin D₃ 1-alpha hydroxylase (CYP27B1) usually within the kidneys. The main regulatory pathway modulating the expression of this enzyme is the parathyroid hormone which upregulates 1 alpha hydroxylase in calcium and vitamin D deficiency. More recently many different cell types have been shown to express CYP27B producing high local concentrations of vitamin D providing a role in autocrine and paracrine signalling ^(91, 92).

25(OH)D is the major circulating form of vitamin D and levels of this form are of clinical use being indicative of an individual's vitamin D status with serum concentrations of 30-50 ng/ml considered as sufficient ^(92, 93). Vitamin D circulates bound to vitamin D binding protein (DBP) also known as group-specific component (Gc) which is an α₂ macroglobulin belonging to the serum albumin superfamily. The major function of DBP is the binding, transport and solubilisation of vitamin D and its metabolites ⁽⁹⁴⁾, however it is a multifunctional protein also involved in macrophage activation and bone resorption. Large amounts of vitamin D in the circulation can saturate the vitamin D binding proteins leading to vitamin D toxicity and hypercalcemia with renal failure and hypertension due to the build up of calcium phosphate crystals in the kidney and vasculature.

Being lipophilic, $1,25(\text{OH})_2\text{D}_3$ is able to traverse the cell membrane and acts within the cell by binding to the nuclear vitamin D receptor (VDR). VDR's are ligand activated transcription factors that interact with vitamin D response elements (VDREs) on vitamin D regulated genes either as homodimers or as heterodimers with the retinoid x receptor ⁽⁹⁵⁾. Vitamin D regulates over 200 genes, these include genes for cellular proliferation, differentiation and apoptosis ⁽⁹⁶⁾. Another modulator of vitamin D is the mitochondrial cytochrome P450 24-hydroxylase (CYP24A1) which inactivates calcitriol into water soluble derivatives ⁽⁹¹⁾. Vitamin D binding to the VDR enhances CYP24A1 and downregulates CYP27B1 which is an important negative feedback mechanism regulating vitamin D levels ^(91, 92).

It has been shown that the VDR can elicit both genomic and rapid responses of vitamin D ⁽⁹⁷⁾ however there has also been speculation of a different membrane receptor for this ligand ⁽⁹⁰⁾. The 1,25-membrane associated rapid response to steroids binding (MARRS) is a newly described receptor isolated from chick intestinal basolateral membranes ⁽⁹⁸⁾. It has since been shown to be present in mammalian cells and demonstrates rapid responses to vitamin D ⁽⁹⁸⁾. It is identical to ERp57, GRp58, PDIA3 (endoplasmic reticulum protein of 57 kDa, glucose regulated protein of 58 kDa and Protein disulfide isomerase associated 3 respectively) ⁽⁹⁸⁾. It is a glycoprotein specific thiol oxidoreductase important in the endoplasmic reticulum for quality control of newly synthesised glycoproteins but has been most widely studied for its role in the immune system in the assembly of the heavy chain of the major histocompatibility complex (MHC) class I molecules ⁽⁹⁹⁾.

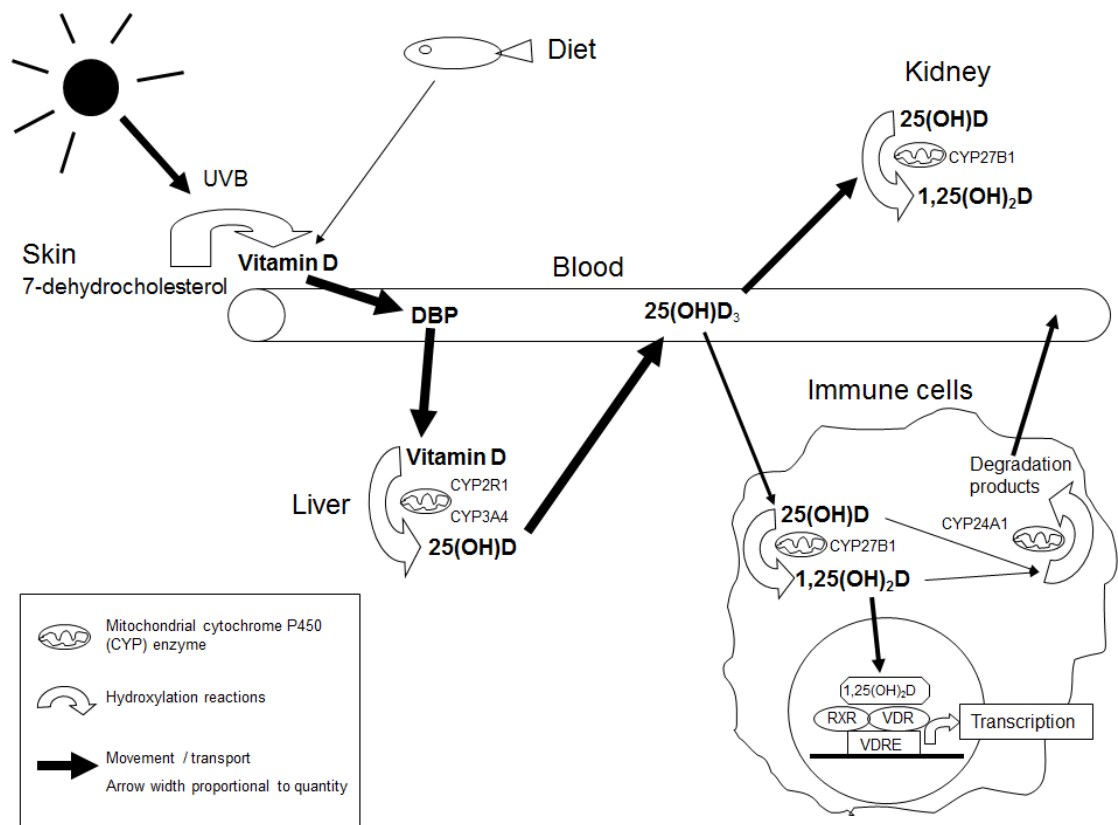


Figure 1.4 – Simplified version of vitamin D metabolism

1,25(OH)₂D metabolites can be sourced from the diet but the majority comes from the action of UVB on 7-dehydrocholesterol within the skin. Metabolites are transported around the body bound to the vitamin D binding protein (DBP), hydroxylated in the liver and then the kidneys to produce the active 1,25(OH)₂D form. Immune cells have also been shown to express the CYP27 enzymes required to activate 25(OH)D into 1,25(OH)₂D, which binds to vitamin D receptor, a ligand activated transcription factor and regulates the expression of genes containing vitamin D response elements (VDRE). 1,25(OH)₂D and 25(OH)D are degraded by CYP24 enzymes.

1.2.2 Immunomodulation

The discovery of the expression of the VDR and hydroxylase enzymes by immune cells led to a surge of research into the area. It is now apparent that circulating 1,25(OH)₂D₃ is not the likely source of vitamin D activity as levels are only around 0.1 nmol/L whereas *in vitro* studies suggest levels of 1-100 nmol/L are required for its modulatory activity⁽¹⁰⁰⁾. It is likely that high levels of circulating 25(OH)D are converted locally within the cellular microenvironment by the expression of CYP enzymes found in the mitochondria of macrophages and dendritic cells⁽¹⁰¹⁾. Upregulation of CYP27B1 and the VDR enables the activated macrophage to use 25(OH)D as a substrate for production of the active form after internalisation of the vitamin D binding protein (DBP) attached to 25(OH)D from the extracellular fluid. VDR is constitutively expressed on APC's and dendritic cells suggesting a role in antigen presentation⁽⁸⁶⁾, and also by macrophages and activated lymphocytes⁽⁸⁸⁾. Antigen-presenting cells, essential for the initiation and maintenance of cell-mediated immune responses, can be inhibited directly by vitamin D. The expression

of MHC class II and co-stimulatory receptors is inhibited, as is the maturation of dendritic cells ⁽¹⁰²⁾. The inflammatory stimuli interferon gamma (IFN- γ) and LPS have been shown to upregulate hydroxylase expression in macrophages ⁽⁹²⁾. Vitamin D degrading enzyme is also expressed by monocytes and macrophages inducible by vitamin D providing a negative feedback loop ⁽⁸⁸⁾.

A well studied area of vitamin D is its effects on T cell responses where it targets transcription of T helper 1 (Th1) cytokines such as IL-2, granulocyte macrophage colony stimulating factor (GM-CSF) and IFN thereby shifting the response from Th1 to Th2 inducing immune tolerance. IL-12 the main controller of Th1 vs Th2 response, is inhibited by vitamin D and this therefore leads to an inhibition of Th1 and activation of Th2 ⁽⁸⁸⁾. Supporting this vitamin D induces the expression of Th2 cytokines IL-4, 5 and 10 ⁽⁸⁸⁾. Conversely, vitamin D insufficiency deregulates the balance between type 1 and 2 immunity leading to over expression of Th1 cytokines. By blocking these it prevents further antigen presentation and recruitment of T lymphocytes. Vitamin D has potent antiproliferative effects on T cells, principally that of the Th subgroup, and suppresses B cell antibody production both directly and indirectly *in vitro* ^(86, 100).

Perhaps one of the most important modulatory actions of 1,25(OH)₂D₃ is its effect on regulatory T cells (Tregs) which prevent the activation of peripheral autoreactive T cells. In the absence of 1,25(OH)₂D₃ the numbers and functions of Tregs are reduced, potentially contributing to the development of autoimmune diseases, such as multiple sclerosis and type 1 diabetes, where low vitamin D status is associated with an increased risk of developing these disorders ^(103, 104). The role of vitamin D in autoimmune diseases has been reviewed recently ⁽¹⁰⁵⁾.

Vitamin D also modulates the expression of a wide range of cytokines including IL-1 β , IL-6, IL-8, IL-10, IL-12 and TNF α ⁽¹⁰⁶⁻¹⁰⁸⁾.

There is an expanse of literature on vitamin D modulating the key inflammatory transcription factor NF κ B, with vitamin D inhibiting NF κ B activity ^(36, 108, 109), increasing I κ B α expression ⁽¹¹⁰⁾, increasing I κ B α mRNA stability and decreasing I κ B α

phosphorylation⁽¹¹¹⁾. Vitamin D also activates NFκB activity increasing IKK^(112, 113). So vitamin D has been shown to both inhibit and activate NFκB activity which is important in different cell types for it to modulate immune responses aiding the clearance of pathogens by increasing the expression of anti-microbial peptides, but limiting local tissue damage by decreasing pro-inflammatory cytokine production⁽¹¹⁴⁾. NFκB has been shown to inhibit VDR function and integrate into the VDR transcriptional complex⁽¹¹⁵⁾. On a transcriptional level, NFκB downregulates 1α hydroxylase activity during chronic inflammation⁽⁹⁰⁾, suggesting that it acts to prevent vitamin D activation locally so that it is unable to act in an immunomodulatory capacity.

Other pathways modulated by vitamin D include p38 MAPK⁽¹¹⁶⁾, PI3K⁽¹¹⁷⁾, JNK⁽¹¹⁸⁾, ERK⁽¹¹⁹⁾, Rho kinase⁽¹²⁰⁻¹²²⁾.

1.3 Vitamin D in lung infection and disease

In addition to immune cells, respiratory epithelial cells can also constitutively convert inactive 25(OH)D to 1,25(OH)₂D₃, enabling high local concentrations of active vitamin D to increase the expression of vitamin D-regulated genes in the lung with important innate immune functions⁽¹²³⁾. Black et al (2005) found a dose responsive relationship between serum concentrations of 25(OH)D and FEV1. Vitamin D deficiency has also been shown to increase the risk of upper respiratory tract infections, tuberculosis and to decrease the FEV1 in asthma and respiratory diseases⁽⁸⁵⁾. Low serum levels of 25(OH)D in lung transplant recipients have been associated with increased incidence of acute rejection and infection.⁽¹²⁴⁾

A large cross-sectional study has shown that vitamin D intakes and higher serum levels are associated with improved lung function in adults⁽¹²⁵⁾ and similar findings have been reported in adolescents⁽¹²⁶⁾. Many recent literature reviews have highlighted the potential of vitamin D in lung diseases including asthma, cystic fibrosis, tuberculosis and respiratory tract infections which have led the way to investigations into lung cancer and COPD⁽¹²⁷⁾.

1.3.1 Tuberculosis

Exposure to sunlight has been known for more than 100 years to help with the treatment of tuberculosis ⁽⁸⁵⁾, although the first indicator of vitamin D having anti-microbial activity against *Mycobacterium tuberculosis* was from studies in the 1980s, where adding vitamin D to monocytes and macrophages infected with *M. tuberculosis* showed that the bacterial load was reduced ⁽¹²⁸⁾. More than 60 years ago clinical studies were carried out administering oral vitamin D as a treatment for mycobacterial infections with high success rates ⁽¹²⁹⁾. A recent meta-analysis found that low serum 25(OH)D levels are associated with higher risk of active tuberculosis ⁽¹³⁰⁾, and several studies have associated low serum 25(OH)D levels with increased susceptibility to tuberculosis and disease progression ⁽¹²⁸⁾.

Tuberculosis patients administered vitamin D or placebo following the sixth week of standard tuberculosis treatment had higher sputum conversion and radiological improvement (100%) compared to a placebo group (76.7%) ⁽¹³¹⁾. Addition of 1,25(OH)₂D₃ to primary human macrophages infected with virulent *M. tuberculosis* reduced the number of viable bacilli ⁽¹³²⁾. Adding a single oral dose (2.5 mg) of vitamin D to the treatment regimen of patients with tuberculosis enhanced significantly the ability of the participants' whole blood to restrict growth of mycobacteria *in vitro* without affecting antigen-stimulated IFN- γ responses ⁽¹³³⁾. A recent randomised controlled trial of adjunctive vitamin D in adults with sputum smear-positive pulmonary tuberculosis showed that vitamin D significantly hastened sputum culture conversion but only in a specific subset of the study population with the tt genotype of Taq1 VDR polymorphism ⁽¹³⁴⁾.

The immune system is able to detect invading pathogens such as *M. tuberculosis* via pathogen-associated molecular patterns (PAMPs); structural proteins expressed by the pathogen which are detected by Toll-like receptors (TLRs) in the host. PAMPs shed from *M. tuberculosis* interact with the TLR2/1 dimer on macrophages, resulting in the up-regulation of both CYP27B1 and VDR ^(96, 135). It has been shown recently that IL-15 is responsible for the induction of CYP27B1, leading to bioconversion of 25(OH)D to 1,25(OH)₂D₃, VDR activation and induction of cathelicidin ⁽¹³⁶⁾. The cathelicidin gene encodes an anti-microbial peptide, LL-37, and this gene, in humans (but not in mice), contains a vitamin D response element.

Therefore binding of vitamin D leads to LL-37-mediated killing of *M. tuberculosis* ⁽¹²⁹⁾. The cathelicidin gene is expressed in respiratory epithelial cells ⁽¹²³⁾ and vitamin D induction of cathelicidin has been shown in a number of cell lines including bronchial epithelial cells ⁽¹³⁷⁾. $1,25(\text{OH})_2\text{D}_3$ modulates the balance in cytokine production towards an anti-inflammatory profile by repression of TLR2, TLR4 and by increasing cathelicidin ⁽¹³⁸⁾. $1,25(\text{OH})_2\text{D}_3$ modulates *M. tuberculosis* induced cytokines in peripheral blood mononuclear cells (PBMC) by dose dependently suppressing IL12p40 and IFN γ ⁽¹³⁹⁾. It also attenuates *M. tuberculosis* induced MMP-7 and MMP-10 expression, and inhibited MMP-9 expression, secretion and activity irrespective of infection in PBMC ⁽¹⁴⁰⁾. In addition, $1,25(\text{OH})_2\text{D}_3$ increases TIMP1 expression in PBMC from patients with tuberculosis compared to healthy controls ⁽¹⁴¹⁾.

1.3.2 Influenza and the common cold

Influenza A virus causes severe epidemics of respiratory illness in humans and is transmitted through airborne droplets and by direct contact. It is characterized by acute neutrophil infiltration and narrowing of the bronchioles ⁽¹⁴²⁾. Controversy remains concerning whether there is a direct link between the seasonality of influenza and vitamin D deficiency, which is also observed more commonly in winter ⁽⁸⁹⁾. Influenza infection involves both innate and adaptive arms of the immune system. Although vitamin D can inhibit pro-inflammatory cytokine release by macrophages, its ability to up-regulate the expression of anti-microbial peptides is relevant, as these peptides can also exhibit anti-viral activity. In addition, viral infection increases activation of vitamin D and increases cathelicidin production further ⁽¹²³⁾. Not only do immune cells secrete these anti-microbial peptides, but epithelial cells present in the upper and lower airways can also secrete them as a host defence mechanism against infection ⁽⁸⁹⁾. Upper respiratory tract infections (URTI), or 'common colds', are the most widespread of infectious diseases, with more than 200 viruses contributing to the clinical symptoms. Early epidemiological studies found a strong association between rickets and RTI ⁽¹⁴³⁾, and a recent large cross-sectional study of the US population reported that vitamin D status is associated inversely with recent URTI and that the association may be stronger in those with respiratory diseases, such as asthma ⁽¹⁴⁴⁾. A recent study of young

Finnish men serving on a military base observed an association between low vitamin D status and days of absence from duty due to physician-diagnosed acute respiratory tract infections ⁽⁹³⁾. In addition, a recent double blind randomised control trial evaluated vitamin D supplementation on the incidence of seasonal influenza A in schoolchildren. Children supplemented with vitamin D had a reduction in influenza A incidence and this was more prominent in those who had not taking other vitamin D supplements. This suggests vitamin D supplementation of children during winter may reduce the incidence of this infection ⁽¹⁴⁵⁾. Further randomized controlled trials are needed to examine the direct effect of vitamin D supplementation and to establish the optimal serum levels of 25(OH)D to aid prevention of RTI.

1.3.3 Cystic fibrosis

Cystic fibrosis is a hereditary disease which not only causes mucous hypersecretion within the lungs and resulting airway obstruction and inflammation, but also affects other systems including pancreatic secretions. Due to the increase in mucous, patients are prone to frequent infections and reduced pancreatic secretions result in patients having problems with malabsorption of fat-soluble vitamins such as vitamin D. Several studies have shown that patients with cystic fibrosis have reduced circulating levels of 25(OH)D despite supplementation ⁽¹⁴⁶⁾, suggesting that either higher than normal levels of supplementation are needed to increase serum concentrations or that there is insufficient conversion of the dietary vitamin D to 25(OH)D. It has been reported recently that portable tanning devices can improve the vitamin D status of patients with cystic fibrosis (CF) during the winter months ⁽¹⁴⁷⁾ but caution is required to avoid overexposure to UVB radiation. DBP has also been associated with cystic fibrosis with a healthy level in the circulation defined as 300–600 mg/l, and low concentrations have been reported in acute respiratory distress syndrome and in the sera of patients with CF ^(94, 148). There is also a higher proportion of low bone density and osteoporosis within this population group. In the previously mentioned study showing that cathelicidin can be induced by vitamin D in primary bronchial epithelial cells, cells from patients with CF also showed increased cathelicidin expression ⁽¹⁴⁹⁾, suggesting that vitamin D can augment anti-bacterial activity in airway epithelia in cystic fibrosis.

A recent cellular study using cells from patients with cystic fibrosis showed that treatment with vitamin D prior to exposure to *P. aeruginosa* LPS and conditioned media produced lower levels of IL-6 and IL-8 expression with a decrease in I κ B α phosphorylation, increased total I κ B α and increase in cathelicidin mRNA and protein ⁽¹⁵⁰⁾. This may provide a novel therapy for prevention and treatment of airway infections in this disorder.

1.3.4 Asthma

Asthma and atopic diseases are characterized by inflammatory responses initiated and sustained by inappropriate Th lymphocyte responses of the Th2 phenotype. Direct evidence for a role of vitamin D in asthma comes from studies showing that VDR variants are a risk factor for asthma ⁽¹⁵¹⁾. An inverse association between maternal intakes of vitamin D during pregnancy and early childhood wheezing has been reported in studies from the United States ⁽¹⁵²⁾ and the United Kingdom ⁽¹⁵³⁾. As stated above, vitamin D is associated with skewing the immune response to a Th2 phenotype, leaving the possibility that vitamin D may potentiate Th2 responses in adult asthmatic patients and clinical trials are required to address this concern. On the other hand, the potential for vitamin D to increase pulmonary defence against respiratory infections may, in the same way as in COPD, reduce the triggering of asthma exacerbations caused by RTI ⁽¹⁵⁴⁾. This is supported by the previously mentioned particularly strong negative association between vitamin D status and URTI in individuals with asthma in the Third National Health and Nutrition Examination Survey (NHANES III) study ⁽¹⁴⁴⁾.

Glucocorticoids are the most effective anti-inflammatory treatments available for many immune diseases, including asthma. However, glucocorticoid resistance or insensitivity in some patients with asthma represents an important barrier to effective treatment and accounts for significant health-care costs ⁽¹⁵⁵⁾. Recently, some evidence has emerged that administration of vitamin D to glucocorticoid-resistant asthmatic patients can enhance subsequent responsiveness to dexamethasone by restoring the defective IL-10 response to glucocorticoids by CD4+ T cells in these individuals ⁽¹⁵⁶⁾. This finding provides encouragement to

undertake trials of vitamin D in overcoming glucocorticoid resistance in both asthma and a number of other inflammatory diseases ⁽¹⁵⁵⁾.

In addition, in asthma there is a degree of airway remodelling characterised by the metaplasia of mucous glands, basement membrane thickening, increased ECM deposition, subepithelial fibrosis and smooth muscle hypertrophy/hyperplasia ^(157, 158). 'A disintegrin and metalloproteinase-33' (ADAM33) has been identified as a novel asthma susceptibility gene by genome-wide screening, and is now known to play an important role in airway remodelling. Its level of expression is associated with asthma development and severity and it declines with therapeutic interventions ⁽¹⁵¹⁾. *In vitro* studies have shown that 1,25(OH)₂D₃ has a direct anti-proliferative effect on human airway smooth muscle cells and can inhibit the expression of ADAM33 ⁽¹⁵¹⁾, suggesting a further beneficial role for vitamin D in the prevention and treatment of asthma.

1.3.5 Lung cancer

Lung cancer is the leading cause of mortality from cancer worldwide (WHO), and is responsible for over twice the number of deaths than any other cancer. It has a poor prognosis, with only a 10% survival rate at 5 years ⁽¹⁵⁹⁾. COPD is the single most important risk factor for the development of lung cancer after smoking exposure ⁽¹⁶⁰⁾. Lung cancer is thought to be driven by DNA damage caused by excessive inflammation and lack of repair processes within the lung, resulting in genomic instability ⁽¹⁶⁰⁻¹⁶²⁾. Around 50-70% of patients with lung cancer suffer from COPD: however, a causal relationship between the two has not yet been established ⁽¹⁶³⁾.

The effects of vitamin D in lung cancer have been reviewed extensively ⁽¹⁶⁴⁾. 1,25(OH)₂D₃ and analogues significantly inhibit cell proliferation in a number of lung cancer cell lines ⁽¹⁶⁵⁻¹⁶⁷⁾. In addition, 1,25(OH)₂D₃ has also been shown to reduce tumour volume, metastasis and angiogenesis in different animal models of lung cancer ⁽¹⁶⁸⁻¹⁷³⁾.

In the UK there is a significant gradient in UVB exposure from north to south and a better lung cancer survival rate in patients with higher exposure ^(174, 175). However,

there is no clear finding from association studies with increased circulating 25(OH)D associated with improved survival in lung cancer patients ⁽¹⁷⁶⁾ and no association between 25(OH)D status and total or lung cancer mortality in 16,818 subjects from the third National Health and Nutrition Examination Survey ⁽¹⁷⁷⁾.

Vitamin D has many antitumorigenic properties, and some types of lung cancer have developed resistance to vitamin D by modulating expression of CYP27B1, CYP24A1 and the VDR. In contrast to normal airway epithelial cells, some lung cancer cell lines have been shown to express very low CYP27B1 or not at all ^(123, 178). Several studies have demonstrated that CYP24A1 is overexpressed and VDR reduced in lung tumor tissue compared with normal tissue ^(179, 180). Also higher VDR expression has been shown to correlate with longer survival in lung adenocarcinoma ⁽¹⁸¹⁾. Serum DBP levels are a significant independent factor associated with better cancer outcome in operated lung cancer patients ⁽¹⁸²⁾.

1.3.6 COPD

In comparison to research in other lung diseases, vitamin D in COPD is under-researched. Most of the studies on vitamin D and COPD are suggestive with vitamin D found to modulate mediators important in COPD pathology in other disease types.

A randomised single centre double blind placebo controlled trial of 182 patients with moderate to very severe COPD giving high dose vitamin D looked at the time to first exacerbation as a primary outcome. There was no reduction in incidence of exacerbations on vitamin D treatment, although a small subset of participants with severe vitamin D deficiency at baseline had a significant reduction in exacerbations. This does suggest that monitoring vitamin D deficiency in this population is very important and also that perhaps patients with severe COPD taking maximum tolerated doses of other medication are no longer responsive to vitamin D treatment and that vitamin D supplementation needs to be commenced earlier in the disease ⁽¹⁸³⁾. Lower serum levels of 25(OH)D concentrations are significantly associated with an increased risk of COPD ⁽¹⁸⁴⁾

DBP is a highly polymorphic gene and it has been demonstrated that polymorphisms in DBP are significantly correlated with susceptibility and severity of COPD⁽⁹⁴⁾. DBP has also been reported to be involved in the chemotactic response of complement attracting neutrophils to the site of inflammation, an important step in the pathology of COPD⁽⁹⁴⁾.

Vitamin D plays a role in influencing skeletal muscle function, with deficiency resulting in muscle weakness, and VDRs are present in this tissue⁽¹⁸⁵⁾. Polymorphisms in the VDR can influence muscle weakness⁽¹⁸⁶⁾, therefore vitamin D may not only be beneficial in the respiratory pathology but also the systemic symptoms of COPD.

Franco et al (2009) found that 51% of the COPD patients that they tested had osteoporosis and a low vitamin D status, and that the prevalence of osteoporosis was correlated with the severity of COPD⁽¹⁸⁷⁾. This suggests that vitamin D may be beneficial in patients with COPD and so the future may involve vitamin D supplementation in order to test this⁽¹⁸⁸⁾. In one study the prevalence of osteoporosis in COPD patients increased over a 3 year period and risk factors for the development of osteoporosis included vitamin D deficiency⁽¹⁸⁹⁾.

On a cellular and molecular level, $1,25(\text{OH})_2\text{D}_3$ has a number of effects that may be relevant to COPD such as inhibiting the formation of MMP's as well as influencing collagen synthesis suggesting it could influence tissue remodelling and alveolar destruction. *Song et al (2007)* have shown that $1,25(\text{OH})_2\text{D}_3$ has a direct antiproliferative effect on human airway smooth muscle cells and an inhibitory effect on MMP-9 mRNA and protein levels suggesting a role in preventing airway remodelling⁽¹⁹⁰⁾. Vitamin D modulates airway smooth muscle function in COPD which has been reviewed⁽¹⁹¹⁾.

Vitamin D also induces the expression of the anti-microbial peptide cathelicidin in both alveolar macrophages and airway epithelial cells which may be important in the innate response to infection in exacerbations of COPD^(137, 149). Recent research has shown that respiratory epithelial cells constitutively activate vitamin D enabling

high local concentrations of active vitamin D to develop suggesting that vitamin D is required by these cells for certain functions ⁽¹²³⁾. As in asthma, steroid resistance is also prominent in COPD and therefore the potential of vitamin D to restore steroid sensitivity would also be beneficial ⁽¹⁹²⁾.

As vitamin D has been shown to have numerous immunomodulatory actions which may make it beneficial in the inflammation of COPD, and respiratory epithelial cells have been shown to constitutively activate vitamin D and are an important source of inflammatory mediators and proteases involved in the pathology of COPD, vitamin D may be able to beneficially modulate inflammation, protease destruction and oxidative stress in airway epithelial cells from patients with COPD.

There is therefore broader potential for vitamin D in prevention and treatment of this disease.

1.4 Aims

The hypothesis is that vitamin D will beneficially modulate the expression of inflammatory mediators and also oxidative stress, both important in COPD pathology, in human airway epithelial cells.

Therefore the aims of this study were:

- To characterise the expression of inflammatory mediators important in COPD pathology in human airway epithelial cells and find a suitable model cell line of primary small airway epithelial cells.
- To investigate the effects of vitamin D on the expression of inflammatory mediators important in COPD pathology in primary airway epithelial cells and lung epithelial cell lines both basally and in response to inflammatory stimuli.
- To examine the effects of vitamin D on oxidative stress in primary airway epithelial cells and cell lines.
- To determine the mechanism of action of vitamin D in airway epithelial cells.

Chapter 2:

Material and Methods

2.1 Materials

2.1.1 General Materials

Recombinant Human TNF α was purchased from R&D Systems (Abingdon, UK), LPS (*E.coli* 0111:B4), 1,25(OH) $_2$ D $_3$, LY294002, SB203580, PD98059 and MG132 were purchased from Calbiochem (Merck Chemicals Ltd, Nottingham, UK). LPS (*Pseudomonas aeruginosa* serotype 10) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (UK, Poole). Y27632 and SP600125 were purchased from Tocris Bioscience (Bristol, UK).

All general laboratory agents and consumables were purchased from Thermo Fisher Scientific (Loughborough, UK) unless otherwise stated.

2.1.2 Cell Culture

2.1.2.1 Consumables

T25 and T75 tissue culture flasks, cryovials and stripettes were purchased from NUNC. 0.45 μ M filters and syringes were purchased from Sigma Aldrich, 15 and 50 mL falcon tubes were purchased from BD Biosciences (Oxford, UK). Eppendorfs and pipette tips were purchased from Starlabs (Milton Keynes, UK).

2.1.2.2 Cell Culture Reagents

Foetal calf serum (FCS) was purchased from Biosera (East Sussex, UK). Trypan blue 0.4% and bovine serum albumin (BSA) were purchased from Sigma Aldrich. RPMI 1640 media, RPMI 1640 phenol red free media, MEM, 10,000 U/mL penicillin, 10,000 μ g/mL streptomycin, 200 mM L-glutamine, 0.25% trypsin-EDTA and LHC basal medium 1x liquid were purchased from Invitrogen (Paisley, UK). SAGM and SABM, HBSS and TE for primary cells were purchased from Lonza (Slough, UK). Vitrogen and human fibronectin were purchased from BD Biosciences.

2.1.3 Nucleic acid isolation

Tri-reagent solution was purchased from Applied Biosystems (Warrington, UK), 1-bromo-3-chloro propane (BCP), absolute ethanol, and 2-propanol were purchased from Sigma Aldrich.

Cells to cDNA lysis buffer, DNase I and 10 x DNase I buffer were obtained from Applied Biosystems. RNase inhibitor was purchased from Promega (Southampton, UK). Mouse moloney murine leukaemia virus reverse transcriptase , 5 x forward strand buffer, 0.1 M DTT, and random primers were procured from Invitrogen and dNTP mix was purchased from Bioline (London, UK).

2.1.4 qRT-PCR

SYBR green jumpstart readymix was purchased from Sigma Aldrich, TaqMan reverse transcription reagents from Applied Biosystems, specific primer sequences were purchased from Invitrogen. Ready-made primers, 0.1 and 0.2 mL PCR tubes were purchased from Qiagen (Crawley, UK).

2.1.5 Protein Detection

2.1.5.1 Western Blotting

NuPAGE LDS sample buffer (4x), NuPAGE reducing agent (10x), 4-12% Bis-Tris gel, 20 x transfer buffer, 20 x MOPS SDS running buffer and NuPAGE antioxidant were purchased from Invitrogen. Broad range molecular weight marker was purchased from BIO-RAD (Hertfordshire, UK). Supersignal west dura ECL was purchased from Thermofisher Scientific and RapidStep ECL reagent was from Merck Millipore (Nottingham, UK).

Rabbit monoclonal anti-Nrf2 antibody was procured from Abcam (Cambridge, UK). Goat anti-rabbit HRP antibody was purchased from Santa Cruz. Monoclonal anti- β -actin antibody was purchased from Sigma Aldrich. Polyclonal goat anti-mouse HRP antibody was from Dako (Ely, UK). Re-blot Plus antibody stripping solution was purchased from Millipore.

2.1.5.2 Flow cytometric bead array

The flow cytometric bead array was purchased from BD Bioscience and contained: human IL-1 β , IL-6, IL-8, IL-10, IL-12p70, and TNF α capture beads, human inflammatory cytokine PE detection reagent, human inflammatory cytokine standards, cytometer setup beads, PE positive control detector, FITC positive control detector, wash buffer and assay diluent.

2.1.5.3 Gelatin Zymography

Tris base, HCl, NaCl, CaCl₂, Tween-20, SDS, ammonium persulfate, TEMED, methanol and glycine were purchased from Thermofisher Scientific. 30% acrylamide: 0.8% bis-acrylamide was purchased from BIO-RAD. Gelatin, Triton-X100, bromophenol blue, glycerol, coomassie brilliant blue and acetic acid were obtained from Sigma Aldrich. MMP9 control was from R&D systems.

2.1.6 Reactive oxygen species assay

2, 7'-dichlorodihydrofluoresceindiacetate (H₂DCFDA) was obtained from Invitrogen. 96 well clear bottom black sided fluorescence plates were purchased from Thermofisher Scientific.

2.1.7 Cytotoxicity/Proliferation Assay

Cell Titer 96 AQueous One Solution Reagent was purchased from Promega. Tert-Butyl hydroperoxide (tBHP) and hydrogen peroxide (H₂O₂) were obtained from Sigma Aldrich.

2.1.8 ELISA

96 well flat bottom Immuno Maxisorp ELISA plate were from Thermofisher Scientific, 0.05% Tween 20 and H₂SO₄ was obtained from Sigma Aldrich. Primary mouse anti-MUC5AC clone 45M1 antibody was from Invitrogen. Secondary goat anti-mouse HRP antibody was purchased from Dako. 3, 3', 5, 5' – tetramethylbenzidine peroxide solution (TMB substrate) was obtained from BD Biosciences.

2.1.9 Immunohistochemistry for MUC5AC expression

Microscope slides and glass cover slips were from Thermofisher Scientific. Porcine gelatine, paraformaldehyde, Triton X-100 and 4', 6-diamidino-2-phenylindole (DAPI) were from Sigma Aldrich. Mouse anti-human MUC5AC clone 45M1 antibody, goat anti-mouse alexofluor 488 nm antibody and goat serum were from Invitrogen. Goat IgG was purchased from Santa Cruz (Middlesex, UK). Fluoromount G was from Cambridge Biosciences (Cambridge, UK).

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Heat Inactivation of FCS

FCS was defrosted in a 37°C water bath and then placed at 57°C for 30 minutes to heat inactivate the serum before separating into 50 mL aliquots for storage at -20°C to avoid freeze-thaw cycles.

2.2.1.2 Preparation of cell culture media

All media supplements (and 0.25% Trypsin-EDTA) were defrosted in a 37°C water bath and then separated into 5 mL aliquots to store at -20°C. Before addition to media, all supplements including FCS were first filter sterilised using a 0.45 µM filter.

2.2.1.3 Cells

2.2.1.3.1 A549

A549 were purchased from the European Collection of Cell Cultures (ECACC) (Salisbury, UK) and are a human alveolar carcinoma cell line derived from a 58-year-old caucasian male ⁽¹⁹³⁾ (ECACC). They are used as an example of an alveolar type II cell line and were originally developed to study surfactant synthesis and secretion ⁽¹⁹³⁾. A549 were cultured in RPMI-1640 media containing 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 10% FCS. At 80-90% confluency, cells were passaged by firstly washing with phosphate buffered saline solution (PBS) followed by addition of 5 mL 0.25% trypsin-EDTA 1x solution for 30 seconds and then removed and the cells placed at 37°C for 3-5 minutes for cell detachment. Cells were resuspended in 5 mL fresh media before cell counting. Cells were maintained between 3×10^5 and 1×10^6 cells/mL in 75cm² flasks at 37°C and 5% CO₂ and were routinely passaged every 3-4 days between passage 90-120.

2.2.1.3.2 NCI-H292

NCI-H292 were purchased from ECACC and are derived from a cervical node metastasis of pulmonary mucoepidermoid carcinoma of a 32 year old female (ECACC). They were originally used for its ease of viral transfection ⁽¹⁹⁴⁾ though

there are many studies now on this cell line particularly with respect to mucin production.

NCI-H292 were cultured in the same media as A549 and using the same procedure. Cells were maintained between 3×10^5 and 1×10^6 cells/mL in 75 cm² flasks at 37°C and 5% CO₂ and were routinely passaged every 3-4 days between passage numbers 11-30.

2.2.1.3.3 16HBE14o-

16HBE14o- cells were obtained from Dr. Gruenert from the University of California, San Francisco USA. 16HBE14o- are derived from SV-40 transformation of cells obtained from human bronchial epithelium^(22, 195). 16HBE14o- were cultured in MEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 10% FCS. T75 flasks were pre-coated with 2 mL of fibronectin coating solution (see appendix) overnight and stored at room temperature before use. At 80-90% confluency, cells were passaged by firstly washing with hepes buffered saline (HBS) followed by addition of 5 mL 0.25% trypsin EDTA 1x solution for 30 seconds. Trypsin was then removed and the cells placed at 37°C for 3-5 minutes for cells to lift off the flask and then resuspended in 5 mL fresh media before cell counting. Cells were used between passage number 11-33, maintained at 37°C and 5% CO₂ and were routinely passaged every 3-4 days.

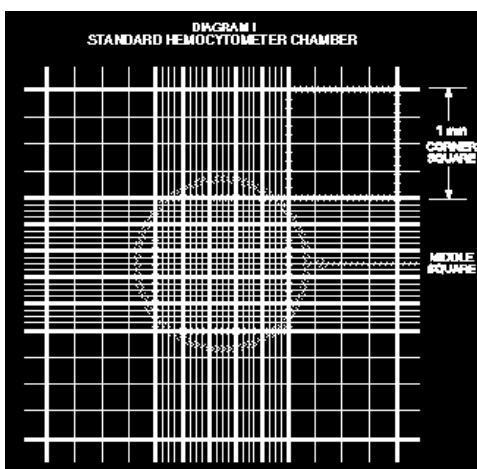
2.2.1.3.4 SAEC

Human small airway epithelial cells (SAEC) were purchased from Lonza and certificate of analysis stated they were derived from a 6 year old male, tissue acquisition number 14289. SAEC were cultured in small airway cell basal medium 500 mL (SABM) containing the small airway cell growth medium SingleQuot kit supplements and growth factors at the following volumes: bovine pituitary extract 2 mL, epinephrine 0.5 mL, gentamicin sulfate amphoterin-B 0.5 mL, triiodothyronine T3 0.5 mL, retinoic Acid 0.5 mL, bovine serum albumin (BSA) fatty acid free 5 mL, insulin 0.5 mL, human recombinant epidermal growth factor in buffered BSA solution 0.5 mL, transferrin 0.5 mL, and hydrocortisone 0.5 mL (Lonza).

At 80-90% confluency, SAEC were passaged by washing with 5 mL HBS followed by addition of primary cell TE and visualising the cells under the light microscope to determine when they are detached (around 2 minutes) and then trypsin neutralising solution was added immediately. Cells were maintained at 37°C and 5% CO₂ between 4 x 10⁵ – 1 x 10⁶ cells/mL and used between passages 1-3. Media was changed every other day.

2.2.1.4 Cell enumeration and viability

Epithelial cells were diluted 1/2 with 0.4% trypan blue solution and 10 µL was added onto a Neubauer haemocytometer (Thermofisher Scientific) and viewed with a light microscope. For cell enumeration, cells were counted in each of the four 1 mm corner squares illustrated in the diagram below (Sigma Aldrich). Cells on the outer edge were counted only on the top and left side lines and not the bottom and right. Each corner square of the haemocytometer represents 0.1 mm³ which is equal to 10⁻⁴ cm³. As 1 cm³ is equal to 1 mL the average cell count x dilution factor x 10⁴ gives the number of cells/mL.



Trypan blue dye only enters cells in which the membrane is not intact and therefore the cells are not viable. In order to calculate cell viability the number of blue stained cells is divided by the total number of cells and multiplied by 100 to represent a percentage.

2.2.1.5 Cryopreservation

Before freezing, cells were counted as above.

-2.2.1.5.1 A549 and NCI-H292

Cell freezing media was produced from making a solution of filter sterilised FCS with 10% DMSO. 2-5 x 10⁶ cells were put into a falcon tube and centrifuged at 2,000 rpm for 5 minutes to form a cell pellet. The supernatant was removed and the cell pellet was resuspended in 1 mL freshly made freezing media and transferred to a cryovial

which was placed on ice and frozen slowly in an insulated container. Cryovials were placed into -80°C for at least a day before transferring to liquid nitrogen.

-2.2.1.5.2 Cryopreservation of 16HBE14o-

The same procedure to that above was used however the cell freezing media consisted of 50% FCS, 40% MEM and 10% DMSO.

-2.2.1.5.3 Cryopreservation of SAEC

The same procedure to that above was used however the cell freezing media consisted of 80% SABM, 10% FCS and 10% DMSO.

2.2.1.6 Cell stimulations

Before addition of any stimulus, cells seeded in tissue culture plates were rested for at least 18 hours.

2.2.1.6.1 LPS

LPS was made up in RPMI 1640 media by vortexing for 3 minutes, heating at 37°C for 10 minutes, vortex for 3 minutes and split into aliquots stored at -20°C with the current vial stored at 4°C to avoid freeze thaw cycles, at a 1 or 5 mg/mL stock. LPS was further diluted in media to use at a final concentration of 10 µg/mL.

2.2.1.6.2 TNF α

Human recombinant TNF α was reconstituted in PBS at 10 µg/mL stock solution and aliquoted into eppendorfs stored at -80°C. TNF α was further diluted in media to a final concentration of 10 ng/mL in cell stimulations.

2.2.1.6.3 Vitamin D

1,25 dihydroxyvitamin D₃ (1,25(OH)₂ D₃) was reconstituted in DMSO and kept at a stock concentration of 1 x 10⁻⁴ M. Aliquots were made in amber screw cap glass vials and stored at -80°C. For use, 1,25(OH)₂ D₃ was further diluted in media to a final concentration of 1 x 10^{-7 to -10} M for experiments with a final concentration of DMSO ≤0.1% to ensure no adverse effects on the cells.

2.2.1.6.4 Inhibitors

LY294002, SB203580, PD98059, MG132, and SP600125 were reconstituted in DMSO at stock concentrations of 10-100 mM and split into aliquots protected from light

and stored in -20°C . Y27632 was reconstituted in PBS. Inhibitors were further diluted to a final concentration of $0.1\text{-}10\ \mu\text{M}$ in cell culture media.

2.2.1.6.5 tBHP and H_2O_2

Both tBHP and H_2O_2 were diluted in PBS from stock solutions to a concentration of $400\ \text{mM}$ and then further diluted in cell culture media for a final concentration of $100\text{-}400\ \mu\text{M}$.

2.2.2 Nucleic Acid Isolation

2.2.2.1 RNA extraction using Tri-reagent

Tri-Reagent solution was used to lyse and extract RNA from cell lines according to the manufacturer's instructions. The method is one of phase separation where the RNA separates into the aqueous phase on addition of BCP and then is precipitated out with 2-propanol and finally washed with ethanol.

Briefly, $1\ \text{mL/well}$ Tri-reagent was added to cells cultured in a 6 well plate and incubated for 5 minutes at room temperature before harvesting into eppendorfs and then stored at -80°C . Following thawing, $100\ \mu\text{L}$ BCP was added to each tube, shaken for 10 seconds and then incubated at room temperature for 10 minutes. Tubes were then centrifuged at $12,000\ \times\ g$ for 20 minutes at 4°C . The aqueous phase was transferred to a fresh tube and $500\ \mu\text{L}$ 2-Propanol was added to each tube, vortexed for 10 seconds then incubated at room temperature for 10 minutes. Tubes were then centrifuged at $12,000\ \times\ g$ for 15 minutes at 4°C and the supernatant was removed from the pellet. $1\ \text{mL}$ 70% ethanol was added to each tube and then centrifuged at $12,000\ \times\ g$ for 10 minutes. The ethanol was removed and the pellet was allowed to air dry for 10 minutes. Isolated RNA was solubilised in $20\text{-}100\ \mu\text{L}$ nuclease free water and stored at -80°C for at least one night before RNA quantification.

2.2.2.2 RNA quantification

UV spectroscopy can be used to quantify the concentration of RNA within a given sample. The Nanodrop 1000 spectrophotometer from Thermofisher Scientific enables quantification of RNA within just $1\ \mu\text{L}$ of sample from and therefore provides a quick and easy method of quantification.

The concentration can be calculated using the Beer Lambert law:

$$A = \epsilon cl$$

A = absorbance in arbitrary units

ϵ = extinction coefficient

c = concentration (M)

l = path length in cm

It is known that the extinction coefficient of RNA is $0.025 \text{ (mg/mL)}^{-1} \text{ cm}^{-1}$ (Ambion) and that an absorbance reading of 1 at 260 nm is $\sim 40 \text{ }\mu\text{g/mL}$ single stranded RNA therefore the software is able to calculate the quantity of RNA within a given sample.

Absorbance readings at 230, 260 and 280 nm are taken and the ratio of 260/280 and 260/230 are an indicator of RNA purity with values 1.8-2.0 considered as purified RNA.

2.2.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

2.2.3.1 cDNA synthesis from RNA extracts

50-100 ng/ μL RNA produced from Tri-reagent extraction was used to synthesise cDNA using Taqman Reverse Transcriptase Reagents as per manufacturer's instructions (see appendix). These were run on a PTC-100 Peltier Thermal Cycler for 10 minutes at 21°C, 15 minutes at 42°C, 5 minutes at 99°C and 5 minutes at 4°C. cDNA was then diluted with 15 μL H₂O before use.

2.2.3.2 cDNA synthesis directly from cell cultures

Primary SAEC do not grow well in culture and it is difficult to produce enough cells to seed in a 6 well plate for experiments and therefore difficult to extract enough RNA for PCR. An alternative method for extracting RNA to cDNA in a few steps in cells grown in a 96 well plate was used for SAEC (see appendix). Briefly, cells were washed twice in ice cold PBS before addition of 30 μL cells to cDNA lysis buffer and transferred into 0.2 mL PCR tubes. Lysates were incubated at 75°C for 15 minutes and then frozen until required. On use 1 μL DNase 1 and 3 μL 10 x DNase 1 buffer was added to each sample and incubated at 37°C for 15 mins and then 75°C for 5

mins. From this stock of DNase treated RNA, 8 μ L was transferred to a fresh tube and the rest was frozen for future use.

1 μ L of random hexamers and 3 μ L dNTPs was added to each sample and then incubated at 75°C for 5 minutes. A mastermix of reagents was made comprising the following volumes per sample; 4 μ L 5 x RT buffer, 2 μ L 0.1M DTT, 1 μ L ddH₂O, 0.5 μ L MMLV reverse transcriptase enzyme and 1 μ L RNase inhibitor and then 8.5 μ L was added to each sample followed by incubation at 37°C for 50 minutes and 75°C for 15 minutes.

This cDNA was diluted with 30 μ L of H₂O before use for qRT-PCR.

2.2.3.3 qRT-PCR

5 μ L cDNA (produced from either of the above methods) and 15 μ L SYBR green mastermix was used to quantify gene expression as per the manufacturer's instructions (see appendix) on a Qiagen Q10 RT-PCR detection system using the following cycles: 1 cycle of 2 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 40 seconds at 60°C, followed by a melting analysis ramping from 60°C to 94°C to determine primer dissociation curves (see appendix).

2.2.3.4 Primer design

Primers were designed using the Roche Universal Probe library (www.roche-applied-science.com/sis/rtpcr/upl/ezhome.html) which automatically blasts target nucleotide sequences in PubMed to identify sequences which give a single product to avoid unspecific binding and primer dimers. Amplicons were 50-120 base pairs in length with primers of 18-22 bases in length, a 50% G/C content and spanned an intron-exon boundary to prevent replication of genomic DNA.

IL-1 β	F = 5'-GGA CAA GCT GAG GAA GAT GC-3'
	R = 5'-TCG TTA TCC CAT GTG TCG AA-3'
IL-6	Qiagen QuantiTect primer assay Hs_IL6_1_SG
IL-8	Qiagen QuantiTect primer assay Hs_IL8_1_SG
IL-10	Qiagen QuantiTect primer assay Hs_IL10_1_SG
IL-12p70	Qiagen QuantiTect primer assay Hs_IL12B_1_SG
TNF α	F = 5'-GCCCAGGCAGTCAGATCATC-3'
	R = 5'-CGGTTCCAGCCACTGGAGCT-3'
GAPDH	F = 5'-AACAGCCTCAAGATCATCAGCA-3'
	R = 5'-TGCTAAGCAGTTGGTGGTGC-3'
TGF- β 1	F = 5'-GGGACTATCCACCTGCAAGA-3'
	R = 5'-CCTCCTTGCGTAGTAGTCG-3'
ICAM-1	F = 5'-TAGCCAACCAATGTGCTATT-3'
	R = 5'-CAGCGTAGGGTAAGGTTCTTG-3'
MUC5AC	Qiagen QuantiTect primer assay Hs_LOC100132635_1_SG
MMP-1	F = 5'-GCTAACCTTTGATGCTATAACTACGA-3'
	R = 5'-TTTGTGCGCATGTAGAATCTG-3'
MMP-9	F = 5'-GAACCAATCTCACCGACAGG-3'
	R = 5'-GCCACCCGAGTGTAACCATA-3'
MMP-12	F = 5'-GGAATCCTAGCCCATGCTTT-3'
	R = 5'-GCAGTGAGGAACAAGTTGGTG-3'
VDR	F = 5'-CTTCTCTGGGGACTCCTCCT-3'
	R = 5'-TGGACGAGTCCATCATGTCT-3'
CYP24	F = 5'-CATCATGGCCATCAAACAAT-3'
	R = 5'-GCAGCTCGACTGGAGTGAC-3'
Thioredoxin reductase I	F = 5'-GGGCAATTTATTGGTCCTCA-3'
	R = 5'-GGTCTTTCACCAAGTGGCAAT-3'
HO-1	F = 5'-ATGGCCTCCCTGTACCACATC-3'
	R = 5'-TGTTGCGCTCAATCTCCTCCT-3'
Nrf2	F = 5'-AACCACCCTGAAAGCACAGC-3'
	R = 5'-TGAAATGCCGGAGTCAGAATC-3'
NQO1	F = 5'-CGCAGACCTTGTGATATTCCAG-3'
	R = 5'-TCCTATGAACACTCGCTCAAACC-3'
Ferritin H	F = 5'-ACCAACGAGGTGGCCGAAT-3'
	R = 5'-TTCAGCCCGCTCTCCCAGT-3'
Table 2.1 – Primers for qRT-PCR (100 μ M)	

2.2.3.5 Analysis

Gene expression was quantified from a standard curve (see appendix) giving copies/reaction of mRNA and then made relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a house keeping gene. Fold change was calculated from the 0 values. Efficiencies of reaction were all over 0.75 and R^2 was always ≥ 0.99 .

2.2.4 ROS assay

2, 7'-dichlorodihydrofluoresceindiacetate (H_2DCFDA) is a compound which is taken up by cells and converted into further intermediates by cellular esterases. In the presence of intracellular ROS the intermediate is irreversibly converted into the fluorescent compound 2',7'-dichlorofluorescein (DCF) which is detectable using a simple microplate based assay.

2.2.4.1 Method optimisation:

Different methods have been employed to determine ROS using this compound including flow cytometry. However, with adherent cells in particular the sheer stress of trypsinisation for use in suspension is a cellular stress. Microplate methods have been used in the literature but the exact method is either left out or vague. For optimisation in the respiratory system a number of these methods were tested as described in Chapter 5.

2.2.4.2 Optimised assay procedure

H_2DCFDA was made up fresh in DMSO at a stock of 10 mM and then further diluted in PBS to a final concentration of 10 μM so that the concentration of DMSO is 0.1% and did not have any detrimental effect on the cells. NCI-H292 and A549 cells were plated at 4,000 cells per well on a 96 well flat clear bottomed black sided fluorescence plate and grown to 80% confluency (2-3 days). Any pre-incubations with vitamin D were carried out in this time. Cell media was removed and cells washed with 100 μL PBS before addition of 100 μL 10 μM H_2DCFDA for 40 minutes. The H_2DCFDA was then removed and replaced with 100 μL phenol red free media for 20 minutes to allow cell recovery. Before addition of ROS inducing stimulus, fluorescence was measured on the on POLARstar OPTIMA microplate reader (BMG Labtech) with absorbance at 485 nm and emission at 520 nm.

2.2.5 Cytotoxicity / Proliferation Assay

NCI-H292 and A549 cells were plated at 4,000 cells and 16HBE14o- 10,000 cells per well on a 96 clear bottom and sided microplates and grown to 80% confluency (2-3 days). Any cell treatments were carried out in this time. 20 µl Cell Titer 96 AQueous One Solution Reagent (MTS) was added to each well, incubated in the dark and absorbance read at 490 nm 1 hour (A549) or 3 hours (NCI-H292/16HBE14o-) later on POLARstar OPTIMA microplate reader. The solution contains MTS tetrazolium compound which is reduced by living cells into a coloured formazan product soluble in tissue culture medium. The quantity of formazan is measured by absorbance and is directly proportional to the number of living cells. For statistical analysis the control of cells alone with DMSO was taken as 100% cell proliferation.

2.2.6 Protein Detection

2.2.6.1 Western Blotting

2.2.6.1.1 Extract Preparation

Supernatants from cells in 6 well plates were removed and 200 µL 1:1 PBS: 2x SDS buffer was added to each well. Cells were scraped into eppendorf tubes and covered with parafilm before boiling samples for 5 minutes. Extracts were kept at -80°C until required. For gel loading, samples were reboiled and 18 µL of each sample was mixed with 2 µL of sample reducing agent and the 20 µL added to each well.

2.2.6.1.2 SDS-PAGE

Pre-made NuPAGE 4-12% Bis-Tris gel were rinsed with deionised (di) H₂O, and the gel foot tape and comb were removed. The gel was inserted into the XCell *SureLock* Mini-Cell rig (Invitrogen). 1 x running buffer (appendix) was added to the upper and lower chambers, and 500 µL NuPAGE antioxidant to the upper buffer chamber. Wells of the gel were rinsed out using the running buffer. 5 µL of molecular weight marker and 20 µL of sample was added to each well. The gel was run at 200 V for 50 minutes.

2.2.6.1.3 Gel Transfer

PVDF membrane was pre-soaked in methanol for 30 seconds and then 1 x transfer buffer for at least 15 minutes before transfer. Blotting pads were soaked in 1 x transfer buffer before transfer. The membrane was placed on the back of the gel with soaked filter paper sandwiching either side and then put in the XCell II Blot Module transfer rig (Invitrogen) with the gel closest to the cathode and membrane closest to the anode. The transfer chamber was filled with 1 x transfer buffer and the lower chamber with diH₂O. The transfer was run at 30V for 1 hour.

2.2.6.1.4 Gel staining

Following transfer the gel was rinsed for 3 x 5 minutes in diH₂O before staining with 20 mL Simply Blue safestain for 1 hour and then washed for 1 hour in diH₂O and kept for imaging on G-box imager Syngene (Cambridge, UK).

2.2.6.1.5 Immunoblotting

Following transfer the membrane was placed in blocker (appendix) for 1 hour at room temperature with agitation. Primary antibody was diluted in blocker and incubated with the membrane for 1 hour at room temperature. The membrane was then placed in blocker for 3 x 5 minutes and then washed for 3 x 5 minutes with TBST (appendix). HRP conjugated secondary antibody was diluted in blocker and incubated with the membrane for 30 minutes at room temperature followed by blocking again 3 x 5 minutes in blocker before washing in TBST for 3 x 5 minutes.

Antibodies were diluted in blocker (volume/volume):

Rabbit monoclonal anti-Nrf2 antibody 1/750

Goat anti rabbit HRP antibody 1/1000

Anti-β-actin mouse monoclonal antibody 1/100,000

Polyclonal goat anti-mouse HRP antibody 1/1000

2.2.6.1.6 Chemiluminescence

RapidStep ECL reagent is a substrate for the horse radish peroxidase enzyme which is conjugated to the secondary antibody and incubation for 1 minute allows conversion of the substrate to luminescence which can be measured by traditional

incubation with film or by imaging on G-box imager. Semi-quantitative data was collected by measuring densitometry using image J software.

2.2.6.1.7 Reprobing

Antibodies were stripped off the membrane using Re-blot Plus antibody stripping solution. The membrane was incubated in 10% Re-blot solution in H₂O with gentle agitation for 30 minutes then washed for 2 x 5 minutes in blocker. Then the membrane was treated with primary antibody and the same protocol was followed for immunoblotting (2.2.6.1.5).

2.2.6.2 Flow Cytometric Bead Array

This method was carried out according to manufacturer's instructions. Standards were reconstituted in 2 mL assay diluent and allowed to equilibrate for 15 minutes. A serial dilution of the standards was performed using 300 µL 1:1 with assay diluent from 1:2 to 1:256. Each cytokine capture bead was mixed together using 10 µL of each per test sample and mixed. To each tube 50 µL of capture beads, 50 µL of human inflammatory cytokine PE detection reagent, and 50 µL of standard dilutions or unknown samples was added. Assay tubes were incubated for 3 hours at room temperature protected from light. 1 mL of wash buffer was added to each assay tube and centrifuged at 200 g for 5 minutes. The supernatant was aspirated and the pellet resuspended in 200 µL of wash buffer. Samples were run on the BD FACSAria II flow cytometer with 4 lasers (blue 488 nm, red 633 nm, violet 405 nm and near UV 375 nm).

2.2.6.3 Gelatin Zymography

In this technique, proteins are separated according to their molecular weight as in western blotting but the gel is incubated for 24 hours to allow any active gelatinases to degrade the gelatin within the gel. On staining of the gel for protein there are clear areas where the enzymes have degraded the gelatin and this can qualitatively show the presence of gelatinase but these bands can also be analysed for their intensity on imaging and therefore semi-quantitative assessment can also be performed.

2.2.6.3.1 Gel preparation

Porcine gelatin was dissolved in ddH₂O at 65°C and then cooled to room temperature.

All resolving gel ingredients (see appendix) were combined and poured between glass gel plates. Isopropanol was added on top of the gel to make it air-tight. The gel was allowed to polymerise at room temperature for 30-60 minutes before removal of the isopropanol and rinsing the top of the gel with ddH₂O to remove any residue.

All stacking gel ingredients (see appendix) were mixed together and poured on top of the set resolving gel and a gel comb was placed between the glass plates.

The gel was allowed to polymerise for 30 minutes.

2.2.6.3.2 Extract preparation

Samples were diluted 3:1 with loading buffer (appendix) with and without EDTA for the control gel. EDTA inhibits metalloproteinase activity by blocking the zinc active site. 10 µL of each sample was added to wells and 5 µL of molecular weight marker or MMP9 control. The gel was run at 150V for 120 minutes or 30 minutes after the dye had run off the bottom of the gel.

The normal gel was placed in rinse buffer (appendix) and the EDTA control gel in rinse buffer + EDTA for 2 x 15 minutes on a shaker. Gels were then incubated with/without EDTA in incubation buffer (appendix) at 37°C for 24 hours to allow the enzymes to activate and proteolytically degrade the gelatin within the gel. The gels were then rinsed in dH₂O and stained with staining solution (appendix) overnight to stain all the protein within the gel. The gel was then destained with destain solution (appendix) for up to one hour until the degraded areas are clear. Gels were then imaged on the G-box imager and semi-quantitative data was collected by measuring densitometry using image J software.

2.2.6.4 ELISA

NCI-H292 were grown in 6 well plates and untreated or treated with TNF α , LPS or PMA for 24 hours before harvesting supernatants. 50 µL of each sample supernatant in duplicate were left to dry in 96 well ELISA plate overnight at 42°C.

Wells were then blocked with 2% BSA for 1 hour and then washed 3 x 5 minutes in PBS containing 0.05% Tween 20. Wells were incubated with 1/100 primary mouse anti-MUC5AC antibody in PBS for 1 hour followed by 3 x 5 minute washes in 0.05% Tween 20. Secondary goat anti-mouse HRP at 1/1000 dilution in PBS was then added and incubated for a further hour. The plate was washed 5 times with 0.05% Tween 20 and colorimetric reaction was developed with 100 μ L mix of 1:1 tetramethylbenzine (TMB substrate) and hydrogen peroxide for 20 minutes before stopping the reaction with 50 μ L H₂SO₄ and reading absorbance at 450 nm on the POLARstar OPTIMA microplate reader.

2.2.6.5 Immunohistochemistry for MUC5AC expression

NCI-H292 and A549 cells were grown on cover slips in 6 well plates. Supernatants were removed and cells washed with PBS. Cells were fixed with 4% paraformaldehyde for 15 minutes. Cover slips were washed 3 x 5 minutes in PBS and cells were blocked in goat quench (appendix) for 10 minutes. Cells were then permeabilised with 0.2% Triton X-100 for 20 minutes and then stained with 1/100 mouse anti-human MUC5AC clone 45M1 antibody diluted in blocking buffer (appendix) and then washed 3 x 5 minutes in wash buffer (appendix) before addition of secondary goat anti-mouse Alexafluor 488 nm at 1/1000 diluted in blocking buffer (appendix). Cover slips were washed 3 x 5 minutes in washing buffer and then cell nuclei were stained with 1/100 DAPI in PBS for 15 minutes before a final vigorous wash with PBS 3 x 5 minutes. Cover slips were mounted on slides with 5 μ L Fluoromount G. Images were taken on Zeiss Axioplan II microscope using Axiovision software.

2.2.7 Statistical analysis

All graphs were produced using GraphPad Prism 5 and the mean plus the standard error mean were plotted. Unless otherwise stated, each data set were made relative to their individual controls by dividing each value by the average of the control values, so that in the end the average control value was 100% or 1-fold. Where data from different cell types is presented on the same graph each is relative to its own control so that differences between the cell types could be compared directly. Students t-test was carried out using GraphPad Prism and

Microsoft Excel to compare the means of test against controls. One-way ANOVA with Tukey's multiple comparison test was used to compare different treatment conditions to each other and controls. Two-way ANOVA with Bonferroni post-test was used to compare kinetic induction patterns of the same mediators between the different cell types and also different cellular treatments.

Chapter 3:

Characterisation of airway epithelial cells

3.1 Introduction

The lung is constantly exposed to noxious agents and pathogens from the external environment in the process of inhalation. Airway epithelial cells provide an important barrier to these external stimuli and this layer is constantly shedding and renewing to avoid damage from exposure to potentially carcinogenic particles such as cigarette smoke. The cells are protected by a mucous layer which when damaged allows interaction with pathogens and other environmental stimuli, activating the airway epithelial cells to initiate an immune response with leukocyte infiltration ⁽¹⁹⁶⁾.

There are a wide range of airway epithelial cell lines with which to study airway inflammation, some more widely characterised than others. In this study three widely studied cell lines were used along with primary small airway epithelial cells (SAEC) to compare the basal and inducible expression of inflammatory mediators. The SAEC were the most physiologically relevant cell type used however, these are expensive and difficult to culture. Therefore it was investigated whether more well characterised cell lines would express similar mediators and therefore be useful models of airway inflammation. The A549 adenocarcinoma cell line is the most widely characterised airway epithelial cell line and has been described by some to be a model of alveolar type II cells ⁽¹⁹⁷⁾, although it is now mostly studied for its cancerous properties. The NCI-H292 cell line is derived from a mucoepidermoid carcinoma shown to be like the SAEC in their response to cigarette smoke ⁽¹⁹⁸⁾ and are more extensively studied for their mucous producing properties ⁽²¹⁾. The 16HBE14o- is a virally transformed immortalised bronchial epithelial cell line which has been widely used to study epithelial barrier function and ion transport and are similar to primary bronchial epithelial cells ^(22, 195). These three cell lines have not been directly compared previously.

In this study the expression of pro-inflammatory mediators important in COPD pathology namely IL-1 β , IL-6, IL-8, IL-10, IL-12p70, TNF α , ICAM1, TGF β 1, MMP1, MMP9, MMP12 and MUC5AC were compared for all tested cell types to identify a cell line model of SAEC to then investigate the effects of vitamin D in further studies. Many groups have investigated the effects of cigarette smoke on inflammatory gene expression in airway epithelial cells which is prominent in the pathology of COPD ⁽¹⁹⁹⁾. However, other important factors can activate an

inflammatory response and have been implicated in this disease. Therefore, *Pseudomonas aeruginosa* LPS, a key pathogen in COPD exacerbations known to activate airway inflammation, and TNF α one of the most important factors in COPD pathology, were used to study their effect on inflammation in airway epithelial cells.

3.2 Aims of study

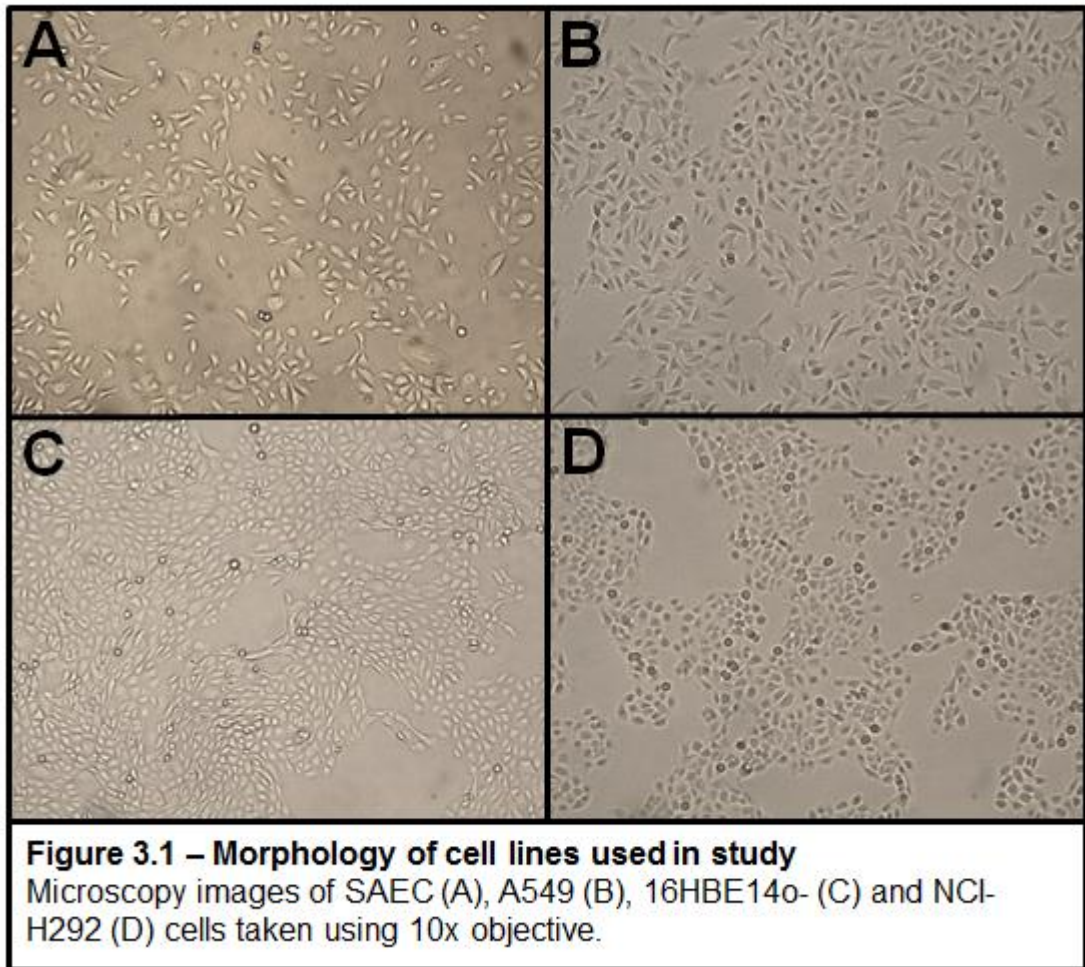
- To characterise the mRNA and protein expression of inflammatory mediators in primary human small airway epithelial cells and three commonly used human airway epithelial cell lines.
- To compare the expression of mediators between the different cell types to identify a robust cell line model to use in future studies

3.3 Results

3.3.1 Morphology of the cell lines used in this study

The cell lines used in this study were derived from different lung locations with primary small airway epithelial cells and the A549 cell line from the small airways, and the NCI-H292 and 16HBE14o- cells from the bronchi. Also, the cell lines have different transformation properties with the 16HBE14o- SV-40 virally transformed for immortalisation, and the A549 and NCI-H292 both being cancerous cell lines. This enabled airway epithelial cells with different cellular characteristics to be compared in their inflammatory gene and protein expression patterns.

Figure 3.1 shows the morphology of the different cells used in the study. The A549 are the closest in morphology to the SAEC, both being alveolar in origin with stretched migratory behaviour congregating in groups and forming a cobblestone like appearance. However, the A549 have different growth characteristics with a greater doubling time than the SAEC. Both the NCI-H292 and 16HBE14o- appear different to alveolar cells and to each other despite both being of bronchial origin. Both are more rounded in morphology than the alveolar cells and tend to clump together in groups which then expand towards other colonies rather than migrate. The NCI-H292 cells also have a greater doubling time than the 16HBE14o- cells, likely due to their malignant properties.



3.3.2 Expression of inflammatory mediators in airway epithelial cells

To study the basal mRNA and protein expression of different mediators SAEC, 16HBE14o-, A549 and NCI-H292 cells were plated out and left to grow to confluency. Supernatants were removed to measure secreted proteins and cells were lysed, RNA extracted, cDNA made and used for qRT-PCR to study mRNA expression. The inflammatory mediators tested were those that relate to COPD pathology, namely: IL-1 β , IL-6, IL-8, TNF α , TGF β 1, ICAM1, MUC5AC, IL-10, IL-12p70, MMP1, MMP9 and MMP12.

Table 3.1 demonstrates that all the cell types tested expressed very similar basal levels of mRNA for IL-1 β , IL-6, IL-8, TNF α , TGF β 1, MMP1, MMP9 and MMP12. IL-10 and IL-12p70 mRNA were not detected in any cell type. ICAM1 mRNA was detected in all but the 16HBE14o- cells. Basal MUC5AC mRNA was expressed in the NCI-H292 and A549.

mRNA	SAEC	16HBE14o-	NCI-H292	A549
IL-1 β	+	+	+	+
IL-6	+	+	+	+
IL-8	+	+	+	+
IL-10	-	-	-	-
IL-12	-	-	-	-
TNF α	+	+	+	+
TGF β 1	+	+	+	+
ICAM1	+	-	+	+
MMP1	+	+	+	+
MMP9	+	+	+	+
MMP12	+	+	+	+
MUC5AC	-	-	+	+

Table 3.1 – Summary of mRNA expression for inflammatory mediators
SAEC, A549, 16HBE14o- and NCI-H292 were left untreated and the mRNA expression of inflammatory mediators IL1 β , IL-6, IL-8, IL-10, IL-12p70, TNF α , TGF β 1, ICAM-1, MMP1, MMP9, MMP12 and MUC5AC was quantified using qRT-PCR.

Where possible the protein expression of mediators was also tested as demonstrated in Table 3.2. Protein expression of cytokines IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α in cell supernatants was detected using a flow cytometric bead array. The key proteins expressed by all of the cell lines were IL-6 and IL-8. IL-1 β was also found to be expressed at low levels in the SAEC but not by the NCI-H292 cell line which was also tested. IL-10, IL-12p70 and TNF α protein was not shown to be expressed basally in any of the cells lines tested.

The basal expression of MMP9 protein has been shown previously in A549, NCI-H292 and SAEC ^(42, 200, 201), however not in the 16HBE14o- cells. Using gelatin zymography, MMP9 enzyme activity was detected in the 16HBE14o- cells.

By developing a MUC5AC ELISA, basal protein secretion was detected in the A549 and NCI-H292 cells.

Protein	SAEC	16HBE14o-	NCI-H292	A549
IL-1 β	+	nc	-	nc
IL-6	+	+	+	+
IL-8	+	+	+	+
IL-10	-	-	-	-
IL-12	-	nc	-	nc
TNF α	-	nc	-	nc
MMP9	+	+	nc	nc
MUC5AC	-	-	+	+

Table 3.2 – Summary of protein expression for inflammatory mediators
SAEC, A549, 16HBE14o- and NCI-H292 were left untreated and the protein expression of inflammatory mediators IL1 β , IL-6, IL-8, IL-10, IL-12p70, TNF α , MMP9 and MUC5AC was quantified using flow cytometric bead array, gelatin zymography and ELISA. Nc = not checked.

3.3.3 Induction of inflammatory mediators by *Pseudomonas aeruginosa* LPS

Gram negative bacteria are important in the pathophysiology of several chronic airway diseases and infection leads to worsening of disease symptoms and chronic inflammation⁽²⁰²⁾. *Pseudomonas Aeruginosa* is one of the most common bacterium isolated in exacerbations of COPD⁽¹⁰⁾. An important virulence factor for this bacterium is LPS found in the outer layer of the bacterial cell membrane. The receptor for LPS is TLR4 which is expressed on the airway epithelial surface⁽²⁰²⁾.

Firstly, the cellular response to LPS treatment was studied in terms of induction of mRNA for inflammatory mediators. Then, the kinetics of mRNA expression in the different cell lines were compared to find a time point with which to target the maximal mRNA induction with vitamin D treatment in future studies.

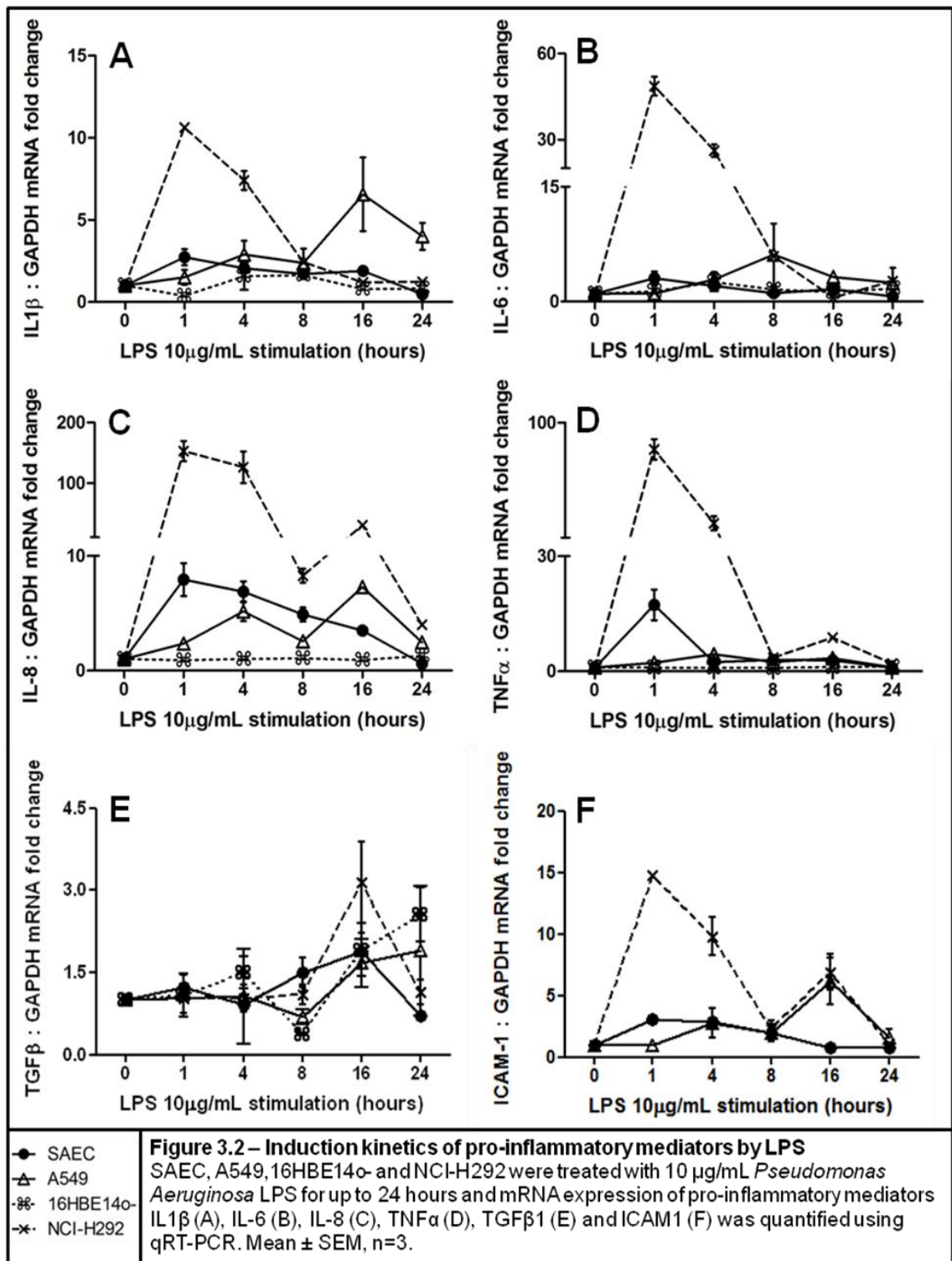
Primary small airway epithelial cells, 16HBE14o-, NCI-H292 and A549 cells were treated with 10 μ g/mL *Pseudomonas Aeruginosa* LPS for up to 24 hours and the kinetics of pro-inflammatory mediators IL-1 β (A), IL-6 (B), IL-8 (C), TNF α (D), TGF β (E) and ICAM1 (F) were quantified using qRT-PCR as shown in Figure 3.2. Surprisingly, the induction kinetics between the different cell lines was quite different showing different sensitivities to LPS treatment. None of the cell lines had a significant induction of TGF β 1 mRNA on LPS treatment.

In the SAEC, with 1 hour of LPS treatment there was a significant induction of IL-1 β (p<0.01), IL-6 (p<0.05), IL-8 (p<0.001), TNF α (p<0.001), and ICAM1 (p<0.05) mRNA expression, with a prolonged significant induction up to 4 hours for IL-8 (p<0.01)

and ICAM1 ($p < 0.05$). The 16HBE14o- cell line was not sensitive to LPS treatment at the concentration used in this study with no significant induction of IL-1 β , IL-6, IL-8, TNF α , or TGF β 1 at any time point 0-24 hours. ICAM1 mRNA was not found to be expressed basally in the 16HBE14o- cell line (Table 3.1) and was not found to be induced on LPS treatment and therefore is not depicted in Figure 3.2F.

The NCI-H292 cell was the most sensitive to LPS treatment with the greatest fold induction of at least ~ 10 fold for all the mediators tested (Figure 3.2). All were significant at 1-4 hours ($p < 0.001$) with a large induction, which then tailed off with IL-1 β , IL-6, IL-8, TNF α and ICAM1 all showing the same kinetic pattern of induction suggesting they are being regulated in the same way. A different kinetic pattern of induction was seen in the A549 cell line with increasing induction between 1-4 hours giving a significant induction of IL-8 at 4 hours ($p < 0.001$) and TNF α at 1 hour ($p < 0.05$) and then a later induction of ICAM1 ($p < 0.05$), IL8 ($p < 0.001$) and IL-1 β ($P < 0.05$).

All of the cell lines tested were significantly different to the SAEC for concentrations of each cytokine at one or more time points via two-way ANOVA. However, the induction kinetics of IL-1 β , IL-6, IL-8, TNF α and TGF β 1 between the NCI-H292 and the SAEC were similar suggesting they may be a useful model in future studies (Figure 3.2).



3.3.4 Induction of mRNA expression for inflammatory mediators by TNF α

TNF α plays a central role in the initiation and maintenance of inflammatory responses in the lung and has been found to be elevated in sputum, breath condensate and the systemic circulation in patients with COPD ^(6, 7). It has also been shown to be responsible for 70% of cigarette induced airspace enlargement and inflammatory cell influx in mice ⁽²⁹⁾. The main source of TNF α is alveolar macrophages and its expression is enhanced by infection. Airway epithelial cells have been shown to secrete the important neutrophil chemoattractant IL-8 in response to TNF α treatment ⁽²⁰³⁾.

As in the previous section, the kinetics of mRNA induction for inflammatory mediators was compared in the different cell lines but in response to TNF α treatment. A concentration of 10 ng/mL was used as this is widely used in the literature to induce an inflammatory response ⁽²⁰⁴⁾.

SAEC, 16HBE14o-, NCI-H292 and A549 cells were treated with 10 ng/mL TNF α for up to 24 hours and the kinetics of IL-1 β (A), IL-6 (B), IL-8 (C), TNF α (D), TGF β 1 (E) and ICAM1 (F) was quantified using qRT-PCR, shown in Figure 3.3.

Similarly to LPS treatment, the highest induction of cytokine mRNA tested occurred at 1 hr although interestingly the cell lines were more similar in their induction kinetics in response to TNF α treatment and this treatment gave a more prolonged induction of mRNA than the LPS treatment.

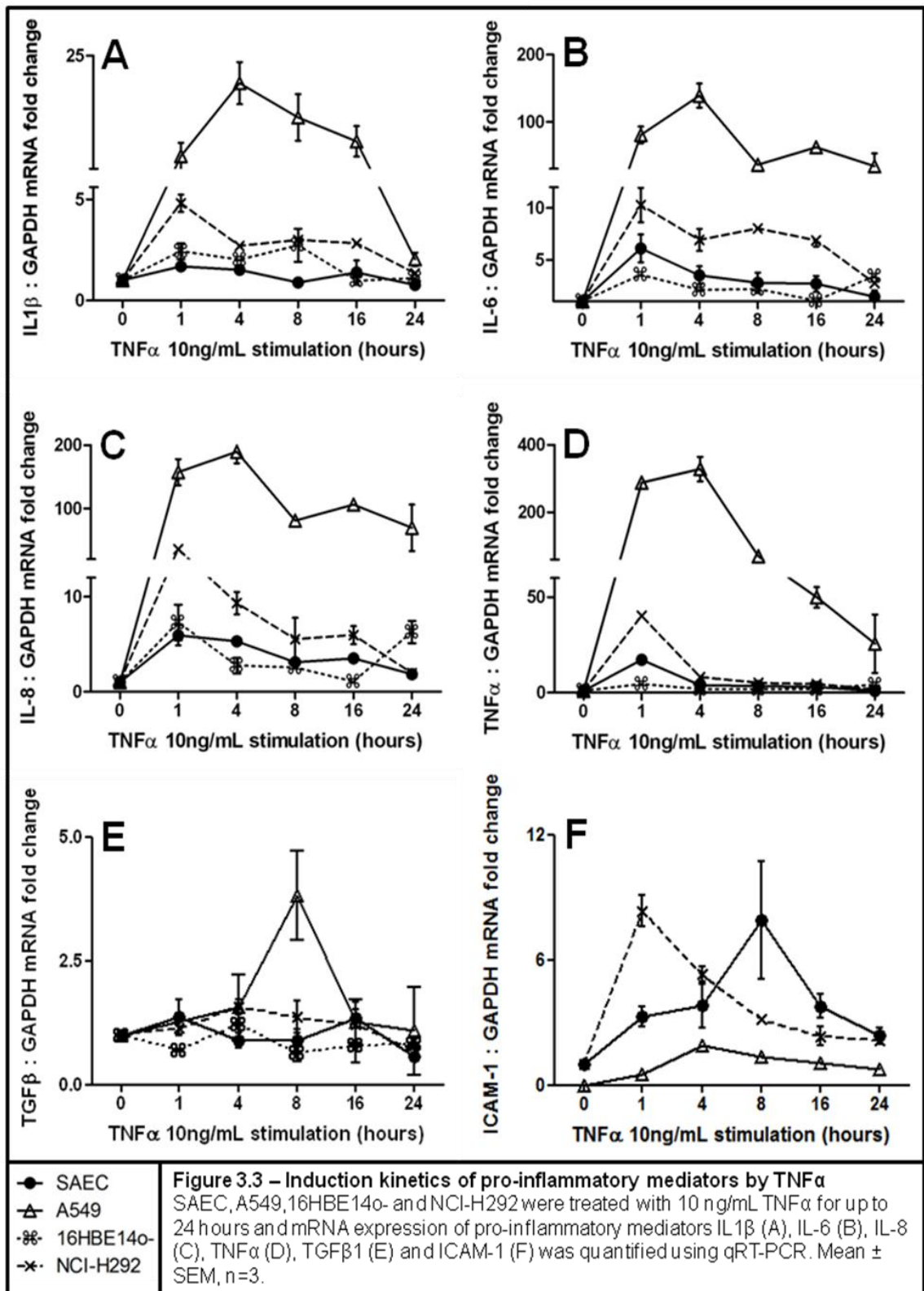
At 1 hr, TNF α induced significant expression of IL-6 ($p < 0.01$), IL-8 ($p < 0.001$) and TNF α ($p < 0.001$) mRNA in the SAEC, but IL-1 β expression although increased at 1 hour was not found to be significant (Figure 3.3). This was similar in the 16HBE14o- with the 1 hour of TNF α treatment inducing significant expression of IL-1 β ($p < 0.05$), IL-6 ($p < 0.001$), IL-8 ($p < 0.01$) and TNF α ($p < 0.001$) mRNA, and also in the NCI-H292 cells a significant induction of IL-1 β ($p < 0.001$), IL-6 ($p < 0.001$), IL-8 ($p < 0.001$), TNF α ($p < 0.001$) and ICAM1 mRNA ($p < 0.001$). In the SAEC, ICAM1 showed different kinetics of induction with 8 hour TNF α treatment giving the peak induction of ICAM1 mRNA ($p < 0.05$).

The A549 were the most responsive to TNF α treatment in terms of the greatest fold induction over basal with over ~ 100 fold induction of IL-6, IL-8 and TNF α mRNA expression. However, the kinetics differed from the other cell lines with 4 hrs giving

the greatest significant induction of IL-1 β ($p < 0.01$), IL-6 ($p < 0.001$), IL-8 ($p < 0.001$), TNF α ($p < 0.001$) and ICAM1 ($p < 0.001$) (Figure 3.3).

Similarly to LPS treatment, TNF α treatment did not induce TGF β 1 mRNA expression in any of the cell types tested.

For TNF α induction of IL-1 β , IL-6, IL-8, and TNF α mRNA the 16HBE14o- and NCI-H292 cells were not significantly different to the SAEC by two-way ANOVA and therefore may be useful models of inflammatory gene expression for future studies.

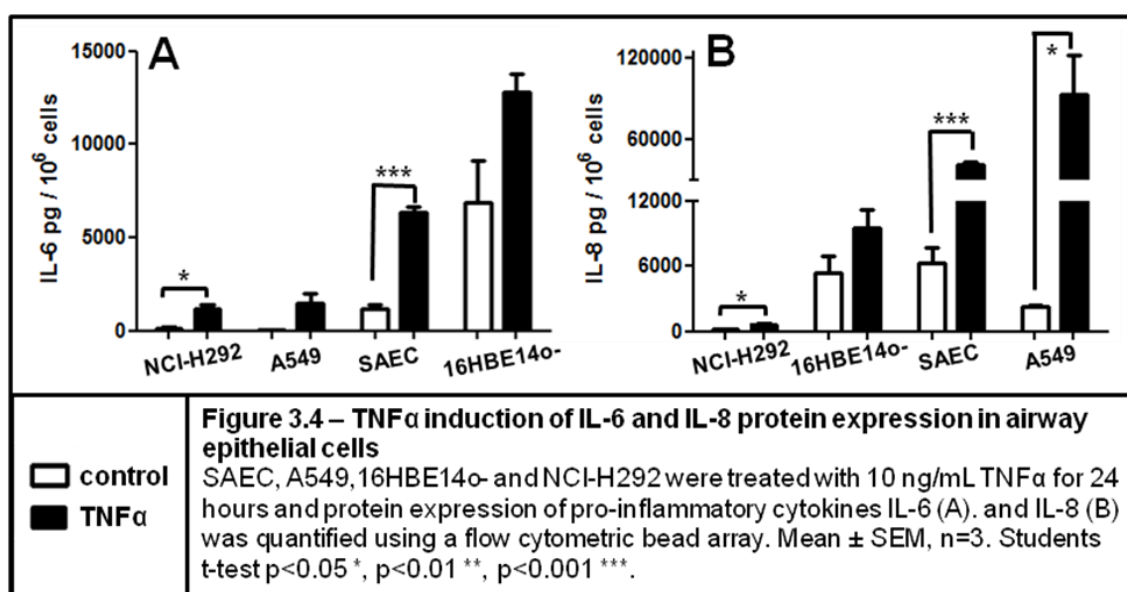


3.3.5 Induction of inflammatory cytokine protein expression by TNF α

A flow cytometric bead array was used to investigate the TNF α induction of IL-1 β , IL-6, IL-8, IL-10 and IL-12p70 in the different cell lines. IL-6 and IL-8 were the key cytokines induced by TNF α treatment (Figure 3.4). This matches the data on basal

protein expression where IL6 and IL8 were the key proteins expressed by the airway epithelial cells (Table 3.2).

TNF α significantly upregulated IL-6 secretion in the NCI-H292 (p<0.05) and SAEC (p<0.001) (Figure 3.4). However, IL-8 protein expression was significantly upregulated by TNF α in the A549 (p<0.05), NCI-H292 (p<0.05) and the SAEC (p<0.001) but not in the 16HBE14o- cells (Figure 3.4). The SAEC had the second greatest expression of both these cytokines and therefore the greatest expression of IL-6 and IL-8 combined. This suggests that they may be more sensitive to treatment with TNF α than the three cell lines tested.



3.3.6 Induction of MMPs and MUC5AC mRNA expression by TNF α

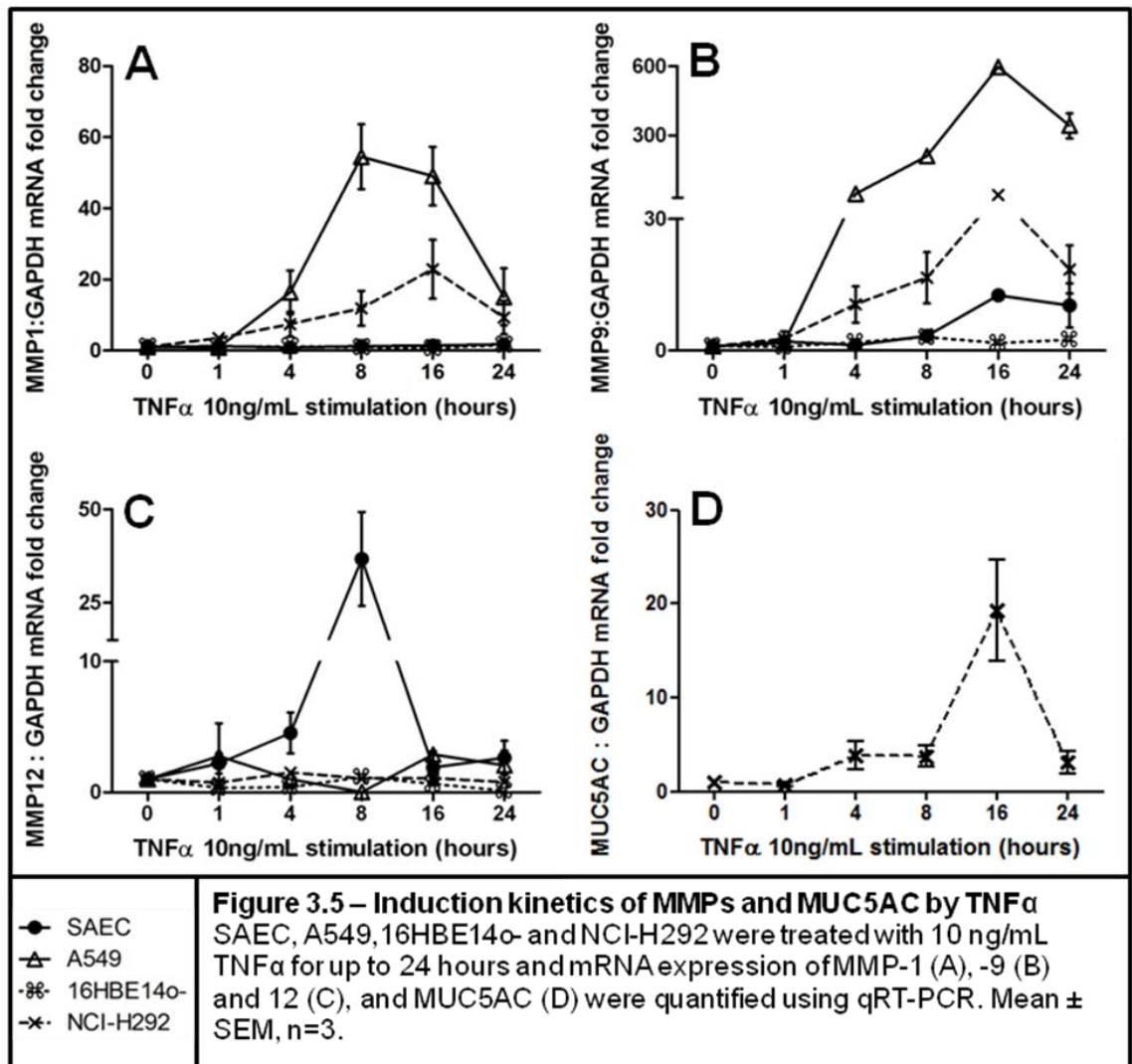
Since TNF α induced similar cytokine expression in the different cell types, it was investigated if the same would be true for MMP and MUC5AC expression. MMPs are important in the remodelling processes within the lung but overexpression in COPD leads to destruction of the alveolar spaces. MUC5AC is also an important airway secretion protecting the epithelial layer. However, in COPD goblet cell hyperplasia and hypersecretion leads to overproduction of MUC5AC and airway obstruction. In this study the TNF α induction of MMPs 1 (A), 9 (B) and 12 (C) and MUC5AC (D) mRNA expression were investigated, shown in Figure 3.5.

TNF α induced MMP1 mRNA expression in A549 and NCI-H292 cells, with a significant induction at 8 hr in A549 (p<0.001) and 16 hr in both A549 and NCI-H292 (p<0.01, p<0.05) respectively (Figure 3.5).

TNF α induced MMP9 mRNA expression in all the cell lines, with a significant induction of MMP9 mRNA at 16 hr in the SAEC ($p < 0.05$), A549 ($p < 0.001$) and NCI-H292 ($p < 0.001$) (Figure 3.5). However, the kinetics were different in the 16HBE14o- with a significant induction at 8 hours of TNF α treatment ($p < 0.001$) (Figure 3.5). For MMP9 expression, the 16HBE14o- and NCI-H292 cells were not significantly different from the SAEC by two way ANOVA.

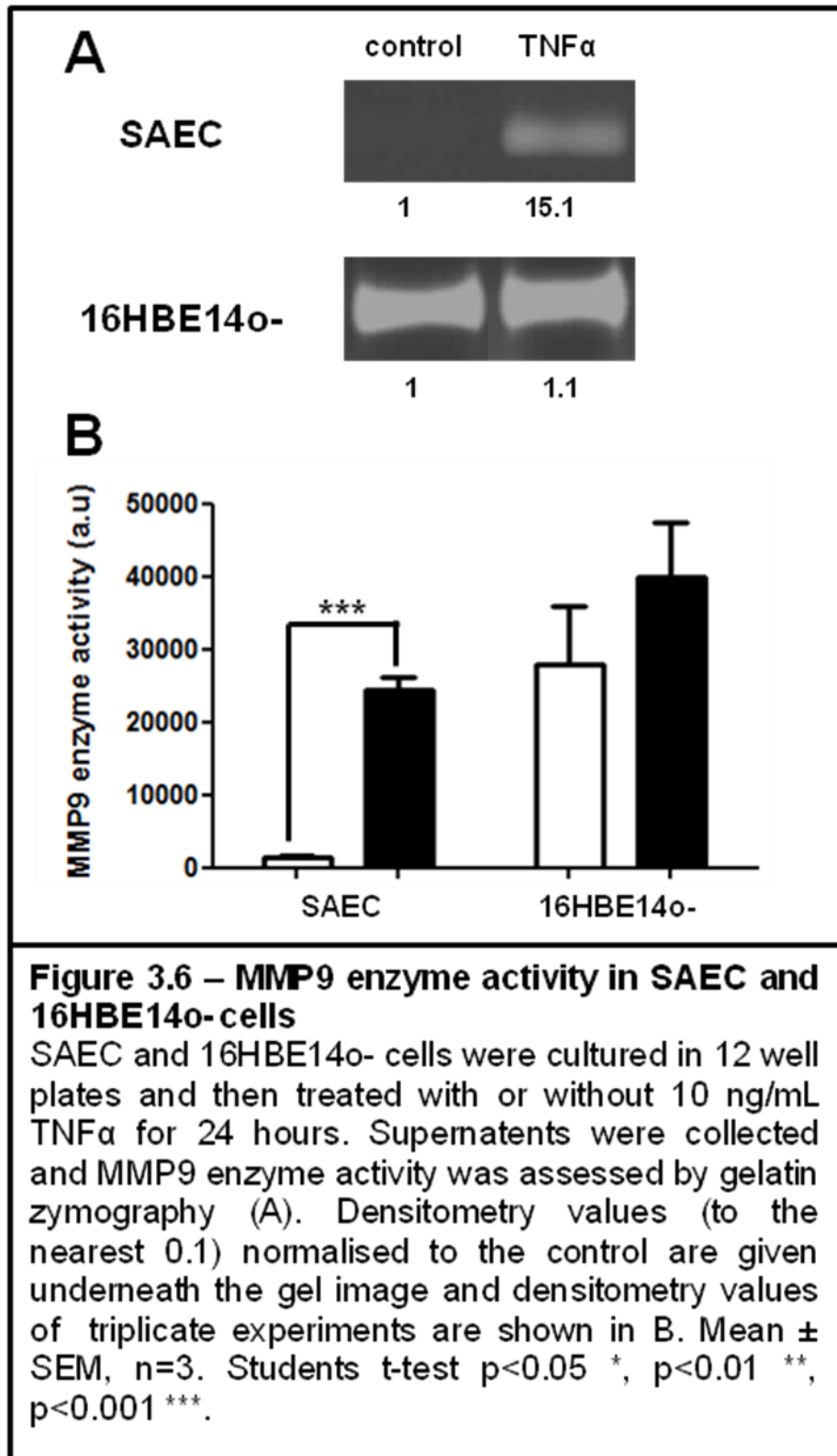
Interestingly, MMP12 was significantly induced by TNF α in the SAEC, peaking at 8 hr ($p < 0.01$), but not in any of the other cell lines (Figure 3.5).

TNF α induction of MUC5AC mRNA expression was studied in the SAEC, 16HBE14o- and NCI-H292 but was only detected in the NCI-H292 cell line with a significant induction at 16 hours ($p < 0.01$) (Figure 3.5).



3.3.7 MMP9 functional activity

TNF α induced MMP9 enzyme activity in the SAEC at 24 hr ($p < 0.001$) shown in Figure 3.6. TNF α had little effect on MMP9 in the 16HBE14o- cells but there was a much higher basal expression (around 5 fold) of MMP9 in this cell line than the SAEC.



3.3.8 MUC5AC expression in A549 and NCI-H292 cells

MUC5AC is the key mucin found upregulated in patients with COPD ⁽⁵⁹⁾ and has been previously shown to be upregulated by PMA and LPS in airway epithelial cells. First the intracellular expression of MUC5AC in A549 and NCI-H292 cells was studied using immunofluorescence shown in Figure 3.7. Distinct populations of MUC5AC expressing cells were identified, making up around 5-10% of the total cell population. In order to investigate the secretion of MUC5AC by the different cell types an ELISA was developed. SAEC and 16HBE14o- did not express MUC5AC protein basally or upon TNF α treatment (data not shown). Upon studying the secretion of MUC5AC in NCI-H292 cell line it was found that 10 ng/mL TNF α did not significantly increase MUC5AC secretion (Figure 3.8). However, LPS and PMA significantly increased MUC5AC secretion in the NCI-H292 cells ($p < 0.05$ and $p < 0.001$ respectively) as shown in Figure 3.8, confirming previous research ^(205, 206).

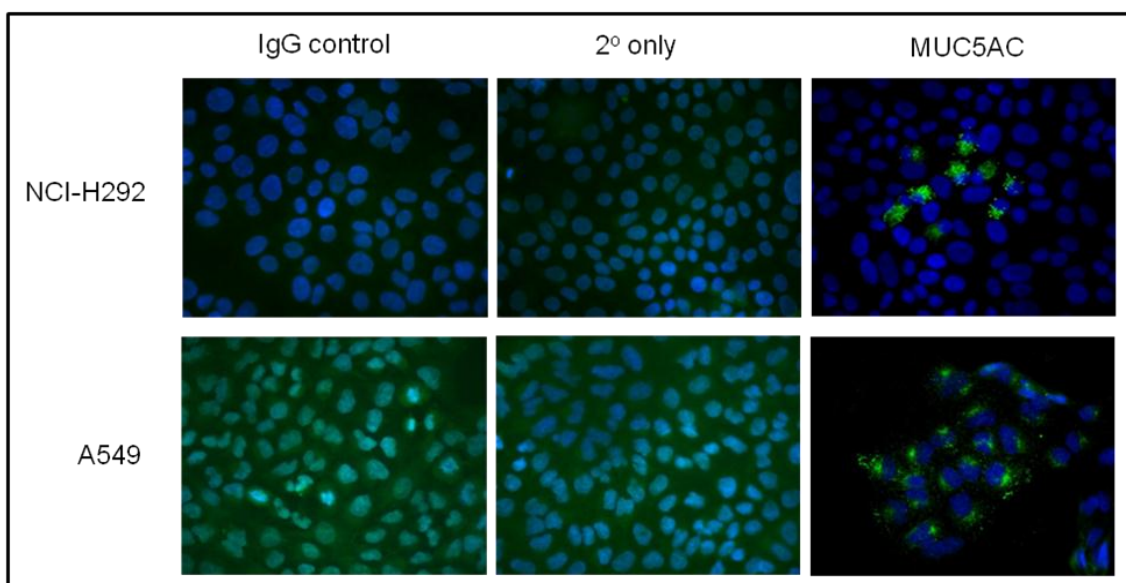
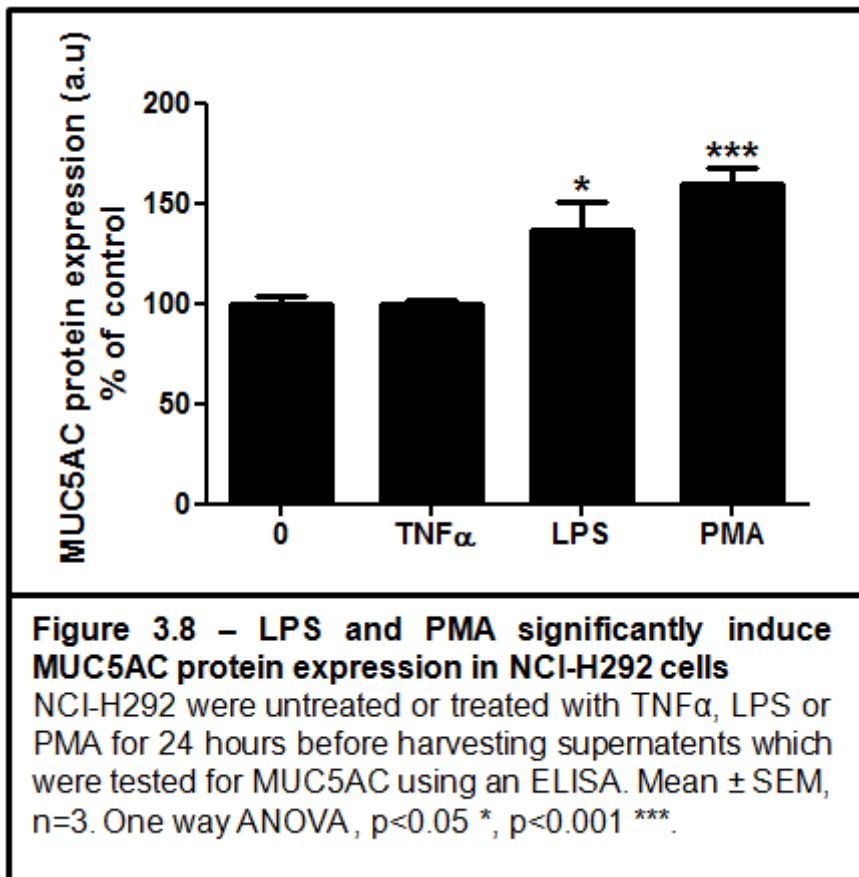


Figure 3.7 – MUC5AC protein expression in NCI-H292 and A549 cells

NCI-H292 and A549 cells were grown on cover slips in 6 well plates and then fixed and permeabilised. Cells were stained with 1/100 Mouse anti-human MUC5AC and then 1/1000 goat anti-mouse secondary Alexafluor 485nm and counter stained with 1/100 DAPI. Blue stain DAPI, green stain MUC5AC. Magnification 40 x.



3.4 Discussion

The novel findings of this study were that IL-6 and IL-8 expression is inducible by TNF α and *Pseudomonas aeruginosa* LPS, and MMP-12 is inducible by TNF α in the SAEC. In addition, IL-6 and IL-8 were inducible by TNF α and LPS in 16HBE14o- cells and they also express and secrete MMP-9. Finally, MUC5AC secretion was not induced by 10 ng/mL TNF α for 24 hours, but 16 hours of treatment gave a significant maximal induction of mRNA in NCI-H292. There was no basal or induced MUC5AC mRNA or protein expression in the SAEC or 16HBE14o-.

This study was novel in the fact that *Pseudomonas aeruginosa* LPS and TNF α , both shown to also be important in COPD, were used as stimuli in the SAEC to look at pro-inflammatory mediators which have not been studied before and also to compare with 3 different cell lines in order to determine if a cell line model could be found to use for future studies. Interestingly, the mediators expressed basally by all the cell types were the same except ICAM1 and MUC5AC (Table 3.1 and 3.2). IL-6 and IL-8 were the key cytokines with basal expression of mRNA and protein in all the different cell types tested.

In exacerbations of COPD the airway epithelium will come under attack from a wide range of different pathogens. The airway epithelium has been shown to express TLRs in order to detect pathogens and elicit an immune response. Previous studies have looked at the effect of LPS on different airway epithelial cells and the expression of its recognition receptor TLR4 on the epithelium⁽²⁰⁷⁾. LPS has previously been shown to upregulate the same mediators as cigarette smoke in the A549, NCI-H292 and SAEC⁽¹⁹⁸⁾, and therefore is a relevant stimulus. Within the literature there appears to be a difference in outcome depending on the type of LPS used. Initially, the LPS strain *E.coli* 0111:B4 was used for this study which did not give a significant induction of cytokines in the A549 cells (data not shown). However, this strain may not be as potent as others in the airways as it is derived from a gut pathogen, with other studies showing it to have little or no effect on cytokine and chemokine expression in the A549 and primary SAEC^(201, 208). There is evidence that different bacterial strains and also subspecies used at the same concentration have different potencies on the induction of IL-8 release in A549 cells^(14, 209). This difference is suggested to be from the lipid A structure in the different strains⁽²⁰⁹⁾.

One study suggests that *Pseudomonas aeruginosa* LPS is the most potent stimuli and with evidence that this is a common pathogen associated with exacerbation of COPD, this stimulus was used ⁽¹⁴⁾. It is possible that LPS derived from known lung pathogens may be more potent in lung epithelial cells whereas LPS from *E.coli* and *Salmonella* may be more potent in the gut epithelium where they are a more physiologically relevant stimulus, although further investigation is required into this hypothesis ⁽²⁰⁷⁾.

Fewer studies have used *Pseudomonas aeruginosa* LPS to activate airway epithelial cells. In this study the A549 were the most insensitive to *Pseudomonas aeruginosa* LPS treatment and the NCI-H292 were highly responsive. This may be down to differences in expression of TLR4 which was not quantified in this study. Previous studies have shown that the A549 express TLR1-6 ⁽²¹⁰⁾, and SAEC have been shown to express mRNA for TLR4 ⁽²⁰¹⁾, and therefore should be able to respond to LPS treatment.

In response to LPS, the maximal induction of IL-1 β , IL-6, IL-8 and TNF α was 1 hr in both the NCI-H292 and SAEC suggesting the NCI-H292 cells may be a useful model for future studies. This 1 hr maximal induction has been shown previously in studies on the NCI-H292 cells where the limited production and transient nature of IL-6 and IL-8 transcripts was found to be overcome with the addition of cycloheximide stabilising transcripts and prolonging induction greater than 1 hr ⁽²¹¹⁻²¹³⁾.

The 16HBE14o- and A549 cells were more insensitive to LPS treatment in this study with little or no significant induction. 1-50 $\mu\text{g}/\text{mL}$ of a different variant of LPS have previously been shown to increase IL-1 β , IL-6, IL-8, TNF α and ICAM1 mRNA and protein in the A549 cells at 24 hours ^(207, 214).

TNF α is an important pro-inflammatory cytokine that is increased in the sputum and systemic circulation of patients with COPD ^(6, 7). It is one of the most important cytokines in COPD pathology and has been shown to drive 70% of cigarette smoke induced emphysema in mice ⁽²⁹⁾. Most of its actions are mediated by binding to its receptor TNFR1, which is expressed in all human tissues and has been shown to be expressed by A549, NCI-H292, and SAEC ⁽²¹⁵⁾. Although to my knowledge there is no literature on TNFR1 expression in 16HBE14o- cells, they did respond to TNF α treatment in this study. TNF α is released by alveolar macrophages in response to

infection and airway epithelial cell lines have been shown to respond to TNF α and also induce TNF α mRNA in response to other stimuli.

In terms of TNF α treatment, the mRNA induction kinetics of pro-inflammatory mediators was more similar between the cell types than with LPS, just with different fold inductions. The NCI-H292, 16HBE14o- and SAEC showed the same kinetic pattern inducing IL-1 β , IL-6, IL-8 and TNF α peaking at 1 hr matching the results for LPS treatment. This suggests that the NCI-H292 and 16HBE14o- cells may be useful models for future studies. In this case the A549 appeared to be the most sensitive to TNF α treatment with the greatest fold induction of cytokine mRNA, although induction peaked at 4 hours suggesting different mRNA regulation to the other cell types. Of the cytokines tested, IL-6 and IL-8 were the only ones detected on TNF α induction (Figure 3.4). An increase in IL-6 and IL-8 was detected in all the cell types and was significant in the NCI-H292 and SAEC. The SAEC had the greatest combined expression of IL-6 and IL-8 protein suggesting they may be more sensitive to TNF α treatment than the other cell lines. However, the levels of TNFR1 expression were not tested in the different cell types which may account for the difference in cellular sensitivities to TNF α treatment.

Different concentrations of TNF α have been tested in the A549 cells with 0.1-25 ng/mL shown to give a dose dependent induction of IL-6, IL-8 and ICAM1 mRNA and protein expression with mRNA expression at 1-4 hours matching the findings of this study^(20, 207, 208, 216-219). TNF α 0.01-10 ng/mL for 24 hours has also been shown to dose dependently increase IL8 protein expression in NCI-H292 cells^(203, 220).

The initial step in the inflammatory process in COPD is overexpression of adhesion molecules leading to excessive transmigration of neutrophils⁽²²¹⁾. ICAM-1 is elevated in patients with COPD and is crucial in the leukocyte transmigration process⁽⁶⁶⁾. In this study ICAM1 mRNA was not found to be expressed in the 16HBE14o- cells. Equally its induction with LPS and TNF α was different in the different cell types. ICAM1 mRNA and protein expression has previously been shown to be induced by 10 ng/mL TNF α for 6-24 hours in A549 cells which fits with the mRNA induction in this study. The signalling pathways involved have been well characterised in the A549 cells^(204, 222-224). The same concentration has been shown to increase soluble ICAM1 expression in NCI-H292 cells⁽²²⁰⁾ and ICAM-1 protein

expression has been shown previously in the 16HBE14o- cells although was not tested in this study.

TGF β 1 is increased in the small airways of patients with COPD and is important in the fibrotic processes ⁽²⁴⁾. TNF α and LPS have been shown to have no effect on TGF β 1 protein or bioactive TGF β 1 in A549 cells ⁽²²⁵⁾ which matches up with the results in this study with neither TNF α or LPS significantly modulating levels of TGF β 1 mRNA in any of the cell types tested at the concentrations used.

MMPs are important in repair and remodelling processes within the lung; however, an imbalance in protease and anti-protease activity is found in COPD leading to alveolar destruction. In patients with emphysema there is an increase in MMP1 and MMP9 concentration in BAL and macrophages. MMP12 also plays a predominant role in the pathogenesis of chronic lung injury in emphysema as it degrades different substrates including elastin which is vital for the elastic recoil of the small airways ⁽²²⁶⁾. The induction of MMPs -1, -9 and 12 by TNF α was investigated in this study. MMP-1 was significantly induced in the two lung cancer cell lines A549 and NCI-H292, which is interesting as MMP-1 is involved in tumor invasion ⁽²²⁷⁾. However, MMP1 is also important for cell detachment and migration which is vital during the constant remodelling and repair pathways within the lung, and is increased on cellular insult ⁽²²⁸⁾. Therefore, the induction in the lung cancer cell types may not be for tumour invasion, but for repair processes associated with the inflammatory insult in cell lines which have already accumulated genetic damage. The expression of MMP-1 mRNA has been shown previously in the SAEC ⁽⁴⁰⁾ and is induced by cigarette smoke, although no effect was seen with TNF α treatment in this study and to my knowledge no-one has investigated the effect of TNF α on MMP-1 in SAEC previously. It has been shown that there is a region in the promoter of MMP-1 in SAEC which is responsive to cigarette smoke and hence gives implications for smoking related diseases ⁽²²⁹⁾.

The induction of MMP-9 mRNA expression was similar in all of the cell types tested in this study with activation at 8-16 hours of TNF α treatment. This suggests that similar signalling pathways may be involved in the induction of MMP-9 mRNA in the different cell types although this would need to be confirmed in future studies.

MMP-9 enzyme activity was also studied in the SAEC and 16HBE14o- cells. TNF α significantly induced MMP9 activity in the SAEC confirming previous studies in primary cells⁽²³⁰⁾ (Figure 3.7). The 16HBE14o- expressed much higher basal levels of MMP9 activity than the SAEC and there was no significant effect with TNF α treatment (Figure 3.7). The induction of MMP-9 by TNF α in the A549 cells was not tested in this study as it has already been well characterised^(42, 231).

In the SAEC, MMP-12 mRNA was increased after 8 hours of TNF α treatment, but not in any of the other cell lines tested, although MMP-12 protein was not tested. The upregulation of MMP12 plays a central role in the transition of emphysema to lung cancer which is facilitated by inflammation⁽²³²⁾. It is interesting that this only occurred in the SAEC.

MUC5AC is a key mucin upregulated in lung disease and is the predominant secretion of the airways^(59, 233). MUC5AC is known to be expressed by A549 and NCI-H292 cells and is inducible in 16HBE14o- cells^{(234) (235)}. In this study, the basal levels of MUC5AC mRNA and protein was detected in NCI-H292 and A549, but not SAEC and 16HBE14o- cells.

Using immunofluorescence, MUC5AC protein expression was detected in the NCI-H292 and A549 cells and was found in specific cell populations representing about 5-10% of the total cells. Previous studies have shown that MUC5AC is exclusively expressed in mucous-secreting goblet cells⁽²³⁶⁾, which supports the fact that in the SAEC and 16HBE14o- cells no basal or induced MUC5AC was detected as they are more homogeneous cell populations without differentiated goblet cells. Although, MUC5AC has been shown to be expressed in the small airways⁽²³⁷⁾. TNF α has been shown previously to induce mRNA for MUC5AC in A549 cells although this was not tested in this study⁽²³⁸⁾. In this study TNF α treatment of NCI-H292 cells for 16 hr gave the maximal induction of MUC5AC mRNA expression returning to basal levels after 24 hr which fits in with other studies⁽²³⁹⁾. By developing an ELISA, MUC5AC secretion was measured in NCI-H292 supernatants⁽²³⁹⁾. Treatment for 24 hr with 10 ng/mL TNF α did not significantly induce MUC5AC secretion. Previous studies have shown a significant induction of MUC5AC secretion in the NCI-H292 cells but at 20 ng/mL of TNF α ⁽²⁴⁰⁾. 50 ng/mL TNF α has been shown to induce MUC5AC expression in cell lysates, not supernatants and hence a measure of intracellular expression not

secretion⁽²⁴¹⁾. This suggests that either a greater concentration of TNF α or a longer treatment time of up to 48 hours is required to give a significant induction of MUC5AC secretion. This study showed a significant induction of MUC5AC secretion on LPS and PMA treatment which has been shown previously in a time and dose dependent manner in the NCI-H292 cells^(21, 242), and was used as a positive control to confirm the ELISA was working.

The mechanism of MUC5AC activation is well defined in the literature in the NCI-H292 cells^(205, 206, 238, 243). In particular, MUC5AC protein expression has been shown to coincide and be dependent on MMP-9 protein expression, and in this study the activation of MUC5AC and MMP-9 mRNA expression was shown to have the same kinetics in the NCI-H292 cells⁽²⁰⁰⁾. In the 16HBE14o- cells, cigarette smoke has been shown to induce MUC5AC expression using immunofluorescence, however, as seen in this study, there was no detectable basal expression of the protein⁽²³⁵⁾.

The results presented in this chapter are not only important to identify the similarities and differences in expression of inflammatory mediators between cell lines and primary cells, but are also important to determine which inflammatory mediators are expressed and induced and at what time points for further studies with vitamin D. Often 2D *in vitro* culture models are used for research without validating in primary cells due to expense and as has been shown in this study and others, cell lines can express different inflammatory mediators and respond differently to treatment with inflammatory stimuli. For example, this study has shown that the kinetics and induction of MMP9 mRNA expression was very similar between the different cell types used whereas the expression of MUC5AC and MMP1 were different. Therefore, it is important to establish where the similarities and differences lie if cell line models are to be used.

There are limitations to this study. When comparing the mRNA and protein expression patterns for the different cell lines, the cell culture media used was not the same for each cell line. Instead, specific media was used for each cell line according to supplier's instructions. Different components of cell culture media can influence the induction of different inflammatory mediators. Arginine is one component which can differ in concentration between culture media types. Reduced bioavailability of arginine leads to exaggerated responses enhancing

inflammation, which has been shown previously with the production of IL-6 and IL-8⁽²⁴⁴⁾. MEM containing arginine has been shown to give similar response to RPMI-1640 which were used in this study for the 16HBE14o-, and the NCI-H292 and A549 cells respectively. It was MEM without arginine, which was not used in the study, that gave an exaggerated response⁽²⁴⁴⁾. However, the SAEC media had many supplements and is very expensive so was only used for the SAEC and therefore the effect of different culture media components accounting for the differences in the expression and induction of inflammatory mediators between the different cell types cannot be ruled out.

Culturing cells in 2D culture as in this study does not completely recapitulate airway epithelia biology⁽²⁴⁵⁾ and thus using 3D air liquid interface culture is important. If the cells used in this study were grown at air liquid interface it is possible that they would behave differently in terms of induction kinetics and the expression of inflammatory mediators. It certainly would make a difference to MUC5AC expression in the SAEC and 16HBE14o- cells as at air liquid interface there would be a ciliated epithelial surface and differentiation into goblet cells. However, the A549 and NCI-H292 cell lines are widely characterised and have been used for many years since their extraction and hence are likely to have been cultured for so many passages in 2D culture that they are too de-differentiated for 3D culture. Equally, there is evidence in the literature that airway epithelial cells derived from different locations, for example nasal vs bronchial, give different responses⁽²⁴⁶⁾. This was highlighted in this study with the differences in the cellular sensitivities to LPS treatment with the A549 being very unresponsive compared to the other cell types and this emphasises the importance of validating experiments in primary cells.

Due to the nature of mRNA and the peak induction at 1 hour demonstrated in this study, a reduction or increase in mRNA may not have a significant effect on protein. Airway epithelial cells in this study only produced protein for IL-6 and IL-8 even though the mRNA for a number of pro-inflammatory mediators was detected and induced. There are a number of possible reasons for this; high mRNA instability, proteins not secreted or secreted before or later than the 24 hour time point used, or the methods used are not sensitive enough to detect them. However, the flow cytometric bead array used is highly sensitive and the 24 hour time point for

protein detection in supernatants is widely used. mRNA instability has been shown previously for transcripts of inflammatory genes so it would not be surprising if cells do not secrete proteins for pro-inflammatory mediators as a mechanism of controlling acute inflammation to limit local tissue damage.

Although cigarette smoke is the key inflammatory stimulus related to COPD pathology, many studies have looked into the effects of cigarette smoke in SAEC and cell lines. LPS has previously been shown to upregulate the same mediators as cigarette smoke in the A549, NCI-H292 and SAEC⁽¹⁹⁸⁾ and TNF α is responsible for both local and systemic inflammation in COPD, so both are physiologically relevant stimulus.

Although no cell line completely matched the induction kinetics of all mediators with the SAEC, the NCI-H292 and 16HBE14o- cells responded similarly in many responses and therefore may be useful models to investigate the effects of vitamin D in future studies before validating in the SAEC. The A549, although of alveolar origin as the SAEC and shown to be a model of ATII in early studies⁽¹⁹⁷⁾, were not a good model of the SAEC in this study, as has been suggested in other more recent studies⁽¹⁹⁸⁾.

3.5 Conclusion

IL-6, IL-8, MMP9 and MUC5AC were found to be the key mediators secreted by some of the airway epithelial cells used in this study. mRNA for IL-1 β , TNF α , TGF β 1, MMP-1, MMP-12, and ICAM-1 were also found to be expressed in some cell types. This suggests along with other indicators in the literature that airway epithelial cells could play an important role in disease pathology by the secretion of pro-inflammatory mediators that have been shown to be increased in the lungs of patients with COPD. There have been no reports comparing the kinetic expression patterns of mediators in these three different cell lines to primary small airway epithelial cells. The key time point for targeting TNF α /LPS induced cytokine mRNA expression was 1 hour in the SAEC, NCI-H292 and 16HBE14o- cells. Although none of the cell types completely mirrored the induction kinetics and protein expression of every single mediator tested basally and in response to LPS/TNF α , the NCI-H292 and 16HBE14o- showed similar kinetics to the SAEC in response to TNF α for some

inflammatory mediators and therefore could be useful for investigating the effects of vitamin D in future studies before validation in the SAEC.

Chapter 4:

Effects of vitamin D on inflammatory mediators in airway epithelial cells

4.1 Introduction

Vitamin D is a steroid hormone initially recognised for its critical role in maintaining calcium and phosphorus homeostasis within the body. However, after the discovery of a vitamin D receptor expressed in most tissues there has been an explosion of interest in other actions of vitamin D. It is now widely accepted that vitamin D has numerous immunomodulatory actions regulating both the innate and adaptive arms of the immune response⁽²⁴⁷⁾. Epidemiological evidence and clinical trials have shown vitamin D to be important in lung function with deficiency associated with reduced lung function⁽¹²⁵⁾. There have also been many associations between vitamin D deficiency and the development of COPD, and also with desensitisation of lung cancers to vitamin D^(164, 248).

At a cellular level, vitamin D has been shown to modulate the response of cells derived from the airways to respiratory syncytial virus infection and also to LPS treatment^(150, 249). In addition, vitamin D has been shown to modulate a wide range of different cellular signalling pathways including p38 MAPK⁽¹¹⁶⁾, PI3K⁽¹¹⁷⁾, JNK⁽¹¹⁸⁾, ERK⁽¹¹⁹⁾, NFκB⁽¹¹²⁾ and Rho Kinase⁽¹²⁰⁾.

The airway epithelium is an important barrier to the external environment and being the first point of contact for noxious substances and pathogens it plays a crucial role in the initiation of the inflammatory response. Airway epithelial cells have been shown to constitutively activate vitamin D. This is the first study to look at the effects of 1,25(OH)₂D₃ in airway epithelial cells on mediators important in COPD pathology. In this study the effect of vitamin D in primary human small airway epithelial cells and cell lines was studied in order to determine how vitamin D may be beneficial in modulating inflammatory mediators important in COPD and lung cancer pathology. Leading on from the characterisation of the inflammatory mediators expressed by widely used airway epithelial cell lines and primary cells, the sensitivity of cell lines to vitamin D treatment was studied along with its ability to modulate key mediators of lung inflammation including IL-1β, IL-6, IL-8, TNFα, MMP9 and MUC5AC.

4.2 Aims of this study

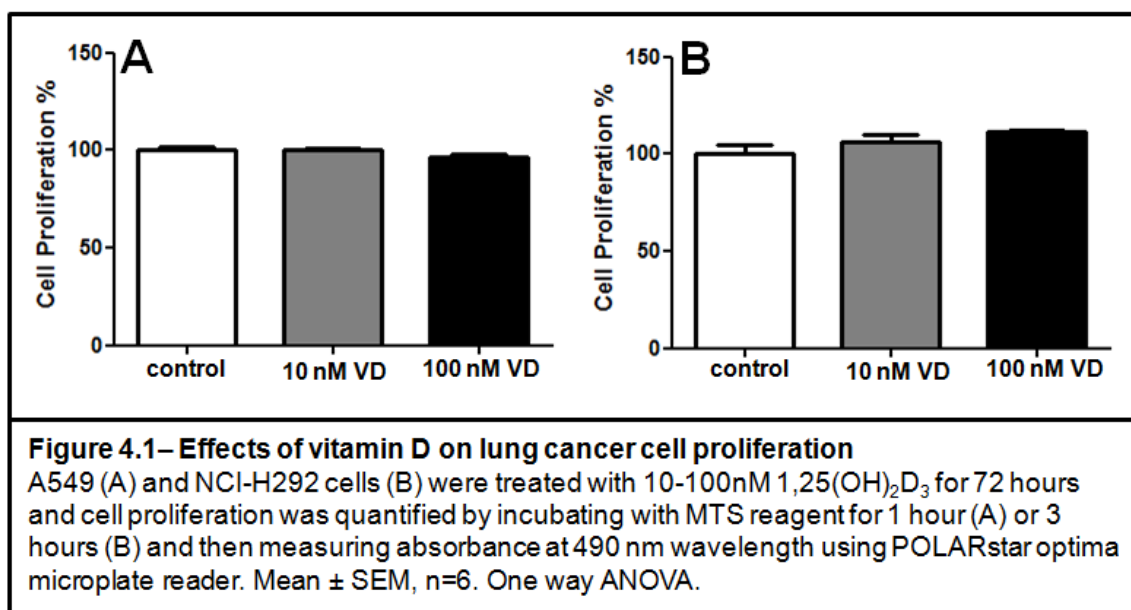
- To determine the sensitivity of primary human small airway epithelial cells and widely used human airway epithelial cell lines to vitamin D treatment
- To investigate the effects of vitamin D on the basal expression of inflammatory mediators in the human airway epithelial cells
- To investigate the effects of vitamin D on the induction of inflammatory mediators by TNF α /LPS in human airway epithelial cells
- To investigate the mechanism of action of vitamin D in human airway epithelial cells

4.3 Results

Part One – Effects of vitamin D on the basal expression of inflammatory mediators in human airway epithelial cells

4.3.1 Effects of vitamin D treatment on cell proliferation in lung cancer cell lines A549 and NCI-H292

Few studies have investigated the effects of vitamin D or its analogs in airway epithelial cells. Previous studies have shown anti-proliferative effects of $1,25(\text{OH})_2\text{D}_3$ in different lung cancer cell lines⁽¹⁶⁵⁻¹⁶⁷⁾. Therefore, the first objective in this study was to determine if any anti-proliferative effects are seen in the lung cancer cell lines used for this study. A549 and NCI-H292 cells were treated with 10 or 100 nM $1,25(\text{OH})_2\text{D}_3$ for 72 hours and cell proliferation was measured using an MTS assay. $1,25(\text{OH})_2\text{D}_3$ treatment had no significant effect on proliferation of either A549 or NCI-H292 cells (Figure 4.1).



4.3.2 Sensitivity of airway epithelial cells studied to vitamin D treatment

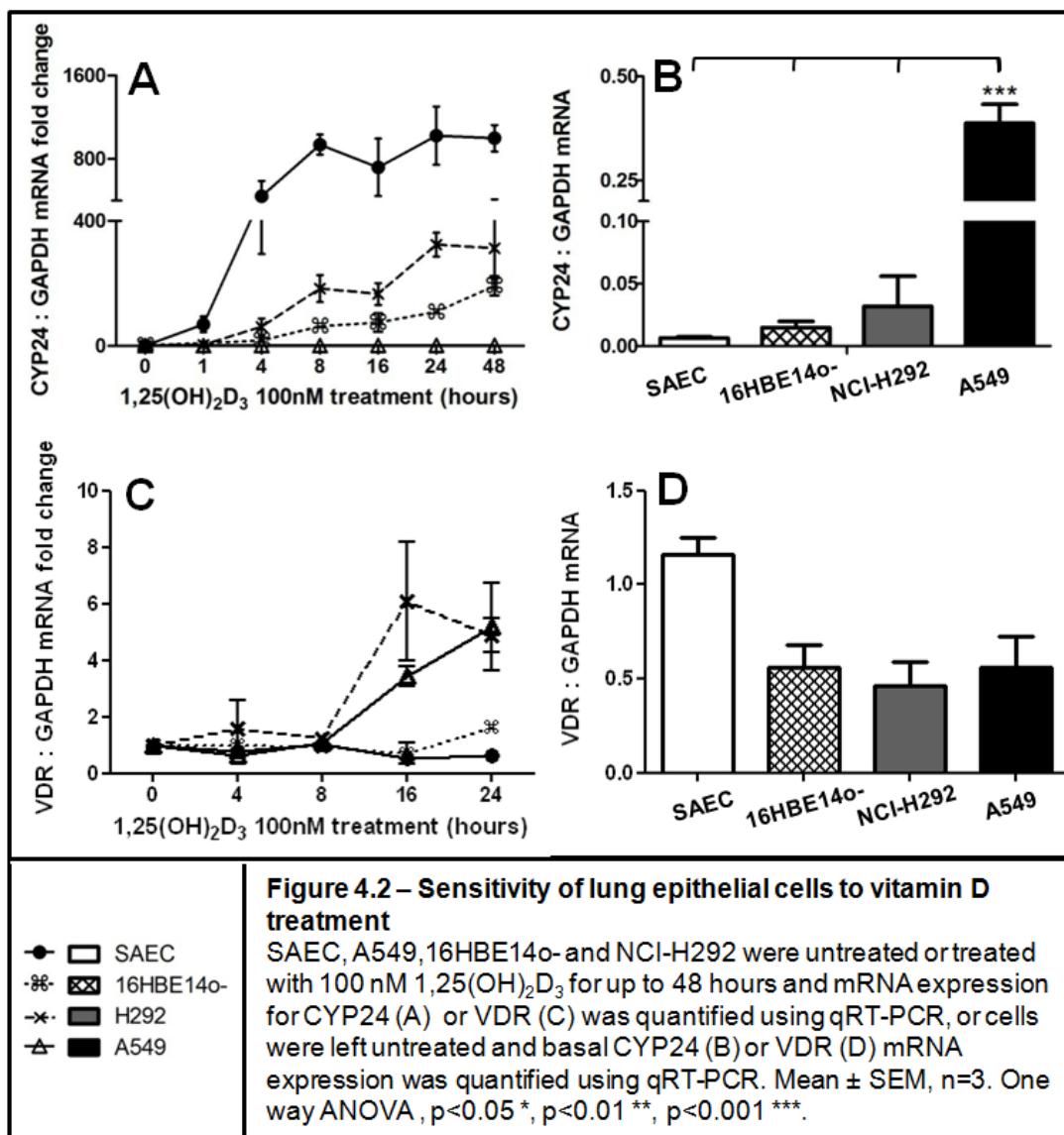
It was next important to establish whether or not all of the cell types used were responsive to vitamin D treatment and expressed the means with which to use vitamin D. The enzyme CYP24 is a key metabolising enzyme of vitamin D and as levels of vitamin D are tightly regulated, addition of vitamin D induces the expression of CYP24 as a gene containing a vitamin D response element regulated by receptor-ligand binding⁽²⁵⁰⁾. The different cell types were treated with $1,25(\text{OH})_2\text{D}_3$ and the induction of CYP24 mRNA expression was measured using qRT-PCR. Primary small airway epithelial cells, with a significant induction of mRNA after

24-48 hours ($p < 0.05$), showed the highest sensitivity to $1,25(\text{OH})_2\text{D}_3$ treatment followed by NCI-H292 24-48 hours ($p < 0.01$) and 16HBE14o- cells 24 hours ($p < 0.05$) and 48 hours ($p < 0.01$) shown in Figure 4.2.

$1,25(\text{OH})_2\text{D}_3$ had no effect on CYP24 expression in A549 cells (Figure 4.2) suggesting that they were not sensitive to $1,25(\text{OH})_2\text{D}_3$ treatment. The basal expression of CYP24 mRNA was also measured and compared among the different cell types (Figure 4.2). A549 were found to express high basal amounts of CYP24 mRNA, which was significantly different to the other cell types tested in the study ($p < 0.001$), suggesting that $1,25(\text{OH})_2\text{D}_3$ may be metabolised before it has an opportunity to act in these cells.

VDR mRNA expression was also studied to note any differences between the cell lines. The expression of VDR mRNA was highest in the primary cells, although there was no significant difference between the basal expression of VDR in the different cell types (Figure 4.2). $1,25(\text{OH})_2\text{D}_3$ significantly induced VDR mRNA expression in the NCI-H292 at 16 hr ($p < 0.05$); however, this was not significant in SAEC and 16HBE14o- cells. Interestingly, VDR expression was significantly increased at 16 ($p < 0.05$) and 24 hours ($p < 0.01$) following $1,25(\text{OH})_2\text{D}_3$ treatment in A549 cells, which may be a mechanism where the cell upregulates receptor expression to make use of any vitamin D that has not been metabolised.

Cancer cells have been shown to dysregulate the vitamin D pathway in order to avoid the anti-cancer and anti-proliferative effects of vitamin D treatment. The CYP24:VDR ratio has been identified as important with some cancers increasing CYP24 expression and decreasing VDR expression to dampen down vitamin D signalling. As such in this study the SAEC had the highest VDR expression and lowest CYP24 expression with the greatest induction of CYP24 on vitamin D treatment. This suggests that they are the most responsive cell type to vitamin D whereas conversely the A549 cells had the greatest basal CYP24 expression and lowest VDR expression suggesting they are the least responsive cell line to vitamin D treatment of those that were used in this study.

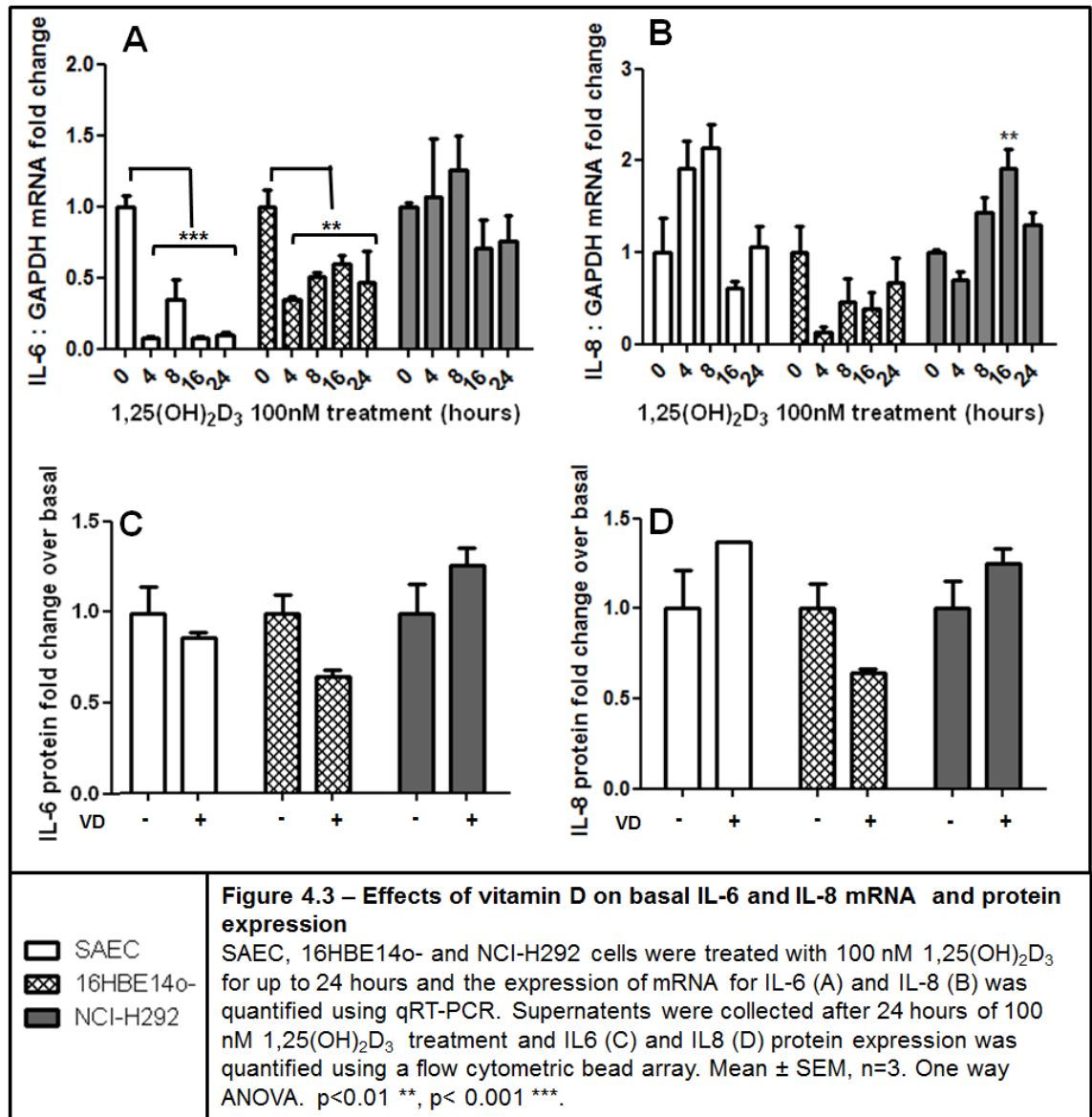


4.3.3 Effects of vitamin D on basal expression of IL-6 and IL-8 in cells responsive to vitamin D treatment

In chapter 3, IL-6 and IL-8 were the key cytokines tested that were detectable at both mRNA and protein levels in the different airway epithelial cells tested. It was firstly investigated if 1,25(OH)₂D₃ had any effect on these mediators in the cell types studied except for in the A549 cells which appear to be the least responsive to vitamin D.

In SAEC and 16HBE14o-, IL-6 mRNA expression was significantly reduced upon treatment with 1,25(OH)₂D₃, (p<0.001) and (p<0.01) respectively for each time point tested shown in Figure 4.3A. IL-8 mRNA was significantly increased at 16 hours in the NCI-H292 (p<0.01) Figure 4.3B. There was no significant difference in protein expression between the different cells on vitamin D treatment however,

there was a trend of a decrease in IL-6 in SAEC and both IL-6 and IL-8 in the 16HBE14o-, but an increase in IL-8 in the SAEC (Figure 4.3C and D).



4.3.4 Mechanism of vitamin D modulating basal IL-6 mRNA expression

The reduction of basal IL-6 mRNA and protein expression by 1,25(OH)₂D₃ was mirrored in the SAEC by the 16HBE14o- and so the mechanism of action was investigated in the 16HBE14o- cells. 1,25(OH)₂D₃ has previously been reported to modulate NFκB^(36, 90, 108-113, 115), p38 MAPK⁽¹¹⁶⁾, PI3K⁽¹¹⁷⁾, JNK⁽¹¹⁸⁾, ERK⁽¹¹⁹⁾ and Rho kinase⁽¹²⁰⁻¹²²⁾ signalling pathways.

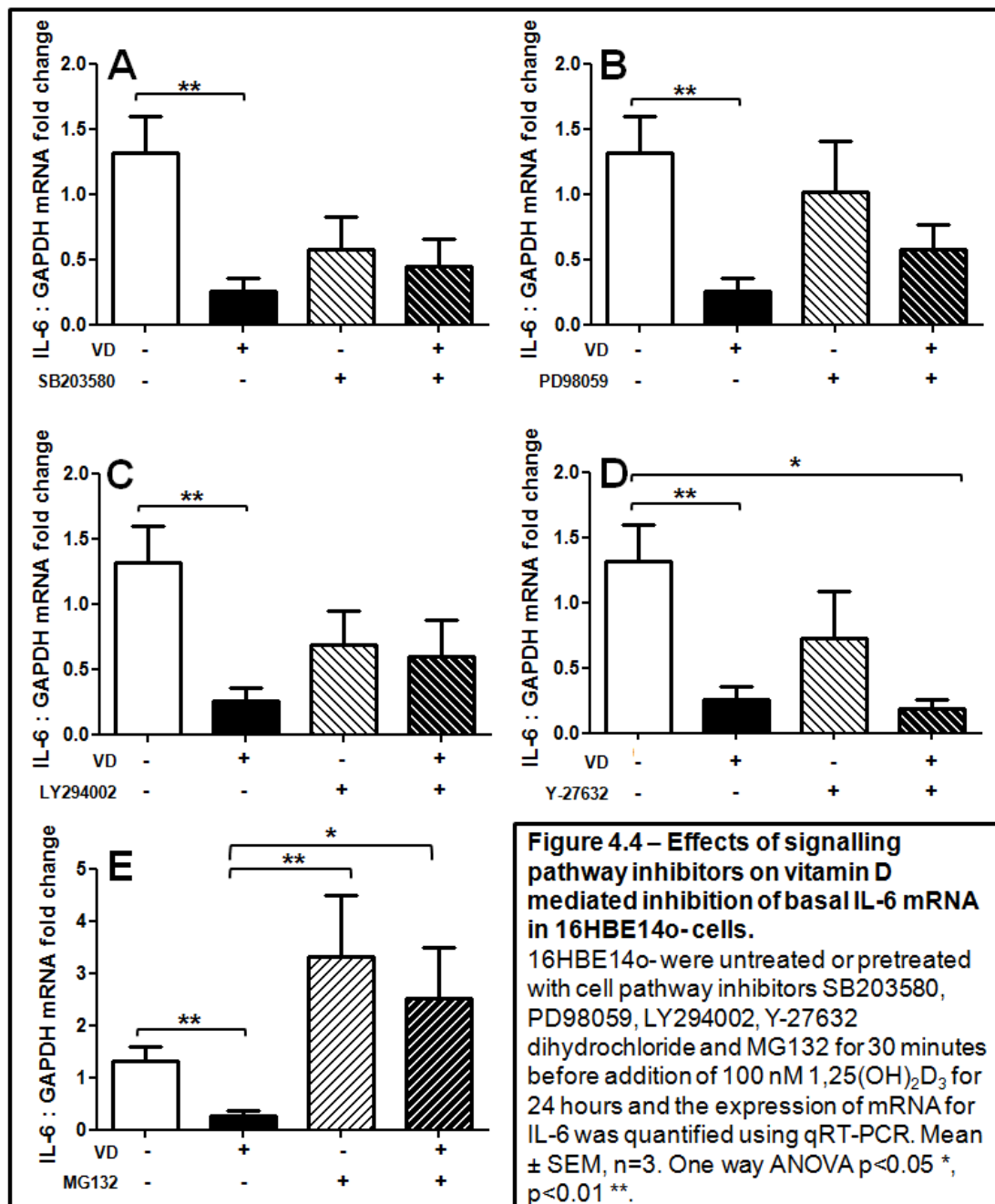
A range of inhibitors of these pathways were used to determine if intracellular kinases were involved in the regulation of IL-6 by 1,25(OH)₂D₃. Firstly, the cytotoxicity of the different signalling pathway inhibitors was tested using a cellular cytotoxicity assay in order to determine a non cytotoxic concentration to use within

the cells (Appendix Figure 7.1). Concentrations of 1-10 μM of each inhibitor was not found to be toxic except with the proteasomal inhibitor MG132 which was significantly cytotoxic at 5 ($p < 0.01$) and 10 μM ($p < 0.001$) (Appendix Figure 7.1). Therefore the maximum tolerated dose of 10 μM was used for each inhibitor except MG132 where 1 μM was used.

Treatment of 16HBE14o- cells with 100 nM $1,25(\text{OH})_2\text{D}_3$ for 24 hours reduced basal IL-6 expression as shown in Figure 4.4 A-E. Treatment of 16HBE14o- with 10 μM SB203580, PD98058 or LY294002, p38 MAPK, MAPKK and PI3K inhibitors respectively, had no significant effect alone or in combination with $1,25(\text{OH})_2\text{D}_3$ on basal IL-6 mRNA expression although data was variable.

Using 10 μM Y-27632 dihydrochloride, a selective inhibitor of the Rho-associated protein kinase (ROCK) p160, had no significant effect on basal IL6 mRNA expression although this was variable. Combined treatment of inhibitor with $1,25(\text{OH})_2\text{D}_3$ significantly reduced IL6 mRNA expression the same as $1,25(\text{OH})_2\text{D}_3$ alone ($p < 0.05$) when compared to basal levels, but not to the inhibitor alone. This suggests $1,25(\text{OH})_2\text{D}_3$ does not regulate ROCK to reduce IL6 expression.

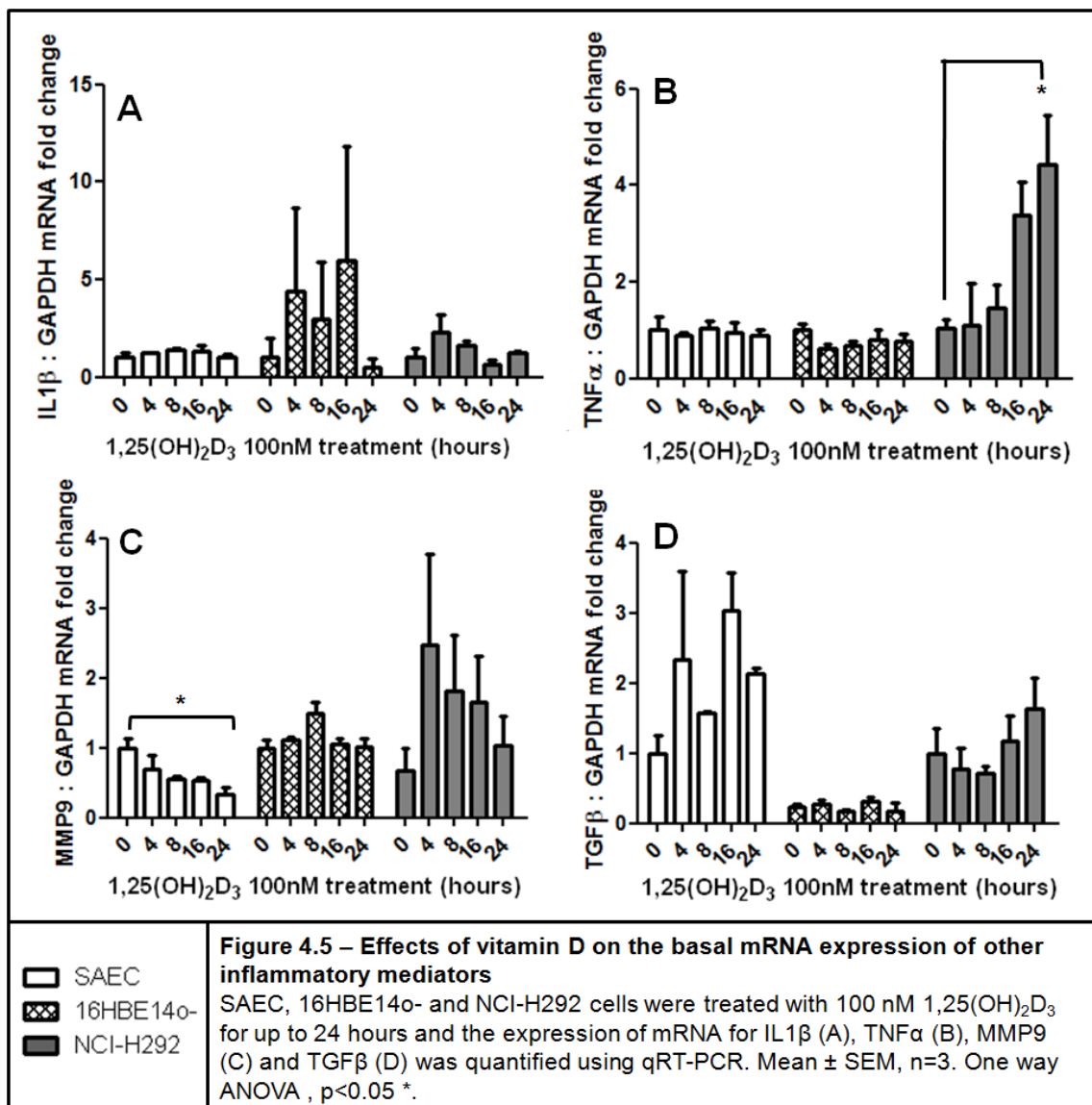
MG132 is a 26S proteasome inhibitor, which also inhibits NF κ B activity by preventing degradation of its inhibitor, I κ B. MG132 increased basal IL-6 mRNA expression and also increased its expression when combined with $1,25(\text{OH})_2\text{D}_3$ although the data was variable and not significant so no conclusions can be drawn.



4.3.5 Effects of vitamin D on basal expression of mRNA for other inflammatory mediators

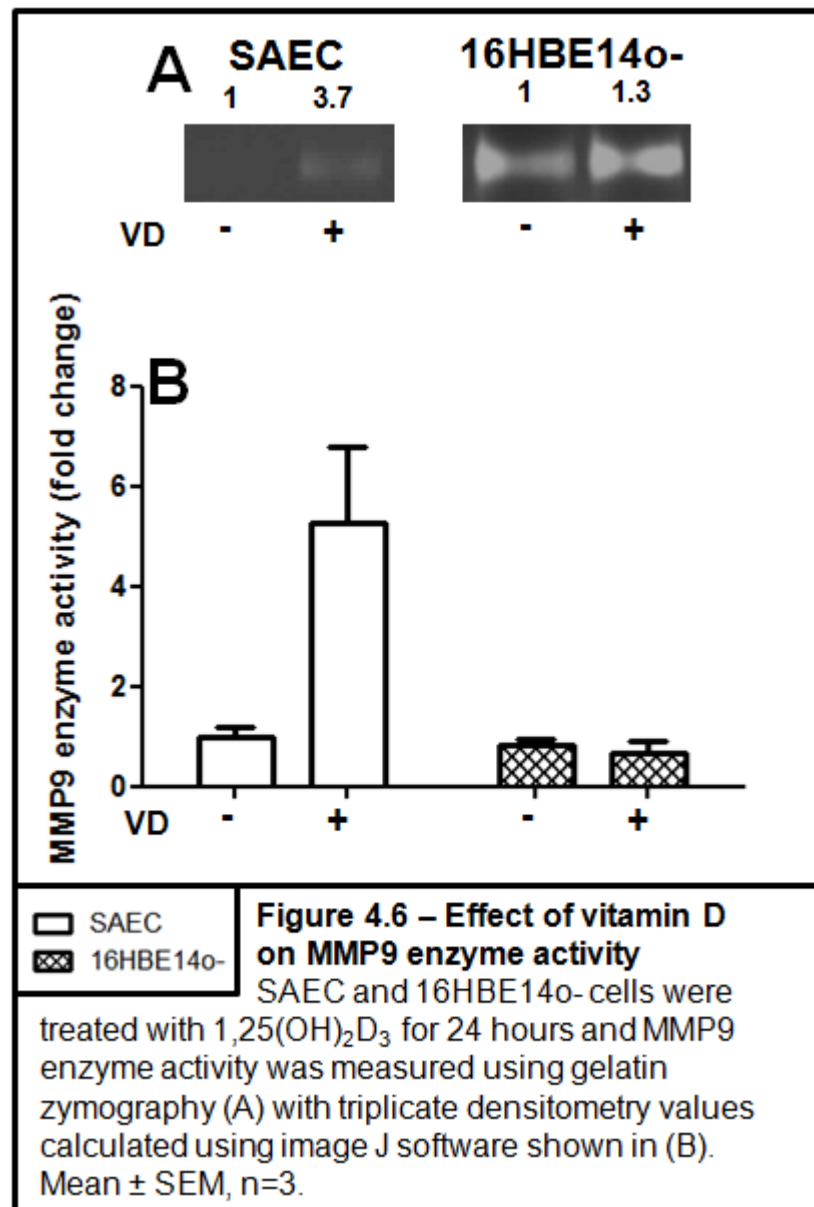
As no conclusive mechanism of action could be determined for the effect of 1,25(OH)₂D₃ in airway epithelial cells, the effects of 1,25(OH)₂D₃ on basal expression for other cytokine mRNA which had been shown to be expressed in airway epithelial cells was tested. This allowed comparison of the effects of 1,25(OH)₂D₃ on different mediators which may point to certain transcription factors or shared signalling intermediates that are modulated by 1,25(OH)₂D₃.

1,25(OH)₂D₃ had no significant effect on IL1β or TGFβ1 mRNA basal expression in any cell type and significantly induced TNFα mRNA after 24 hours of treatment in the NCI-H292 cells (p<0.05) Figure 4.5. 1,25(OH)₂D₃ significantly reduced the expression of MMP9 in the SAEC after 24 hours (p<0.05) but not in the other cell lines. IL-10 and IL-12p70 basal mRNA expression was not detected basally (see section 3.3.2, chapter 3) however, they have been shown previously to contain vitamin D response elements and therefore the effects of 1,25(OH)₂D₃ on the expression of these was investigated, but still no mRNA was detected (data not shown).



4.3.6 Effects of vitamin D on MMP9 enzyme activity in airway epithelial cells

As $1,25(\text{OH})_2\text{D}_3$ significantly reduced MMP9 mRNA in the SAEC, the effects on enzyme activity were investigated. SAEC and 16HBE14o- cells were treated with 100 nM $1,25(\text{OH})_2\text{D}_3$ for 24 hours and the cell supernatants were collected and analysed using gelatin zymography to measure MMP9 enzyme activity. Interestingly, there was no significant difference on $1,25(\text{OH})_2\text{D}_3$ treatment in the 16HBE14o- cells fitting with the mRNA data, however, paradoxically there was an increase in MMP9 enzyme activity in the SAEC after $1,25(\text{OH})_2\text{D}_3$ treatment although this was not significant. Representative gelatin zymography images are shown in Figure 4.6A with the densitometry values of a triplicate experiment shown in Figure 4.6B.



4.3.7 Mechanism of action of vitamin D on induction of TNF α mRNA in NCI-H292 cells As shown previously in Section 4.3.5 and Figure 4.5, 1,25(OH)₂D₃ significantly activates TNF α mRNA expression after 24 hour of treatment in the NCI-H292 cell line. In order to determine a possible mechanism of action, molecular inhibitors of p38 MAPK, ERK MAPK, PI3K, ROCK, JNK and NF κ B were used to try and block this effect. Firstly, the cytotoxicity of the different inhibitors in the NCI-H292 cells was studied to ensure the concentrations used were not cytotoxic (Appendix Figure 7.2).

SB203580 a p38 MAPK inhibitor, LY294002 a PI3K inhibitor and SP600125 a JNK inhibitor, had no significant effect alone but in combination with 1,25(OH)₂D₃ gave a synergistic induction of TNF α ($p < 0.05$ at least) (Figure 4.7 A,C,F) suggesting that these signalling molecules are negative regulators of TNF α mRNA expression. Using a ROCK inhibitor Y-27632 dihydrochloride, PD98059 MAPKK inhibitor and MG132 proteasomal inhibitor the data was more variable (Figure 4.7 B,D,E) and hence was not significantly different so no conclusions can be drawn and further study is required.

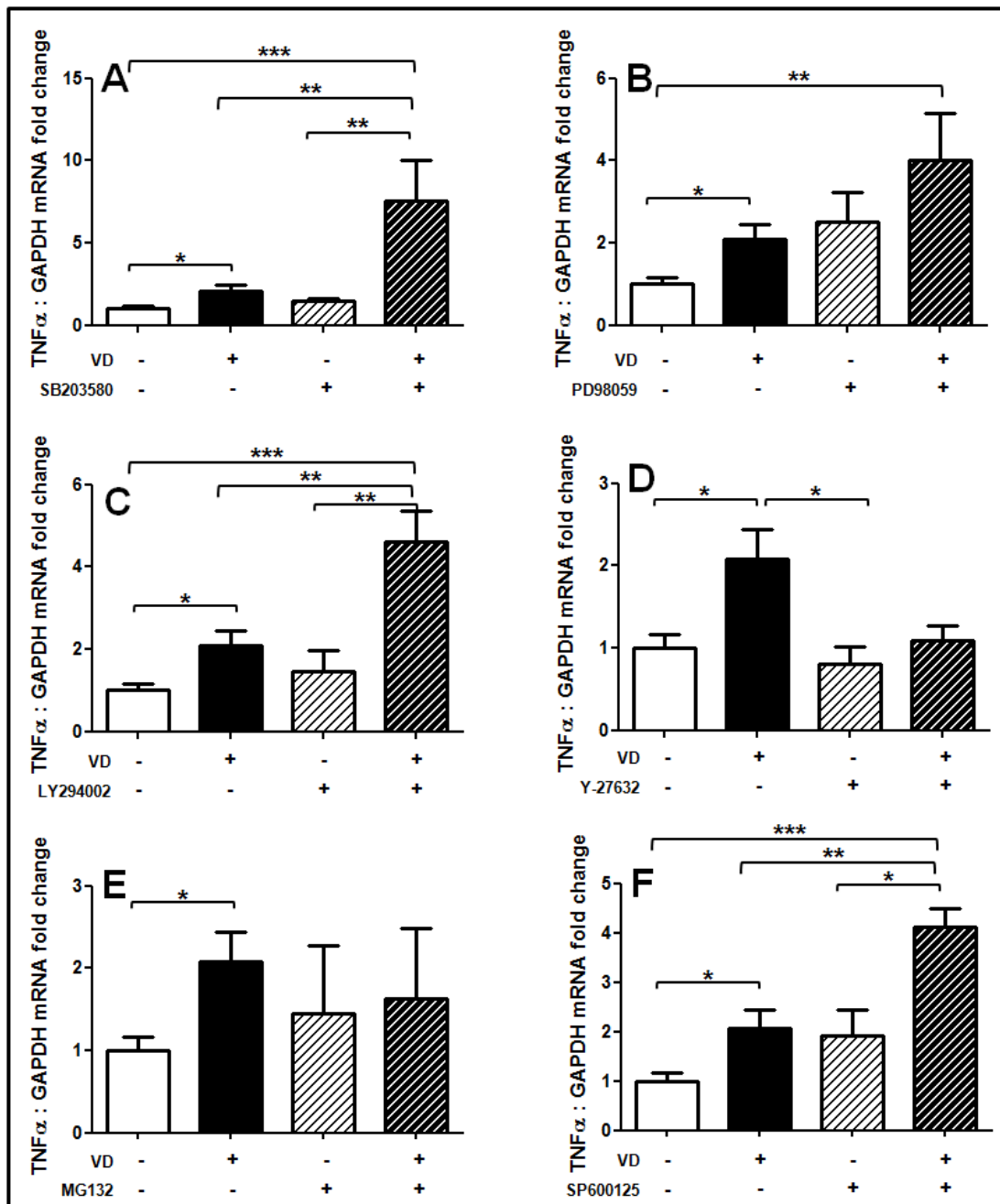


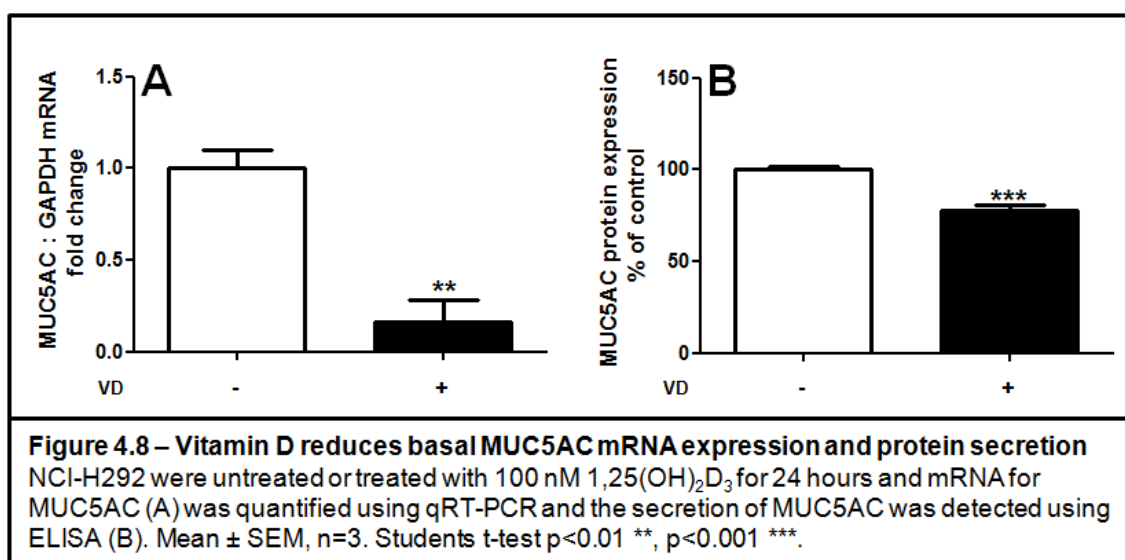
Figure 4.7 – Effect of signalling pathway inhibitors on vitamin D mediated activation of basal TNF α mRNA in NCI-H292 cells.

NCI-H292 cells were untreated or pre-treated with cell pathway inhibitors SB203580 (A), PD98059 (B), LY294002 (C), Y-27632 (D), MG132 (E) and SP600125 (F) for 30 minutes before addition of 100 nM 1,25(OH) $_2$ D $_3$ for 24 hours and the expression of mRNA for TNF α was quantified using qRT-PCR. Mean \pm SEM, n=3. One way ANOVA. p<0.05 *, p<0.01 **, p<0.001 ***.

4.3.8 Effect of vitamin D on the basal expression of MUC5AC in the different cell types

MUC5AC was found to be expressed in the NCI-H292 cell line used in this study which was responsive to $1,25(\text{OH})_2\text{D}_3$ treatment. The effect of $1,25(\text{OH})_2\text{D}_3$ on basal MUC5AC mRNA expression and MUC5AC protein secretion was determined.

$1,25(\text{OH})_2\text{D}_3$ significantly reduced basal MUC5AC mRNA expression after 24 hours of treatment ($p < 0.01$) and significantly reduced basal MUC5AC protein secretion after 24 hours of treatment ($p < 0.001$).



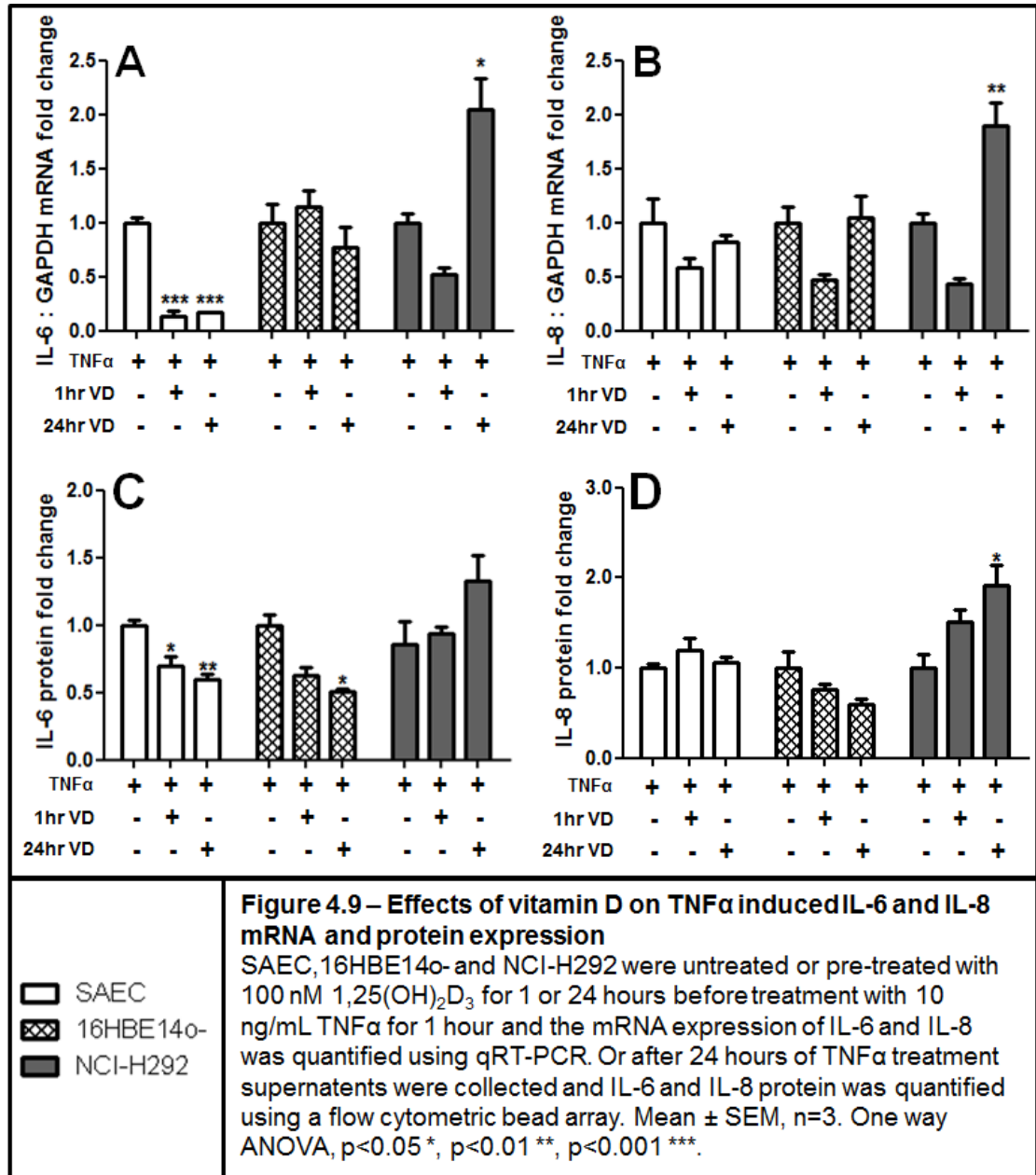
Part Two – Effects of vitamin D on inflammatory responses induced by TNF α or LPS

4.3.9 Effects of vitamin D on TNF α induction of inflammatory mediators

In chapter 3, the induction of inflammatory mediators by TNF α and LPS was investigated and it was concluded that 1 hr of treatment gave peak induction of mRNA expression for the various pro-inflammatory mediators. Therefore, cells were incubated with $1,25(\text{OH})_2\text{D}_3$ for various times and concentrations before the addition of TNF α for 1 hr in the different airway epithelial cells.

In SAEC, pre-incubation with 100 nM $1,25(\text{OH})_2\text{D}_3$ was found to significantly reduce TNF α induced IL-6 mRNA and protein expression at both 1 and 24 hr incubation ($p < 0.05$ at least)(Figure 4.9A and C). A similar effect was found in the 16HBE14o- for IL-6 protein expression (Figure 4.9C). $1,25(\text{OH})_2\text{D}_3$ did not significantly affect TNF α induced IL-8 mRNA and protein expression in the SAEC or 16HBE14o- cells.

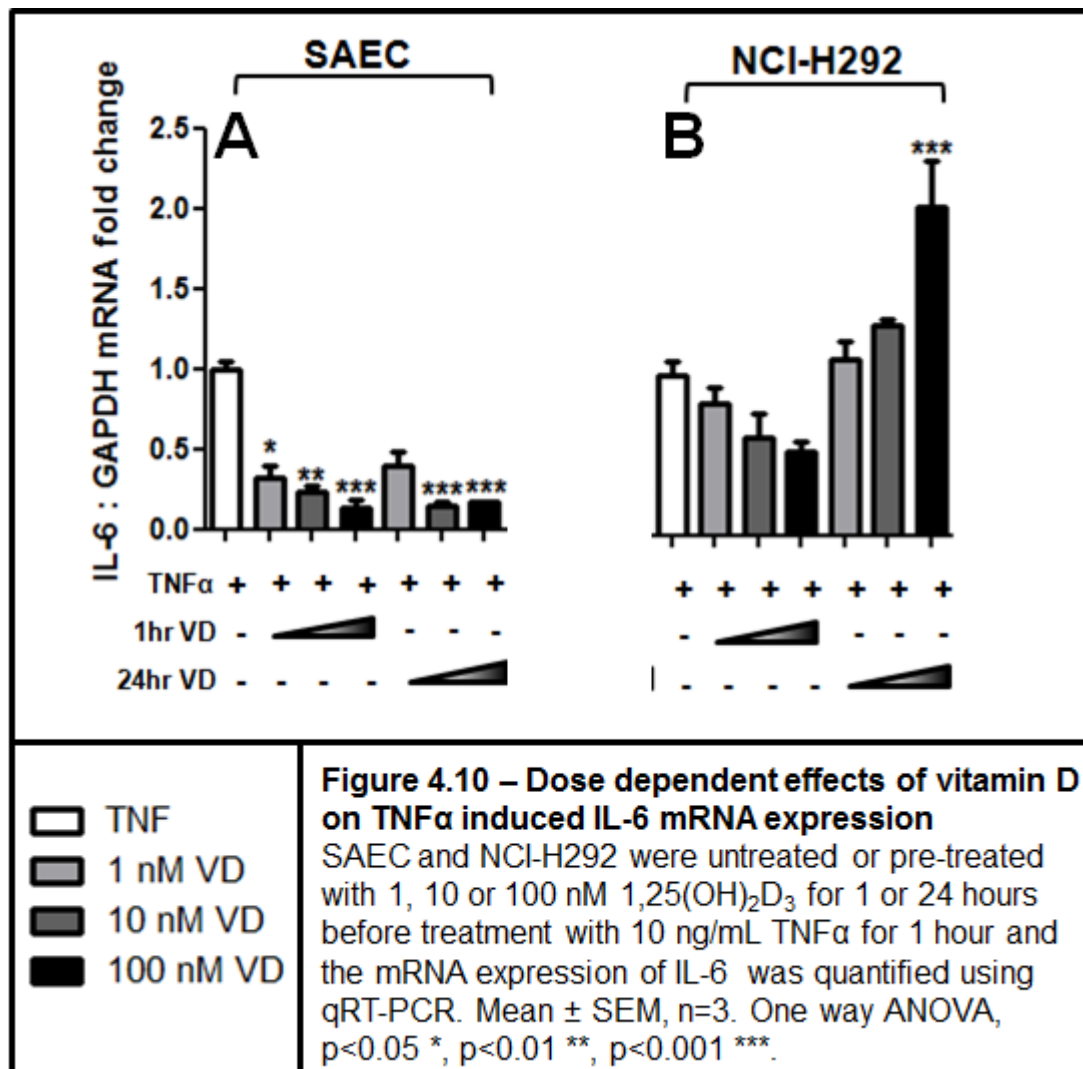
The NCI-H292 cells were also found to have a non significant reduction of IL-6 and IL-8 mRNA with 1 hr pre-incubation, but a significant induction of IL-6 and IL-8 mRNA after 24 hr pre-incubation of 1,25(OH)₂D₃ before TNF α treatment (p<0.05 at least) with a non significant trend of increased IL-6 and a significant increase in IL-8 protein expression (p<0.01) with 24 hr of 1,25(OH)₂D₃ pre-treatment (Figure 4.9A-D).



4.3.10 Dose dependent effects of vitamin D in SAEC and NCI-H292

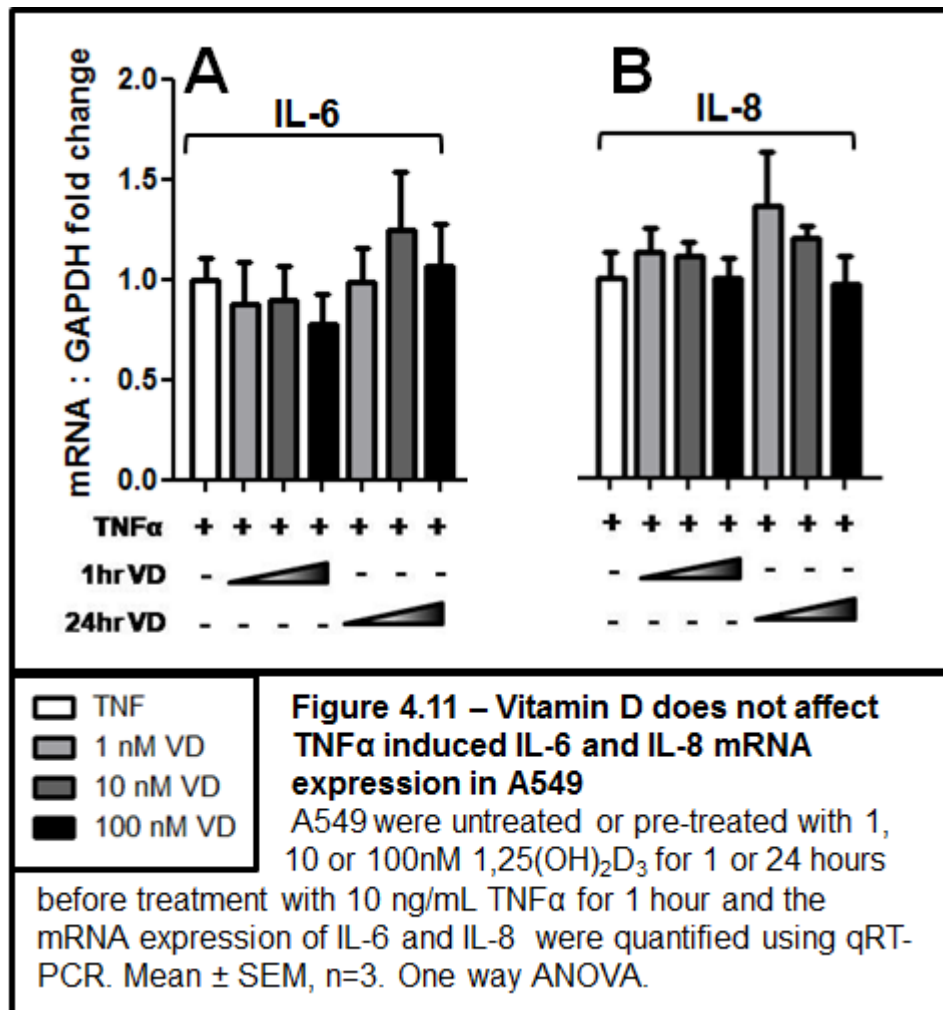
The effects in 4.9 were using the highest dose of 1,25(OH)₂D₃ 100 nM. Different doses were used in order to determine whether or not this was a dose dependent effect. Indeed, using 1-100 nM 1,25(OH)₂D₃ pre-treatment for 1 or 24 hr in the SAEC

leads to a dose dependent reduction in IL-6 mRNA expression. In addition, the non significant reduction in IL-6 expression with 1 hour pre-treatment in the NCI-H292 as well as the significant induction with 24 hour pre-treatment ($p < 0.001$) were also found to be dose dependent (Figure 4.10).



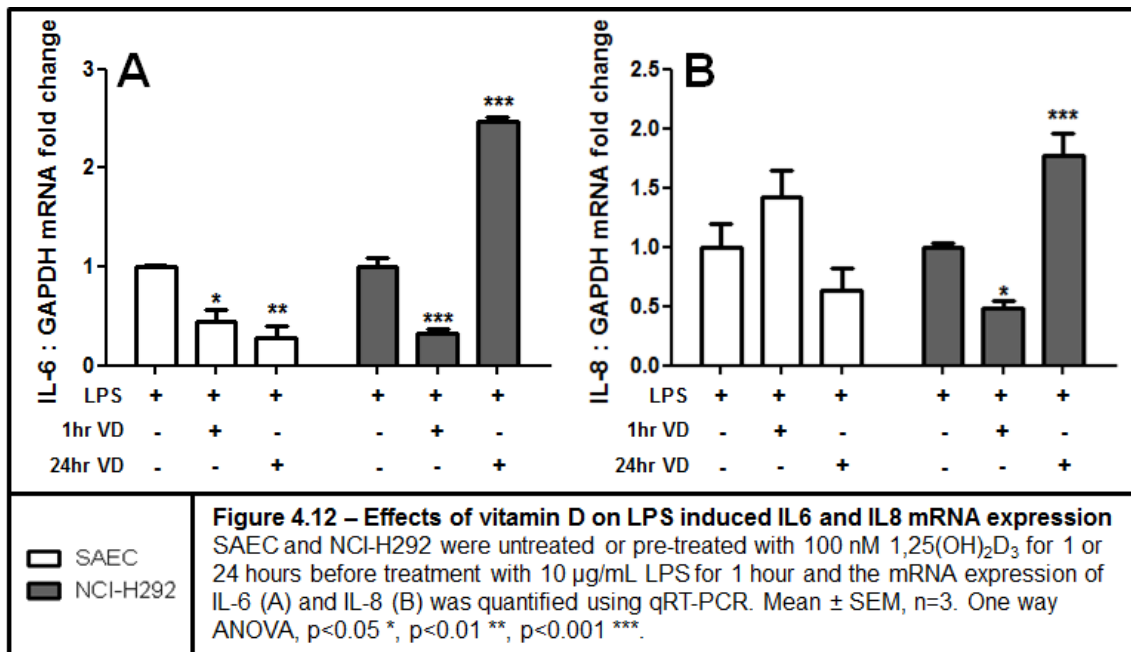
4.3.11 Vitamin D does not affect TNF α induced IL-6 and IL-8 mRNA expression in the CYP24 over-expressing A549 cells

Although A549 cells express high quantities of CYP24, it was investigated whether or not treatment with 1,25(OH) $_2$ D $_3$ in this cell line would have any effect on TNF α induced IL-6/IL-8 expression. 1,25(OH) $_2$ D $_3$ (1-100 nM) for either 1 hr or 24 hr before 1 hr TNF α had no significant effect on IL-6 and IL-8 mRNA expression in the A549, again suggesting that the cells are insensitive to 1,25(OH) $_2$ D $_3$ treatment (Figure 4.11).



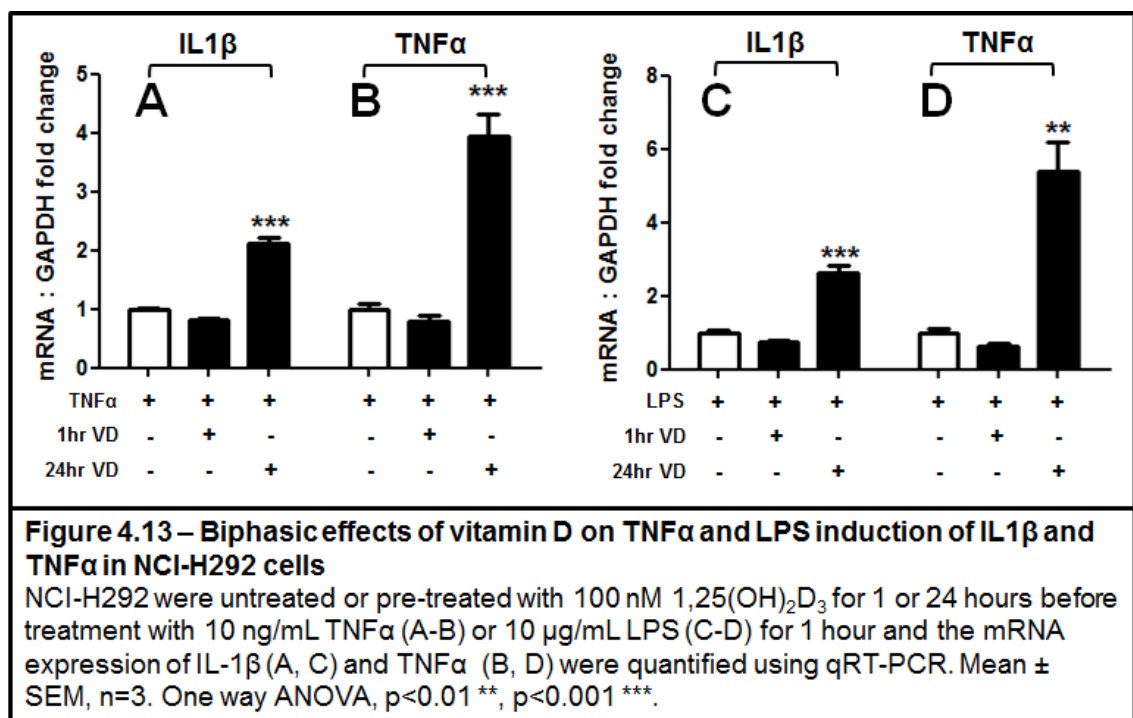
4.3.12 Effects of vitamin D on LPS induced IL-6 and IL-8 mRNA expression

To determine whether the effects of vitamin D on induced expression of cytokines is unique for the TNF α signalling or whether a similar effect would be seen using other inflammatory stimuli, LPS was used and investigated in the SAEC and NCI-H292 cells. Again, pre-treatment of SAEC for 1 or 24 hrs with 100 nM 1,25(OH) $_2$ D $_3$ before LPS treatment for 1 hr significantly reduced IL-6 mRNA expression but had no effect on IL-8 mRNA expression. Similarly, 1 hr pre-treatment of NCI-H292 before LPS treatment gives a significant reduction in IL-6 and IL-8 mRNA expression ($p < 0.001$ and $p < 0.05$ respectively) and 24 hr pre-treatment gives a significant induction of both IL-6 and IL-8 mRNA expression ($p < 0.001$).



4.3.13 Effects of vitamin D on TNF α /LPS induction of mRNA for other inflammatory mediators in NCI-H292 cells

In order to determine whether the biphasic effect found in the NCI-H292 cells were only found with IL-6 and IL-8, the effects of 1,25(OH)₂D₃ pre-treatment on LPS and TNF α induction of IL1 β and TNF α was also studied. Indeed, the same pattern was found with a significant induction with 24 hr pre-treatment (Figure 4.13).



4.3.14 Effects of vitamin D pre-treatment on MMP9 mRNA expression and enzyme activity

In section 4.3.6, $1,25(\text{OH})_2\text{D}_3$ treatment was found to reduce basal MMP9 mRNA expression in SAEC and 16HB14o- but paradoxically increased MMP9 enzyme activity in the SAEC. In this section the effect of $1,25(\text{OH})_2\text{D}_3$ on $\text{TNF}\alpha$ induction of MMP9 mRNA and enzyme activity was investigated. Pre-treatment for 1 or 24 hours with 100 nM $1,25(\text{OH})_2\text{D}_3$ significantly reduced $\text{TNF}\alpha$ induction of MMP9 mRNA expression in the SAEC and the 16HBE14o- cells (Figure 4.14A). This effect was mirrored for enzyme activity in the 16HBE14o- cells with a significant reduction of expression on 24 hours pre-treatment with $1,25(\text{OH})_2\text{D}_3$ (Figure 4.14B and C), although as shown in Figure 3.6 $\text{TNF}\alpha$ did not significantly induce MMP9 enzyme activity in the 16HBE14o- cells. Similar to the basal expression in section 4.4.6, paradoxically there was a synergistic induction of MMP9 enzyme activity in the SAEC on 24 hrs $1,25(\text{OH})_2\text{D}_3$ pre-treatment before $\text{TNF}\alpha$ addition (Figure 4.14B and C).

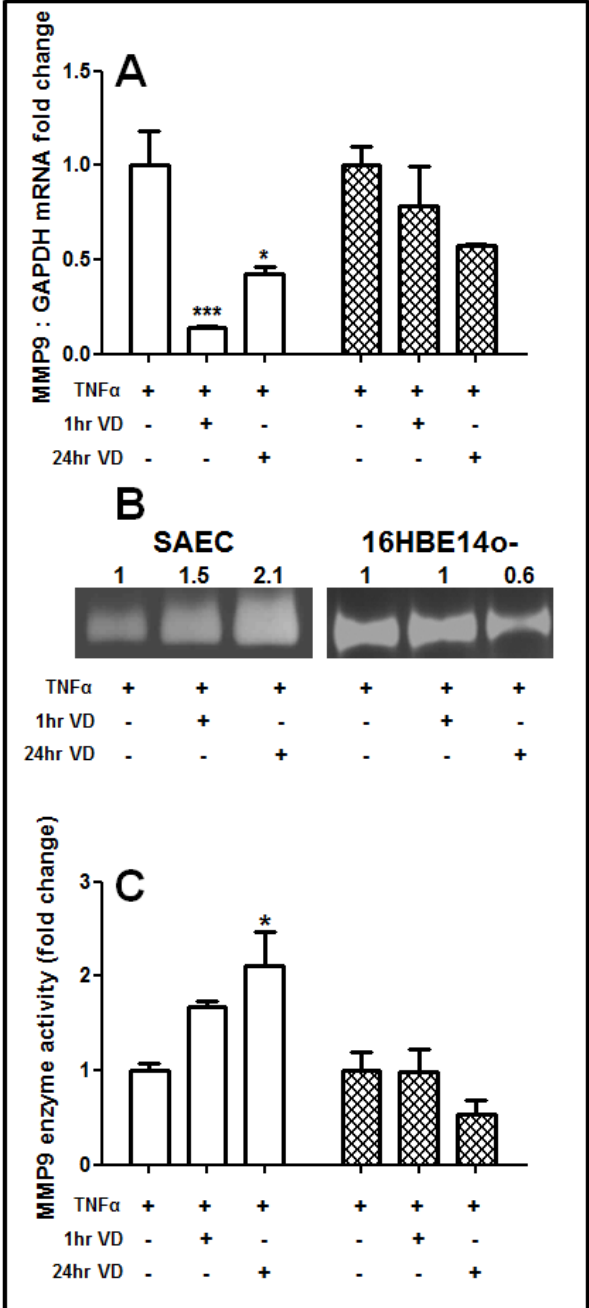


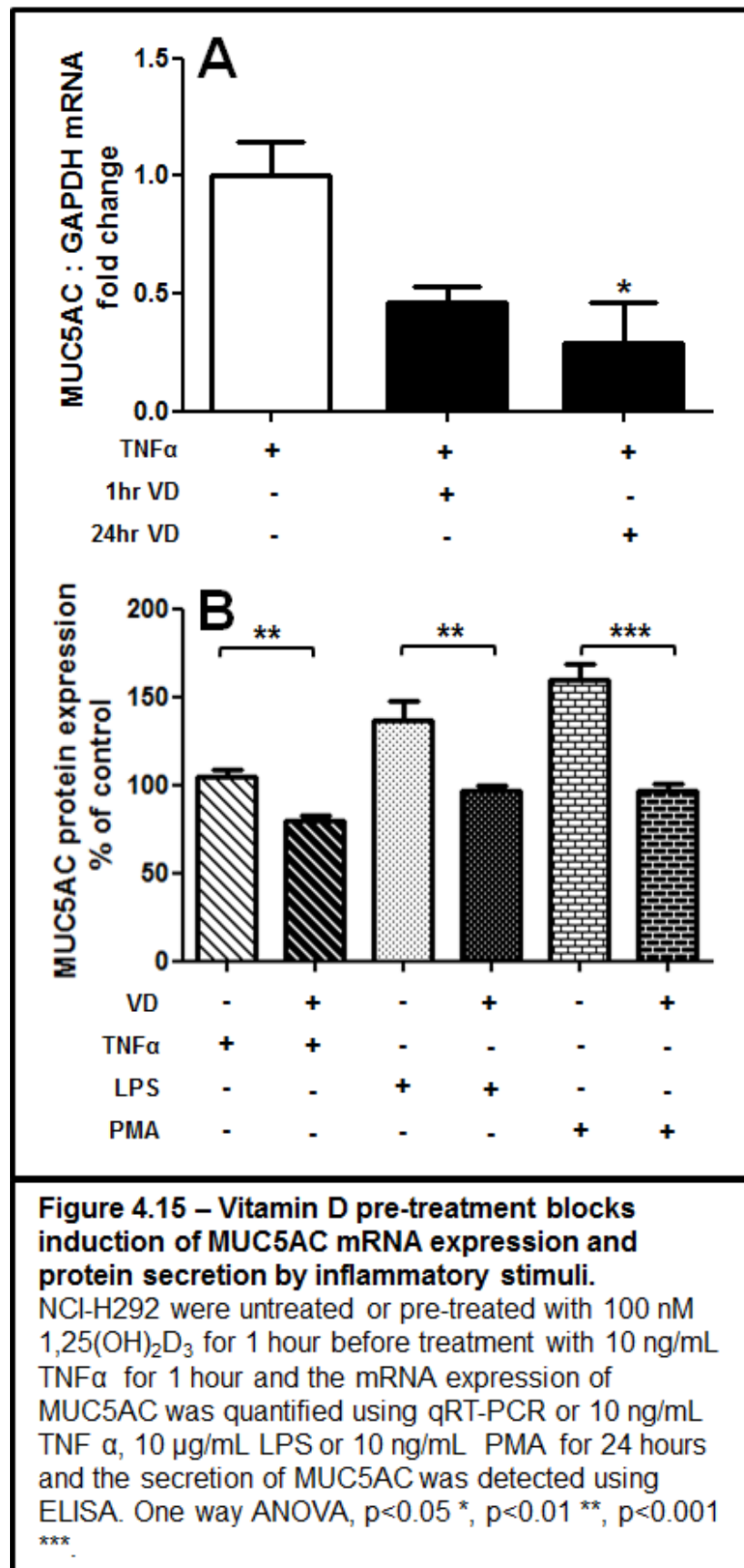
Figure 4.14 – Effect of vitamin D on MMP9 mRNA expression and enzyme activity in SAEC and 16HBE14o- cells

SAEC and 16HBE14o- cells were untreated or treated with 100 nM 1,25(OH)₂D₃ for 1 or 24 hours before treatment of 10 ng/mL TNFα for 1 hour and mRNA (A) was quantified using qRT-PCR, or 24 hours and enzymatic activity was measured in cell supernatants using gelatin zymography (B) with densitometry values for a triplicate experiment shown in (C). Mean ± SEM, n=3. One way ANOVA, p<0.05 *, p<0.001 ***.

4.3.15 Effects of vitamin D pre-treatment on MUC5AC induction by known inducers

Pre-treatment of the NCI-H292 mucous expressing cell line for 24 hours with $1,25(\text{OH})_2\text{D}_3$ gave a significant reduction in $\text{TNF}\alpha$ induced MUC5AC mRNA expression ($p < 0.05$) (Figure 4.15A).

In chapter 3 section 3.3.8, LPS and PMA were found to significantly induce MUC5AC protein secretion. Pre-treatment with $1,25(\text{OH})_2\text{D}_3$ for 24 hours before stimulation with these compounds for 24 hours was found to significantly reduce the induction of MUC5AC protein secretion, even for $\text{TNF}\alpha$ which did not significantly induce expression in section 4.4.8 ($p < 0.01$ at least) (Figure 4.15B).



4.4 Discussion

The novel findings in this study were that vitamin D modulates IL-6 expression in the SAEC, 16HBE14o- and NCI-H292 cells. In addition, vitamin D exhibited a biphasic response in the NCI-H292 cells with a reduction or induction of cytokine mRNA depending on the length of pre-incubation. In the mucous producing NCI-H292 cells, vitamin D was also shown to block MUC5AC basal mRNA expression and protein secretion and its induction by known activators. These findings help to further characterise the potential benefits of vitamin D in inflammatory lung diseases such as chronic obstructive pulmonary disease and lung cancer. The mechanism of action of vitamin D was also studied, but the results were inconclusive and this area requires further investigation in the future.

There are very few studies where vitamin D or its analogs have been used in airway epithelial cells. This is the first study to report the effects of vitamin D in primary small airway epithelial cells. A number of studies have shown an anti-proliferative effect of vitamin D treatment in some lung cancer cell lines expressing the VDR. 1,25(OH)₂D₃ and analogs significantly inhibited cell proliferation in the NCI-H82 and NCI-H209 small cell lung carcinoma and the EBC-1 and H520 non-small cell carcinoma cell lines⁽¹⁶⁵⁻¹⁶⁷⁾. However, in the NCI-H292 and A549 lung cancer cells used in this study, vitamin D was not found to have an anti-proliferative effect (Figure 4.1) despite the NCI-H292 cells being responsive to 1,25(OH)₂D₃ treatment. A recent study has found that vitamin D does have an antiproliferative effect in both the A549 and NCI-H292 cells but this after 7 days of treatment⁽²⁵¹⁾. Inhibiting CYP24 increased this antiproliferative effect.

Previous studies have shown that cancer cells, including those derived from lung tissue, can harbour mutations/alterations dysregulating parts of the vitamin D pathway, by altering expression of the VDR, CYP27 and CYP24. This is thought to be a mechanism by which tumours can evade the anti-cancer properties of vitamin D. In this study, the expression of CYP24 and VDR was compared in all the cell types used to see if they are responsive to vitamin D treatment. All of the cells tested in this study expressed detectable basal levels of CYP24 and VDR mRNA. CYP24 was increased upon treatment with vitamin D in all cells except A549 suggesting they are insensitive to vitamin D treatment, which has previously been shown⁽¹⁷⁹⁾. SAEC had the lowest expression of basal CYP24 mRNA and the greatest VDR mRNA

expression. They also had the greatest sensitivity to vitamin D treatment with the largest fold induction of CYP24 on vitamin D treatment. 16HBE14o- cells were also sensitive to vitamin D with an induction of CYP24 on vitamin D treatment which also is novel. This is the first study to my knowledge to show that primary small airway epithelial cells are sensitive to vitamin D treatment. Previous studies have shown a high expression of CYP24 by the A549 cells, and a relatively high expression in the NCI-H292 and expression of VDR by both cell types which was also found in this study^(123, 179). CYP24 upregulation and VDR down regulation has been shown previously in lung tumours and lung cancer cells in comparison to normal tissue, with 1,25(OH)₂D₃ clearance being enhanced in malignant cells through transcriptional regulation of CYP24 and metabolism of CYP24 mRNA^(106, 179, 180, 252, 253). Although the A549 cells were not found to be sensitive to vitamin D treatment in this study, a recent study has shown that treatment of A549 cells with 1,25(OH)₂D₃ decreases DNA damage⁽²⁵⁴⁾.

IL-6 and IL-8 were the key cytokines of those tested that were detectable at both protein and mRNA levels in the different airway epithelial cells. Interestingly, in this study 1,25(OH)₂D₃ was shown to reduce basal expression of IL-6 mRNA in the SAEC and 16HBE14o- and appeared to increase IL-8 mRNA expression in the SAEC, with no significant effects in the NCI-H292. A previous study on the effect of 1,25(OH)₂D₃ on IL-6 and IL-8 expression in cells from cystic fibrosis showed modulation of both of these mediators⁽¹⁵⁰⁾. To further investigate the reduction in IL-6 mRNA which was mirrored by the 16HBE14o- in the SAEC, the 16HBE14o- were used to study the potential signalling pathways involved.

Previous studies have shown that vitamin D reduces IL-6 production in normal prostate cells by inhibition of p38⁽¹¹⁶⁾ and that induction of IL-6 by inflammatory mediators is regulated by p38⁽²⁵⁵⁾. p38 is important in regulating the biosynthesis of pro-inflammatory cytokines namely IL1 β , TNF α , IL-6 and IL-8 and therefore is a major contributor to inflammation and may play a critical role in the inflamed lung⁽²⁵⁶⁾. It has also been shown to be important in increasing IL-6 synthesis in airway epithelial cells⁽²⁵⁷⁾.

The reduction in IL-6 mRNA expression after treatment with 1,25(OH)₂D₃ was similar to the effect of p38 MAPK and PI3K inhibition, and also similar to that of the

combined treatment of $1,25(\text{OH})_2\text{D}_3$ and either the p38 MAPK or PI3K inhibitor, but these were not significantly different from the basal expression and so no conclusions can be made. Further repetition of this data is required.

Vitamin D has also been shown to modulate the RhoA/Rho Kinase ROCK pathway in different cell types ^(121, 122). In this study, inhibition of ROCK was not found to have any effect on IL-6 expression and did not affect the reduction of IL-6 expression by vitamin D suggesting that this pathway is not the mechanism of action.

A key transcription factor which has been shown in many previous studies to be modulated by vitamin D is NF κ B. In this study, using a proteasome inhibitor MG132 to inhibit activation of NF κ B lead to an increase in basal IL-6 expression but this was not significant and the data were variable and inconclusive. Vitamin D has been shown to modulate NF κ B DNA binding activity by increasing I κ B α by a number of mechanisms – increasing mRNA stability, decreasing phosphorylation, reducing nuclear NF κ B translocation, increasing mRNA transcription, protein translation, post-translational modifications and degradation by the proteasome ^(110, 111, 249, 258). From this there is a suggestion that vitamin D would likely be modulating NF κ B. However, further studies are required to fully determine how vitamin D is modulating these responses in airway epithelial cells.

NF κ B is the key transcription factor controlling the expression of many inflammatory mediators and therefore you might expect that if vitamin D was only modulating this signalling pathway, that it would modulate all the mediators in the same way. In order to determine if the effects of vitamin D were common to specific molecular pathways which were unable to be defined using the inhibitors, the effects of vitamin D on the mRNA expression of other inflammatory mediators were studied. $1,25(\text{OH})_2\text{D}_3$ treatment significantly reduced the expression of MMP9 mRNA expression in the SAEC and significantly induced the expression of TNF α mRNA in the NCI-H292 cell lines. It appears that the effects of vitamin D vary in different cell lines and for different mediators making defining the mechanism of action more complex. However, because these mediators are all regulated by NF κ B and yet vitamin D is having different effects it suggests that if vitamin D is modulating NF κ B it is doing it by different mechanisms to give a different effect, or

that it is not the only pathway that vitamin D is modulating and therefore giving different effects.

IL-10 and IL-12p70 mRNA basal expression was not detected previously in basal airway epithelial cells however, being primary vitamin D target genes under direct transcriptional control of the VDR⁽¹⁰⁶⁾, the expression of these was investigated on vitamin D treatment, but still no mRNA was detected in the cell types tested.

Interestingly, vitamin D treatment in the NCI-H292 cells was found to increase TNF α after 24 hours of treatment. In order to determine a possible mechanism of action molecular inhibitors were used to try and block this effect. Vitamin D has been shown to increase TNF α mRNA expression after 24 hours of treatment in prostate cancer cells. However, only mRNA was increased as additional cofactors were required for protein expression⁽²⁵⁹⁾.

Inhibiting p38 MAPK, PI3K and JNK had no significant effect alone on the induction of TNF α basal mRNA in the NCI-H292 cells but with 1,25(OH)₂D₃ gave a synergistic induction of TNF α suggesting that these pathways are involved in negatively regulating basal TNF α mRNA expression in these cells. p38 MAPK has been shown to be key in regulating mRNA stability so it may be that p38 MAPK reduces TNF α stability and inhibiting it leads to an increase in TNF α mRNA but further investigation into this would be required. Using MAPKK and proteasome inhibitors the data was more variable and hence was not significantly different which could mean that vitamin D does not modulate any of these pathways to increase TNF α but further confirmation is required.

It has already been shown that in CFBE41o- and CFTE29o- cystic fibrosis cells vitamin D has an immunomodulatory action on the induction of IL-6 and IL-8⁽¹⁵⁰⁾ and it was hypothesised in this study that vitamin D would have the same effect in other airway epithelial cells. Vitamin D was found to significantly reduce TNF α induced IL-6 mRNA and protein expression in the SAEC at both 1 and 24 hour preincubation time points, and IL-6 protein in the 16HBE14o-. Interestingly, there was no significant effect of vitamin D on IL-8 protein, but there was a reduction in IL-8 mRNA in the 16HBE14o- and an induction at 24 hours pre-incubation in the NCI-H292 cells.

The reduction of IL-6 mRNA induced by TNF α in the SAEC was dose dependent although this effect could be down to 1,25(OH)₂D₃ reducing basal IL-6 mRNA

expression rather than blocking TNF α signalling (Figure 4.3, 4.10). In addition the biphasic reduction and induction of IL-6 mRNA at the 1 and 24 hour pre-incubations respectively was a dose dependent effect in the NCI-H292 cells (Figure 4.10).

The effect of vitamin D on these mediators in the insensitive A549 cells was also studied and found that there was no effect whatever dose or time point suggesting that CYP24 is preventing the action of vitamin D in this cell line (Figure 4.11). However, in order to prove this further studies would be required to block CYP24 and see if this allows a similar effect of vitamin D in these cells as the SAEC or whether there are other factors at play in this cancer cell lines.

These different modulating effects of 1,25(OH) $_2$ D $_3$ in the SAEC and NCI-H292 cells were also found when using LPS as the inducer of inflammatory cytokines (Figure 4.12). In cystic fibrosis cells, agonists of the VDR were found to inhibit IL-6 and IL-8 protein expression when used 1 hour before exposure to LPS ⁽¹⁵⁰⁾. This fits in with the 1 hour incubation data for SAEC and NCI-H292 cells with IL-6 but not IL-8 expression.

Studies have also shown that vitamin D can reduce cytokine production induced by LPS in a number of different cell types and one of the mechanisms is by reducing TLR4 expression ⁽²⁶⁰⁾. This also fits in with *in vivo* data from a hamster model of acute lung injury (ALI) whereby ALI was induced by lipopolysaccharide (E.coli 0111:B4) inhalation, and vitamin D inhibited neutrophil recruitment in the lung by 40% ⁽²⁶¹⁾.

It is key that studies using vitamin D in other cell types have shown reduction of IL-8 expression as well as IL-6 by vitamin D whereas here we show just modulation of IL-6 suggesting different regulation.

Another novel finding of this study was the biphasic response of vitamin D in NCI-H292 cells. There has been a similar finding in the literature looking at the effect of 1,25(OH) $_2$ D $_3$ on basal expression of cytokines in HL-60 leukaemic cells showing a reduction of mRNA expression at 4 hours and increase at 24 hours for a range of inflammatory mediators. This was found to be due to suppression and then reactivation of NF κ B. The suppression was associated with increased I κ B α and the late reactivation was associated with vitamin D stimulated phosphorylation of I κ B α requiring active AKT and IKK pathways ⁽¹¹²⁾. A biphasic effect has also been found in U937 leukaemic cells where pre-treatment for 3-6 hours with vitamin D before LPS

gave a reduction in TNF α mRNA whereas pretreatment for 12-48 hours gave an induction of TNF α mRNA ⁽¹⁰⁷⁾. From this study it is clear that the biphasic response is cell type specific and it may be an important factor in cancer development.

MMP9 is a key protease upregulated in COPD responsible for destructive processes within the lung. In this study 1,25(OH)₂D₃ was found to reduce MMP9 mRNA expression in the SAEC but paradoxically there was an increase in MMP9 enzyme activity. Equally, pretreatment with vitamin D significantly reduced TNF α induced MMP9 mRNA expression in the SAEC, but synergistically induced MMP9 enzyme activity. Vitamin D has been shown to reduce MMP9 expression *in vitro* in human airway smooth muscle cells ⁽¹⁵¹⁾ and *in vivo* in patients with pulmonary tuberculosis ^(141, 173).

Although the mechanism of action for this was not studied, vitamin D has been shown to attenuate TNF α induction of MMP9 expression in keratinocytes by inhibiting JNK and NF κ B pathways ⁽¹¹⁸⁾. One hypothesis to test in the future that relates to this is the newly defined vitamin D receptor ERp60 which has been found to be present on extracellular matrix vesicles in chondrocytes and causes release of MMPs on vitamin D treatment. Therefore this fits in with the data in the SAEC where MMP9 mRNA was reduced but enzyme activity was increased possibly because MMP9 was stored up and vitamin D treatment caused it to be released from intracellular/extracellular vesicles into the supernatant ⁽²⁶²⁾.

MMP-9 has also been shown to be crucial in secretion of MUC5AC on LPS treatment ⁽²¹⁾. In the mucous producing NCI-H292 cell lines vitamin D treatment was found to reduce basal MUC5AC mRNA and protein expression. Being at both mRNA and protein levels suggests that vitamin D is acting at the transcription factor level or reducing the stability of transcripts in order to reduce expression. Vitamin D pretreatment also blocked the induction of MUC5AC secretion by LPS and PMA. The mechanism of this needs further investigation. The EGFR pathway is key in MUC5AC secretion in this cell type and vitamin D has been shown to modulate this pathway previously in different cancer cell types but further investigation would be required to determine if it is a mechanism in this cell type ⁽²¹⁾. It has also been shown that MUC5AC secretion is inhibited by antagonists of RAR α , and although vitamin D was not tested it is an antagonist of this pathway suggesting that it could be a mechanism of action ⁽²⁶³⁾.

A limitation to the study is that as yet no-one has shown how much active vitamin D can be produced within the lung given sufficient circulating levels of 25(OH)D₃. A range of doses 1-100 nM were tested and a number of effects were shown to be dose dependent giving strength to the evidence, but to reach a statistically significant effect required 100 nM vitamin D. Previous studies have shown the capabilities of airway epithelial cells to convert 25(OH)D to 1,25(OH)₂D and therefore in this study 1,25(OH)₂D was used. It is possible that much higher local transient concentrations of 1,25(OH)₂D₃ could be produced in airway epithelial cells but that has yet to be shown. It is possible that the concentration of 1,25(OH)₂D₃ used in this study is not physiological. However, the main concept of this study was to determine the effects of vitamin D and there is a therapeutic potential to administer high dose vitamin D in an inhaled form directly to the lung epithelium, and doses used in this study could be pharmacological.

The protein expression for different mediators was not measured in every study done and mechanistically only the mRNA was studied. Some of the mechanistic data was more variable and inconclusive and needs further investigation with increasing repetitions and protein data to show by western blotting either changes in the levels of different signalling components, or differences in their phosphorylation status to determine what vitamin D is activating/inactivating.

This study has shown that the A549 cells appear insensitive to vitamin D treatment and this is likely to be due to high expression of CYP24 which has been shown before, but to prove the difference between the cells is due to CYP24 expression, further studies would have to knock out CYP24 and see if vitamin D has the same effect as in the other cell types or vice versa. Another limitation is that this study did not determine the protein expression of CYP24 and VDR. Studies have shown that CYP24 mRNA and protein levels correlate however, the expression of VDR mRNA and protein can be completely independent.

4.5 Conclusions

This study has shown that vitamin D modulates the basal expression and induction of IL-6 in different airway epithelial cells but interestingly not IL-8. Vitamin D was also found to modulate MMP9 enzyme activity and reduce the secretion of MUC5AC. Vitamin D has shown some attributes which overall may be beneficial in lung diseases. Although the expression of inflammatory mediators were very similar between the different airway epithelial cells tested, their response to vitamin D treatment were more different. This could be down to individual variability, or there may be distinct cellular subtypes which can define how vitamin D modulates expression of inflammatory mediators. Further studies are required to further characterise the effects of vitamin D and its mechanism of action which will be crucial to fully determine the benefits of vitamin D.

Chapter Five:

Effects of vitamin D on oxidative stress in airway epithelial cells

Acknowledgements: Alfisal Taymoor, Jade Robinson and Ben Farrington, undergraduate MPharm project students who helped develop the assay and carried out some of the experiments.

5.1 Introduction

COPD is characterised by excessive inflammatory responses enhanced by the generation of oxygen and nitrogen free radicals induced by factors such as cigarette smoke ⁽¹⁹⁹⁾. Oxidative stress and the generation of ROS is a key process in cellular respiration. However, in excess it also drives inflammation and is very important in the pathology of COPD and lung cancer. Patients with COPD show evidence of oxidative stress in their lung tissue ⁽¹⁹⁹⁾. This is predominantly due to the large number of carcinogenic chemicals which induce free radicals and oxidative stress within the lung tissue during smoking. However, exposure to pathogens and also other inflammatory agents can also induce oxidative stress and dysregulate the anti-oxidant/pro-oxidant balance. The main ROS produced are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). A common feature of inflammatory lung disease is the infiltration of inflammatory cells which when activated, generate O_2^- which is converted to H_2O_2 by superoxide dismutase ⁽²⁶⁴⁾. Vitamin D has been shown to be key to innate immune responses by inducing ROS production in alveolar macrophages in order to fight infections ^(114, 265). It has also been shown to have both anti-oxidant and pro-oxidant properties in different cell types ⁽²⁶⁶⁻²⁶⁸⁾. However, it has yet to be seen whether vitamin D has any anti or pro-oxidant effects in the airway epithelium.

In order to determine the effect of vitamin D on oxidative stress in airway epithelial cells, firstly a method was optimised and developed in order to define a method of measuring oxidative stress in the airway epithelium. The effects of vitamin D on oxidative stress and antioxidant gene expression were then studied and possible mechanism of action were investigated using small molecule inhibitors of intracellular signalling pathways.

5.2 Aims

- Develop and optimise an assay in order to measure oxidative stress in airway epithelial cells
- Study the effects of vitamin D on oxidative stress in airway epithelial cells
- Study the effects of vitamin D on antioxidant gene expression in airway epithelial cells and its mechanism of action

5.3 Results

In order to study the effects of vitamin D on oxidative stress in airway epithelial cells it was first necessary to optimise methodology for measuring ROS production. Various methods have been employed in the literature in order to study ROS production in different cellular systems but most rely on detecting specific oxidative species ⁽²⁶⁹⁾. The compound 2, 7'-dichlorodihydrofluoresceindiacetate (H₂DCFDA) was first used in the literature as a method of measuring production of H₂O₂. However, since then it has been found to be reactive to many different radicals and therefore is now used as a measure of oxidative stress within cells ⁽²⁷⁰⁾. H₂DCFDA crosses the cell membrane and is hydrolysed by cellular esterases to a non fluorescent intermediate which in the presence of ROS, is oxidised to highly fluorescent dichlorofluorescein (DCF). Intracellular DCF fluorescence can be quantified as a measure of overall oxidative stress within cells ⁽²⁷⁰⁾. The emitted fluorescence is directly proportional to the concentration of reactive oxygen species. Many different methods have been described using this agent and these depend on the hypothesis tested and cell types used with each optimised for experimental outcome. H₂DCFDA has been used predominantly by flow cytometry to measure ROS but more recently microplate and fluorescence microscopy methods have been developed (Table 5.1). In this study adherent cells were used and trypsinisation of cells into suspension for analysis can potentially change the oxidative state of the cells and therefore the subsequent results ⁽²⁷¹⁾. With a microplate assay you can get a quantitative measurement of fluorescence intensity from the cells in situ and this also allows for a more high-throughput investigation. Table 5.1 summarises the studies which have used this agent with all the variables examined. These include the concentration of H₂DCFDA to use on the cells, the length of incubation with the agent, the loading buffer, whether the cells are loaded with H₂DCFDA before or after the stimulus, at what time point the fluorescence is measured, any recovery period from the cell loading, wash steps and also the method of quantitative analysis.

Cell Type	Concentration DCF	Duration DCF (minutes)	Load or Stimulate first	Detection	Reference
DU145	10 μ M	30	Stimulate	Spectrofluorometer	(272)
Retinal Pigment Epithelium	10 μ M	30	Stimulate	Microplate reader	(273)
Mouse ES cells	10 μ M	30	n/a	Luminometer and plate reader	(274)
Pulmonary artery smooth muscle cells	5 μ M CM-DCF	30	Stimulate	Microscopy	(275)
Mouse cerebral arteries	5 μ M CM-DCF	30	Co-incubate	Microplate	(276)
CRL-1548 Hepatoma cells	20 μ M	30	Load	Microscopy and plate reader	(277)
B14 Chinese Hamster cells	0.25 μ M	60	Co-incubate	Spectrofluorometer	(278)
ARPE-19 cells	10 μ M	20	Load	Spectrofluorometer	(279)
Fibroblasts	10 μ M	60	Load	Microplate reader	(280)
Liver cells	100 μ M	30	Load	Microplate	(281)
Bovine keratinocytes	50 μ M	15	Load	Microplate	(282)
PC12	10 μ M	20	Stimulate	Microplate	(283)
RBC	0.4 mM	15	Load	Flow cytometry	(284)
A549	20 μ M	30	Load	Flow cytometry	(14)
PC12	100 μ M	30	Load	Microplate	(270)
A549	1 μ M CM-H ₂ DCFDA	30	Stimulate	Flow cytometry	(285)
A549	1 mM CM-H ₂ DCFDA	15	Load	Flow cytometry	(286)
A549	10 μ M	30	Load	Flow cytometry	(287)
A549	11 μ M	60	Load	Flow cytometry and microscopy	(288)
A549	10 μ M	60	Stimulate	Flow cytometry	(254)

Table 5.1 - Summary of the literature using H₂DCFDA and their methodology

5.3.1 Optimisation of methodology for the ROS assay

A number of studies have measured ROS production using H₂DCFDA in A549 cells (Table 5.1) therefore A549 were used initially to optimise the conditions for the experiment before testing out on the other cell types. From the literature, the concentration of 10 µM H₂DCFDA was the most common concentration used and had been used previously in the A549. This dose was not found to be cytotoxic to the cells (Appendix Figure 7.4)

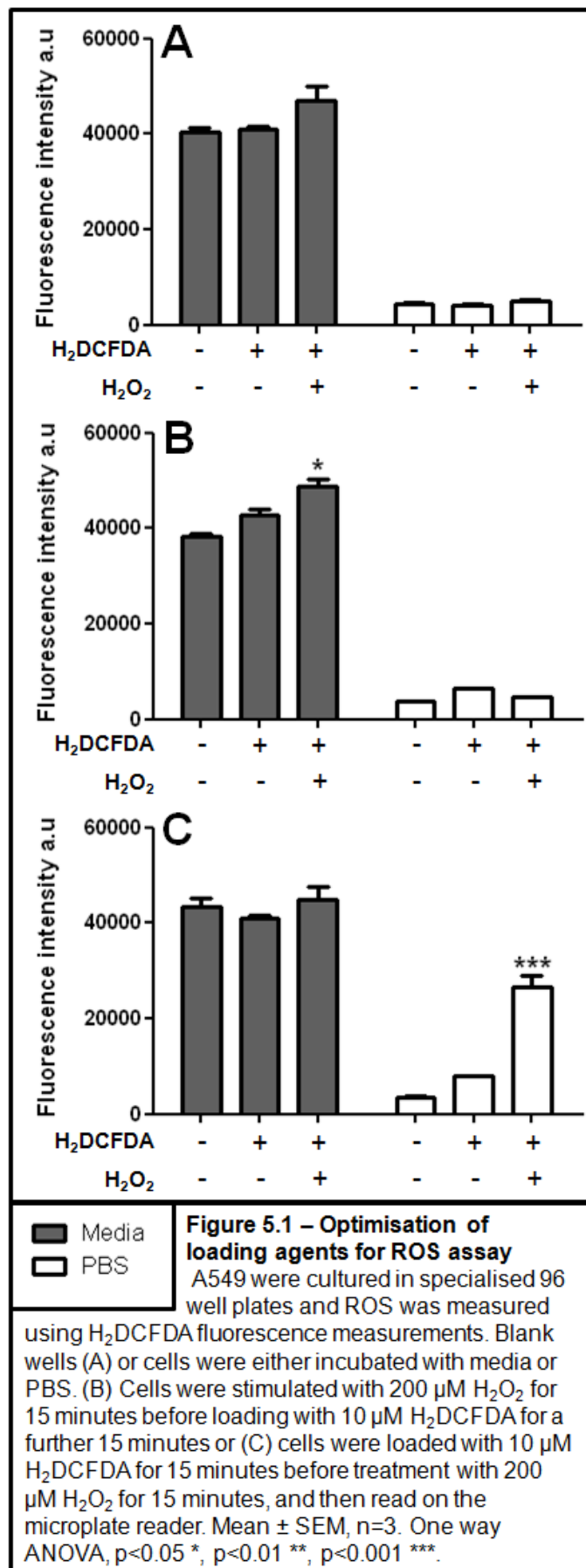
Firstly it was necessary to test the basal fluorescence of the loading agent PBS/media in this assay. Phenol red found in the media can quench fluorescence, and media itself can interact with the H₂DFCDA reagent⁽²⁸⁹⁾ and therefore controls were included of media alone with the agent and cells alone in media with the reagent as a baseline. As shown in Figure 5.1, the media alone gave a much higher background fluorescence than the PBS when read under the same conditions and gain parameters on the microplate reader. Also, when media alone was incubated with H₂O₂ and H₂DCFDA there was a non-significant increase in fluorescence not found with PBS (Figure 5.1 A).

From optimising experiments for loading buffers, it was found that using a phenol red free media gave much lower background fluorescence than regular media making it almost the same as the PBS (data not shown). This was a better alternative to PBS, avoiding any stress from lack of nutrients, for longer experiments.

H₂O₂ is increased in the sputum of patients with COPD and is also detected at higher levels in the exhaled breath condensate in these patients than control patients⁽⁶⁴⁾. H₂O₂ was used as a positive control as a known ROS inducer but is also a physiologically relevant stimulus. The next determinant was whether or not the cells should be loaded before the ROS inducing stimulus, as previous studies have used both methods (Table 5.1). Cells were either stimulated with 200 µM H₂O₂ for 15 minutes before loading with 10 µM H₂DCFDA for 15 minutes or vice versa (Figure 5.1B and C). H₂O₂ significantly induced ($p < 0.05$) ROS in cells with media when stimulated before loading (Figure 5.1B). However, there was a greater significant

effect of ROS induction by H_2O_2 in cells loaded before stimulation with PBS ($p < 0.001$) (Figure 5.1C).

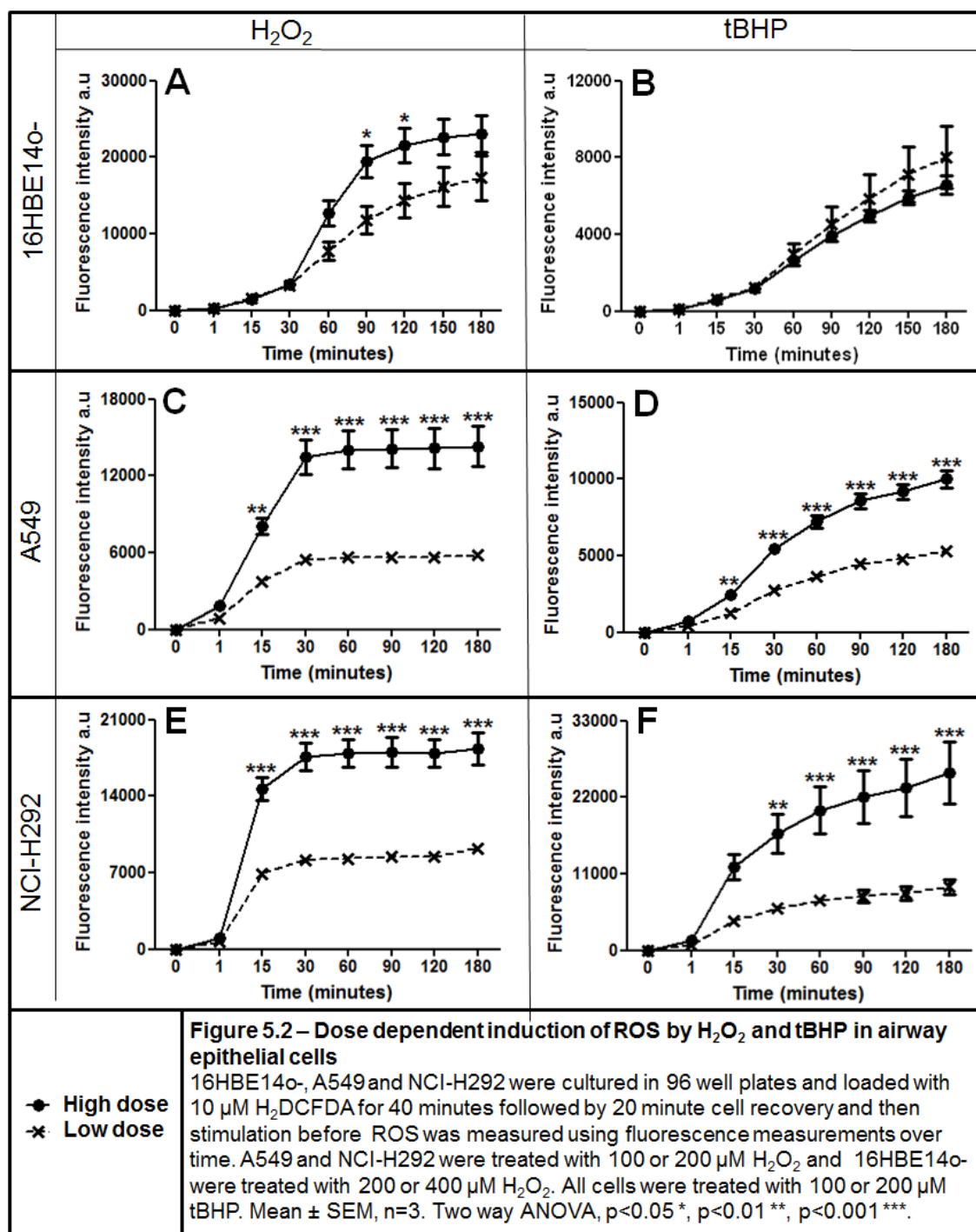
Final optimisation experiments found that the optimal conditions were to load the cells with H_2DCFDA for 40 minutes followed by a 20 minute cell recovery period before treatment with the ROS inducer. This was because some of the cell types were found to change morphology on loading with H_2DCFDA but when the H_2DCFDA was removed and replaced with media, cells recovered after 20 minutes to their original phenotype. This was not down to any cytotoxic effect on the cells (Appendix Figure 7.4).



5.3.2 Induction of ROS by H₂O₂ and tBHP

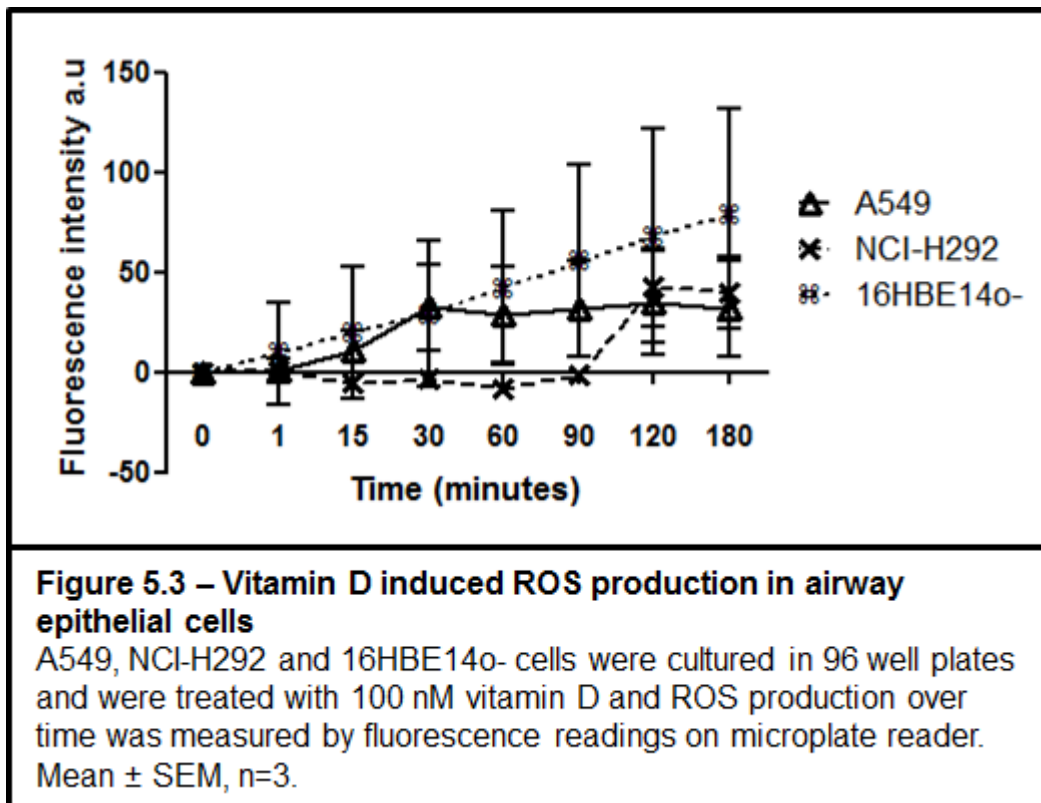
After optimising the conditions for the assay it was then necessary to determine how the fluorescence intensity would be measured. Some studies use one measurement at a certain time point but in this study the fluorescence change over time was measured in order to see the rate of change. As there was no literature on which time point to use, in this study the fluorescence change over time was measured. Any minor change in fluorescence values for the control wells of cells with the H₂DCFDA was subtracted from all the values. Tert-butyl hydroperoxide (tBHP) is a pro-oxidant hepatotoxic compound used as a model to study mechanism of cellular alterations arising from free radical action ⁽²⁹⁰⁾. In order to determine that the assay was sensitive to changes in fluorescence, the H₂O₂ or tBHP were added to the cells at different concentrations to determine if the assay could detect a dose dependent effect.

Treatment of A549, NCI-H292 with 100-200 µM and 16HBE14o- cells with 200-400 µM H₂O₂ and 100-200 µM tBHP gave a significant dose dependent induction of ROS for all except tBHP in the 16HBE14o- cells (Figure 5.2).



5.3.3 Effect of vitamin D on ROS production in airway epithelial cells

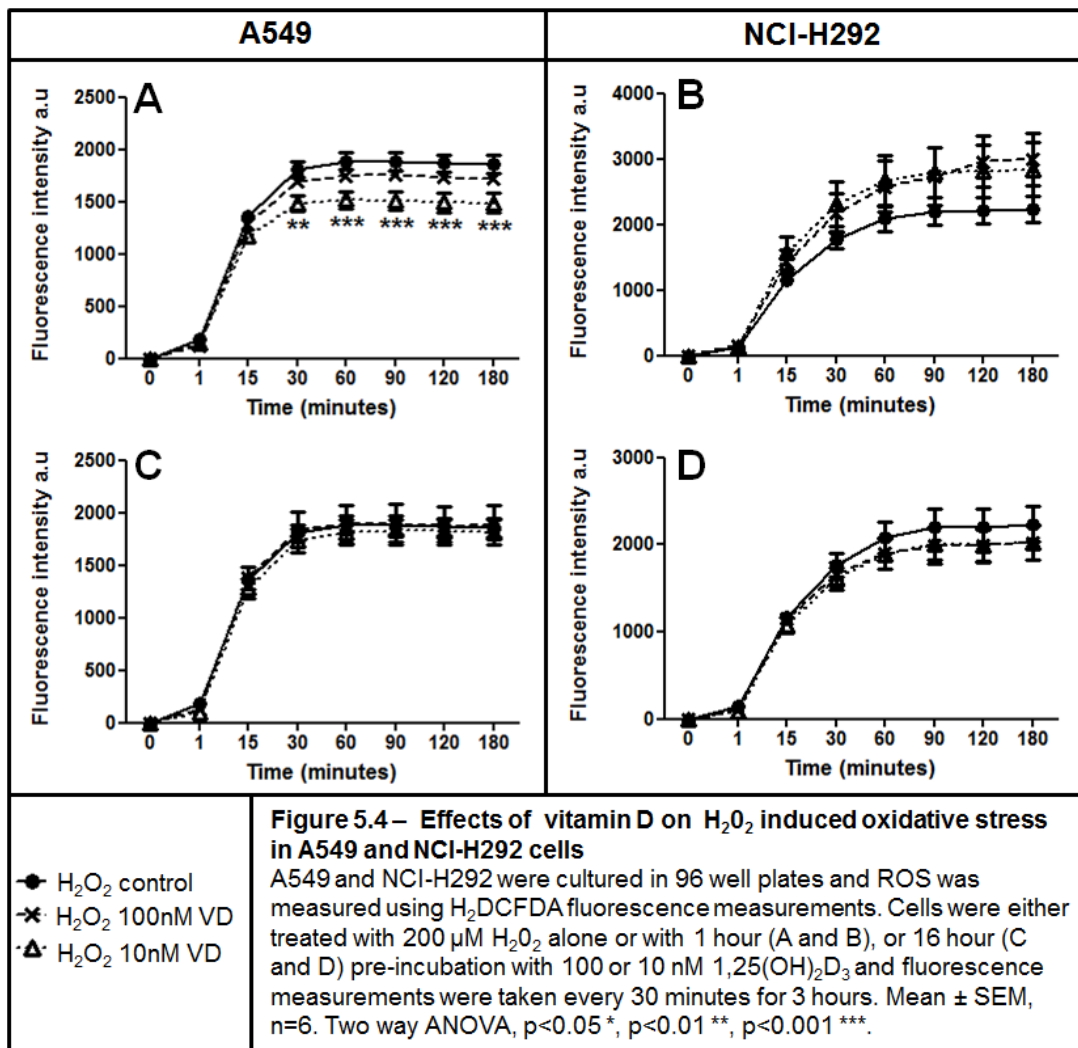
Using the same parameters set up for the ROS producers, the effect of vitamin D alone on ROS production in the different airway epithelial cells was measured over time (Figure 5.3). Although there was a trend of an increase in ROS production after vitamin D treatment for 3 hours, the fluorescence intensity values were very low and variable and were not found to give a significant induction for any of the cell types.



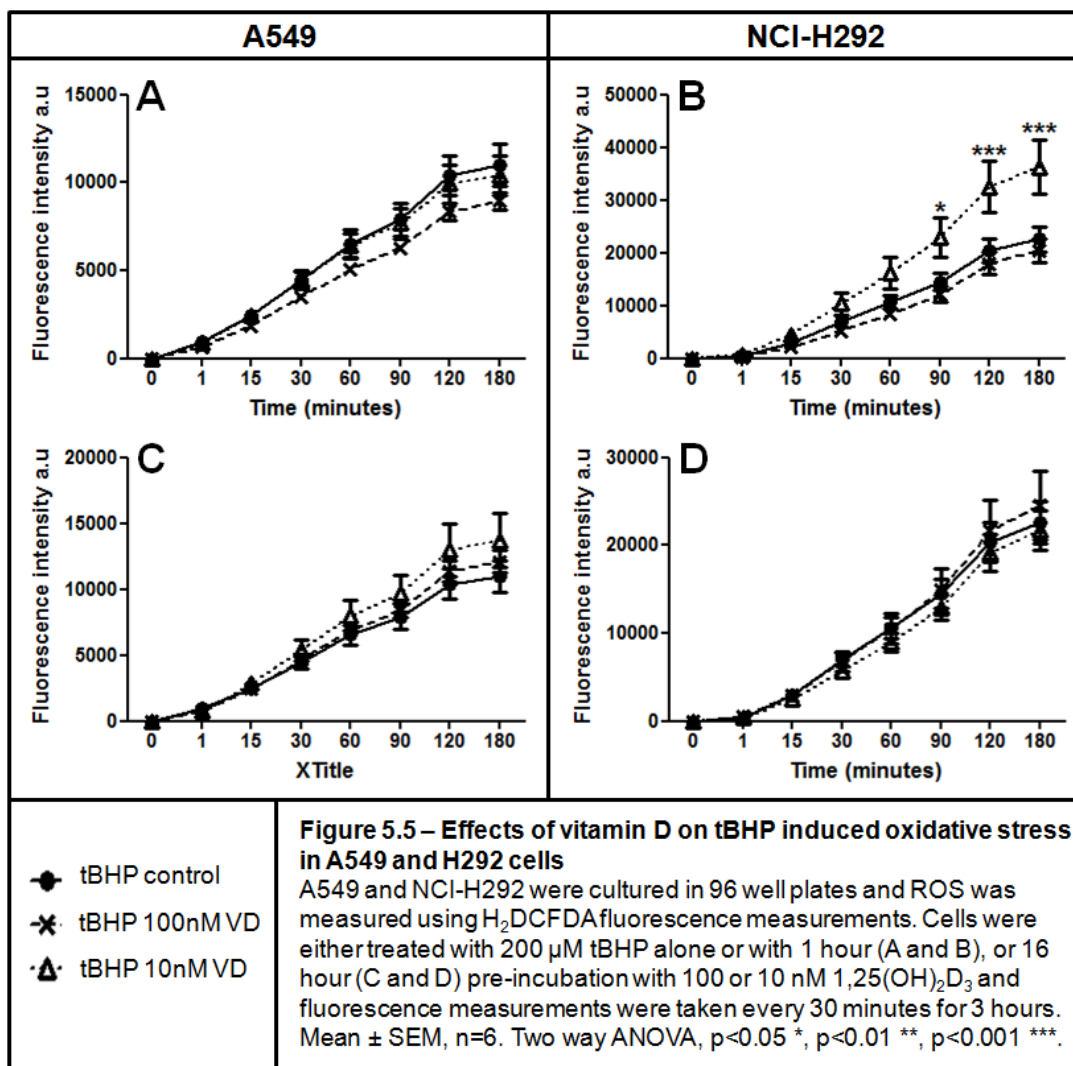
5.3.4 Effect of vitamin D on the induction of ROS by H₂O₂ and tBHP

Vitamin D has been shown to sensitise breast and colon cancer cells to ROS dependent cytotoxicity induced by H₂O₂ ^(266, 267) and also to protect normal prostate cells from oxidative stress induced death but not malignant prostate cells ⁽²⁶⁸⁾.

To look at whether vitamin D exhibited redox activity in airway epithelial cells, the effect of vitamin D on the induction of ROS by H₂O₂ and tBHP was investigated. In chapter 4, vitamin D gave differing effects on cytokine production in different airway epithelial cells depending on whether the cells were pre-incubated for 1 hour or 16-24 hours with vitamin D. Therefore, A549 and NCI-H292 cells were preincubated with vitamin D for 1 or 16 hours before loading the cells with H₂DCFDA and then stimulating with H₂O₂ or tBHP. As shown in Figure 5.4, pre-incubation with vitamin D had no significant effect on the induction of ROS by H₂O₂ except in the A549 when pre-incubation for 1 hour with 10 nM vitamin D significantly decreased oxidative stress (p<0.001).



In contrast, pre-incubation of NCI-H292 for 1 hour with 10 nM vitamin D prior to tBHP stimulation led to a synergistic induction of ROS ($p < 0.001$) (Figure 5.5).

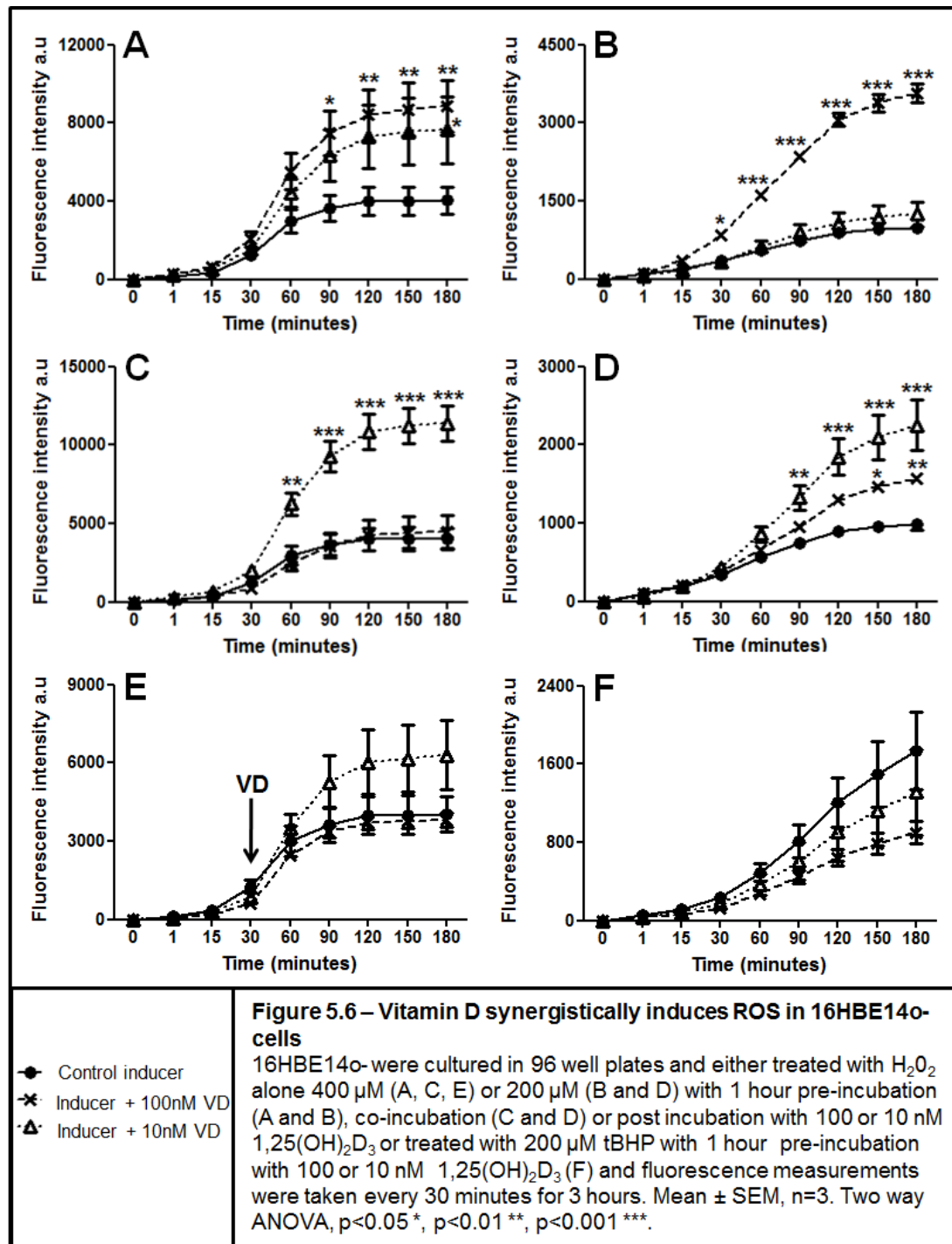


As 1 hour pre-incubation of vitamin D was the only time point found to have a significant effect on ROS induction in the A549 and NCI-H292 cells, this was used for further study in the 16HBE14o- cells, shown in Figure 5.6. Firstly the effect of 1 hour pre-incubation with 10 and 100 nM vitamin D before either 200 or 400 μM H₂O₂ was studied to see if there was a dose dependent effect for both. 100 nM vitamin D significantly increased 200 or 400 μM H₂O₂ induction of ROS (p<0.01) (Figure 5.6 A-B).

The effect of co-incubation of vitamin D and H₂O₂ treatment on ROS production was also examined in 16HBE14o- cells. 10 nM vitamin D co-incubated with either 200 or 400 μM H₂O₂ gave a significant induction of ROS when compared to H₂O₂ alone. With 200 μM, 100 nM vitamin D significantly induced ROS when compared with H₂O₂ alone, but was not as potent as 10 nM vitamin D (Figure 5.6 C-D)

Vitamin D was also added 30 minutes after 400 μM H_2O_2 to see if it would have any effect on the induction of ROS. Addition of 10 nM vitamin D gave a trend of an increase in ROS induction; however this was not significant (Figure 5.6 E).

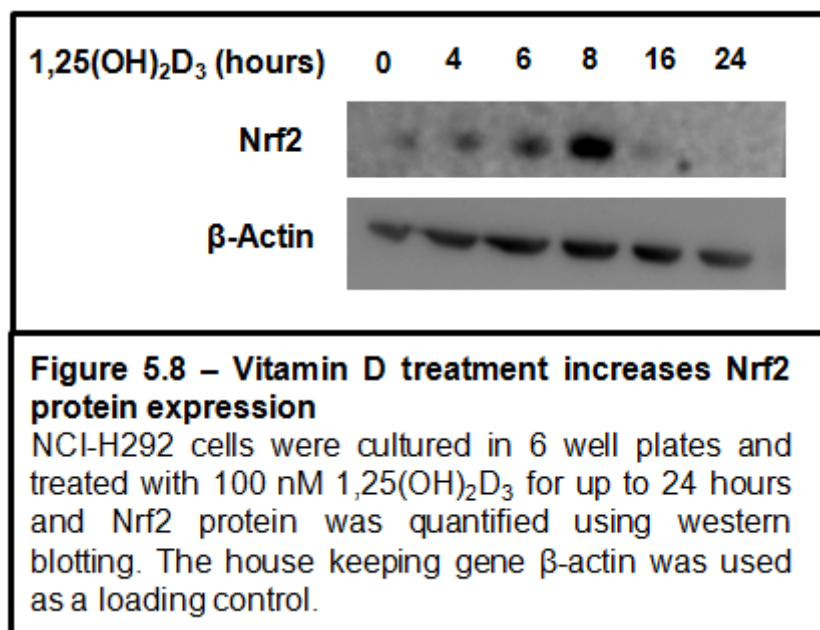
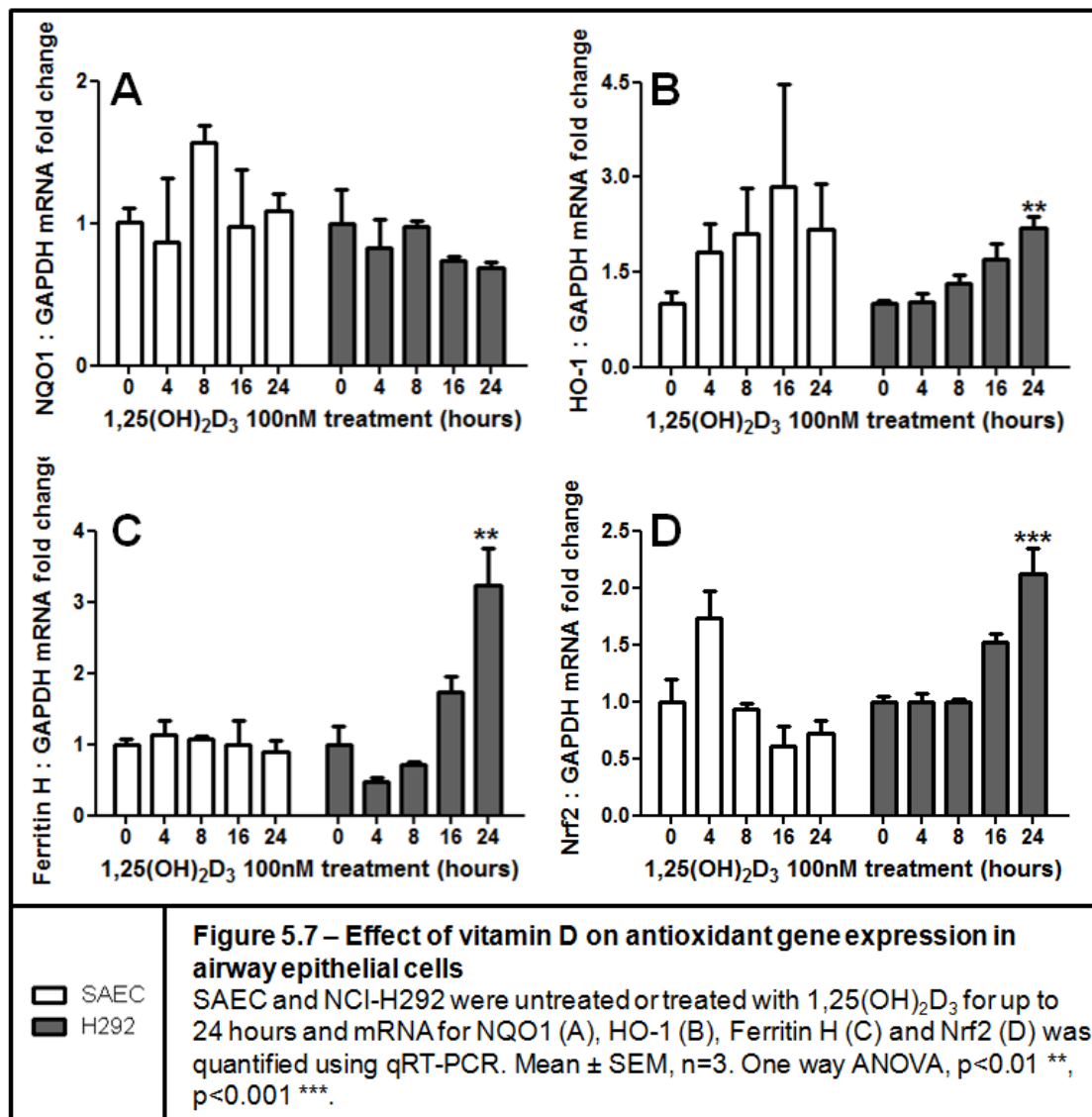
Finally, the effect of vitamin D pre-incubation on induction of ROS by 200 μM tBHP was studied. Vitamin D pre-incubation had no significant effect on the induction of ROS although there was a trend of a decrease in ROS production (Figure 5.6 F).



5.3.5 Effect of vitamin D on genes controlled by the antioxidant response element

In chapter 4 section 4.3.13, vitamin D gave a biphasic response of cytokine production in the NCI-H292 cells, with longer incubation giving an induction of pro-inflammatory cytokines. Vitamin D was also shown to have a pro-oxidant effect in the NCI-H292 with pre-incubation on tBHP treatment. It was hypothesised that vitamin D may also modulate the expression of antioxidant genes either by acting as a pro-oxidant itself or acting in an anti-oxidant way to increase antioxidant genes. The effect of vitamin D treatment on the expression of genes involved in antioxidant responses was studied. Genes investigated were NQO1, a phase II detoxifying enzyme contributing to xenobiotic detoxification, HO-1 and Ferritin H (cytoprotective stress response proteins) and Nrf2, the transcription factor that regulates the expression of a wide range of antioxidant genes ^(67, 199). 100 nM vitamin D treatment did not significantly affect expression of antioxidant genes in SAEC (Figure 5.7). However, in the NCI-H292 cells treatment with 100 nM vitamin D for 24 hours significant increased HO-1, ferritin H and Nrf2 mRNA expression.

The expression of these mediators is regulated by the transcription factor Nrf2. This protein activates critical cellular pathways that protect against oxidative injury and inflammation. Vitamin D also caused a time dependent increase in Nrf2 protein, measured by western blot analysis (Figure 5.8).



5.3.6 Effects of molecular inhibitors on induction of antioxidant genes in NCI-H292 by vitamin D treatment

In order to determine the mechanism of how vitamin D might be inducing HO-1 and ferritin H mRNA expression, inhibitors were used to block different intracellular signalling pathways.

Nrf2 is continually targeted for proteasomal degradation by its cytosolic inhibitor, Keap1. As a result, MG132 should cause a build up of Nrf2 in the cell, and subsequently an increase in the expression of its target genes. MG132 was used as a positive control in this study. Pre-treatment of cells with MG132 for 30 min alone or prior to 24 hr treatment with vitamin D significantly induced HO-1 mRNA expression (Figure 5.9 B).

Y-27632 (a ROCK inhibitor) significantly reduced HO-1 basal expression but not vitamin D-induced expression, suggesting that vitamin D – induced HO-1 does not require ROCK (Figure 5.9A). Inhibiting PI3K had no effect on vitamin D – induced HO-1 mRNA expression with there still being a significant induction suggesting this pathway is not involved. However, inhibition of p38 MAPK, MAPKK, and JNK showed a similar effect to the positive control blocking the significant induction of HO-1 mRNA expression by vitamin D. This suggests that vitamin D could be modulating these pathways, or that the increase of HO-1 by these inhibitors is greater than the effect of vitamin D and therefore vitamin D is not able to increase the expression any further.

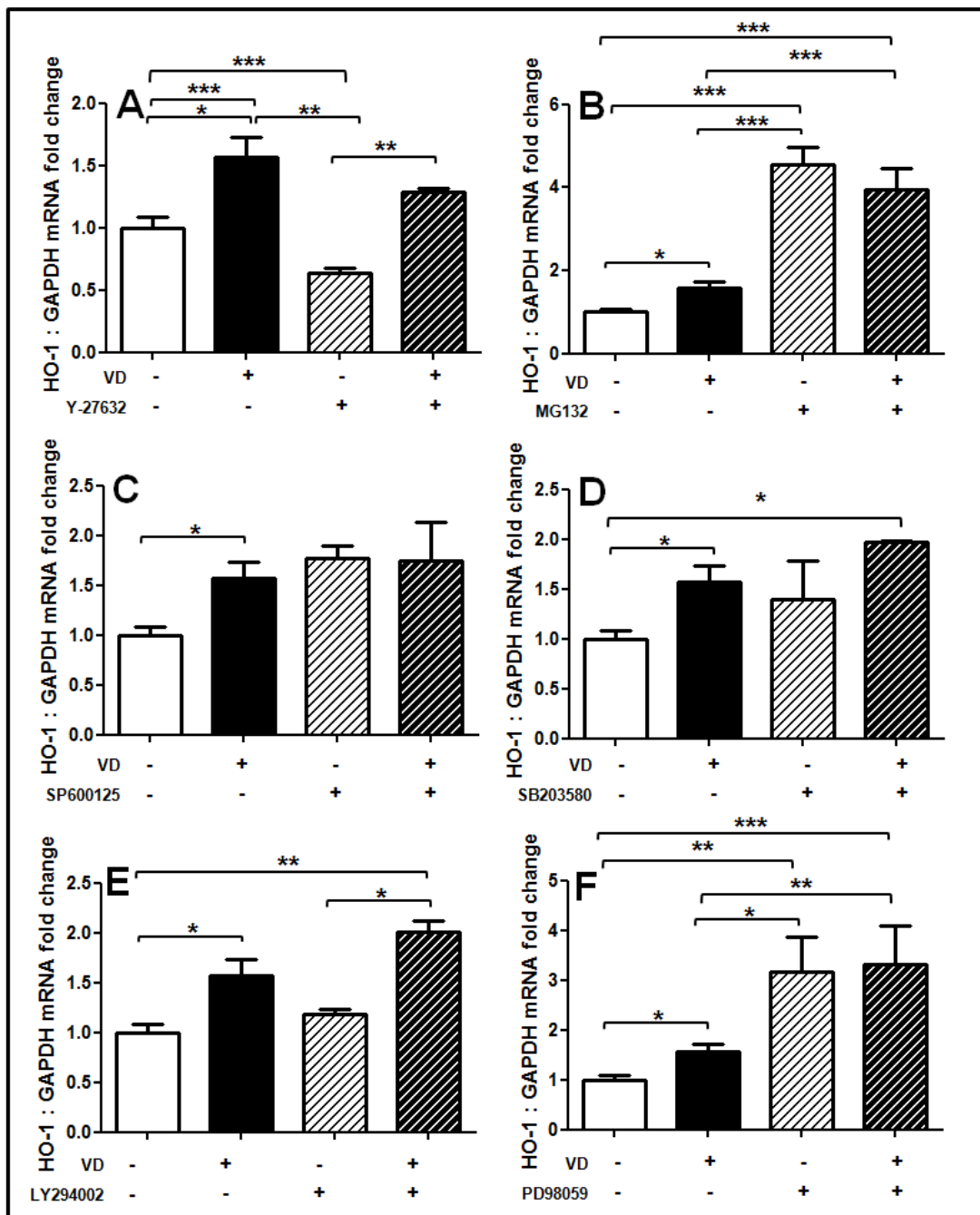


Figure 5.9 – Effect of signalling pathway inhibitors on vitamin D mediated activation of basal HO-1 mRNA in NCI-H292 cells.

NCI-H292 cells were untreated or pre-treated with cell pathway inhibitors Y-27632 (A), MG132 (B), SP600125 (C), SB203580 (D), LY294002 (E) and PD98059 (F) for 30 minutes before addition of 100 nM 1,25(OH)₂D₃ for 24 hours and the expression of mRNA for HO-1 was quantified using qRT-PCR. Mean +/- SEM. n=3. One way ANOVA, p<0.05 *, p<0.01 **, p<0.001 ***.

Treatment of cells with vitamin D significantly induced ferritin H mRNA expression (p<0.05) (Figure 5.10). As seen with HO-1, treatment with the positive control MG132 significantly increased ferritin H mRNA expression (Figure 5.11B).

Treatment with the JNK, PI3K, p38 MAPK and MAPKK inhibitors in combination with vitamin D still gave an increase in Ferritin H expression which was significant with

some of the inhibitors. This suggests that vitamin D is not modulating these pathways to increase Ferritin H expression although further study is required to confirm this.

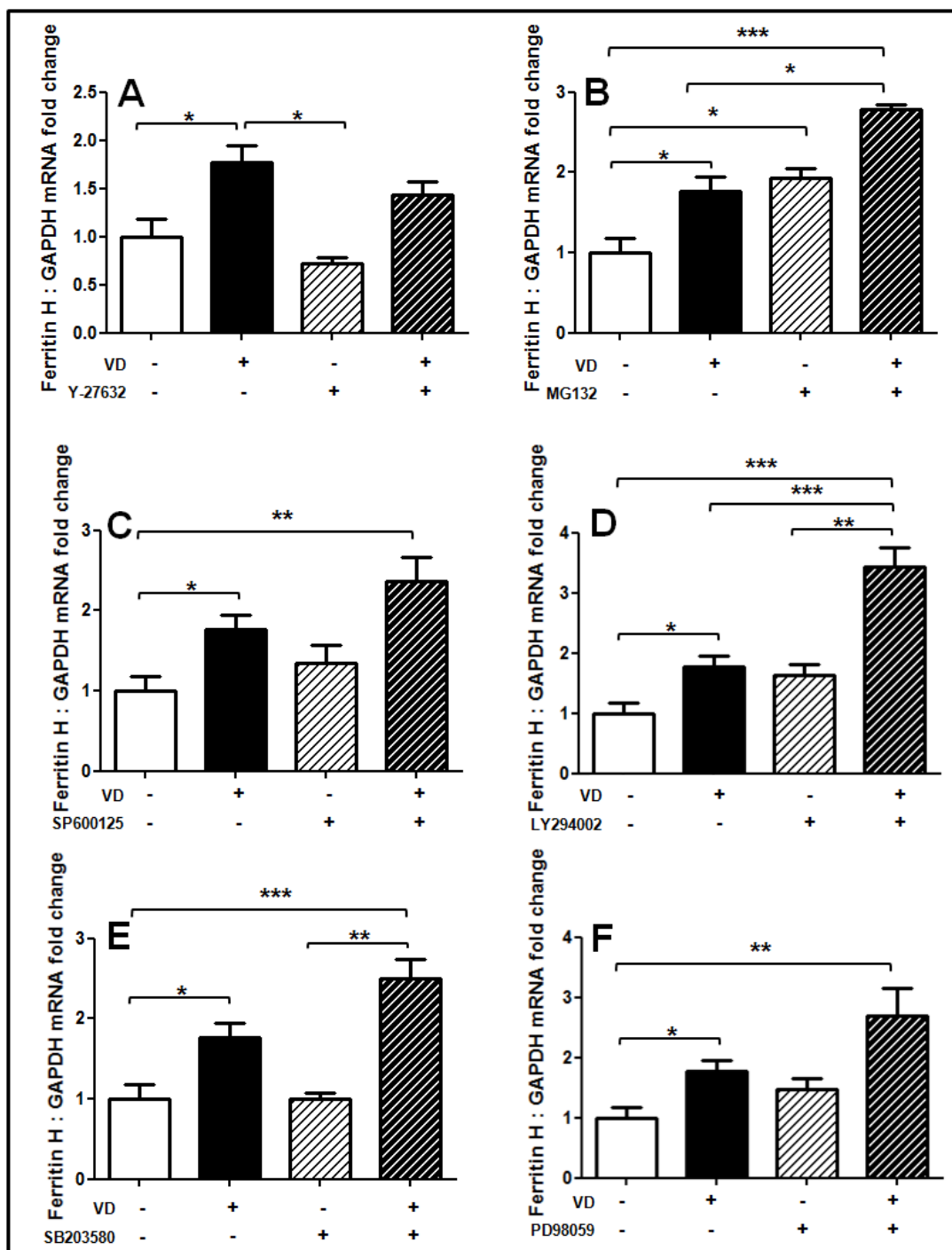


Figure 5.10– Effect of signalling pathway inhibitors on vitamin D mediated activation of basal Ferritin H mRNA in NCI-H292 cells.

NCI-H292 cells were untreated or pre-treated with cell pathway inhibitors Y-27632 (A), MG132 (B), SP600125 (C), SB203580 (D), LY294002 (E) and PD98059 (F) for 30 minutes before addition of 100 nM $1,25(\text{OH})_2\text{D}_3$ for 24 hours and the expression of mRNA for Ferritin H was quantified using qRT-PCR. Mean +/- SEM. n=3. One way ANOVA, p<0.05 *, p<0.01 **, p<0.001 ***.

5.4 Discussion

The novel findings of this study were that vitamin D modulated oxidative stress in different ways in the different cells and was found to significantly induce HO-1 and Ferritin H antioxidant response genes and increase Nrf2 protein expression in the NCI-H292 cells. Methodology was development and optimisation to study and compare oxidative stress in the different airway epithelial cells.

To my knowledge this is the first study to use H₂DCFDA by microplate assay to study oxidative stress in different airway epithelial cells. A number of variables were tested and optimised in the methodology to produce an efficient, reproducible technique allowing high throughput screening. H₂O₂ and tBHP are known inducers of oxidative stress and significantly modulated ROS production in a dose dependent manner, giving strength to the methodology of the assay. Although H₂O₂ is a physiologically relevant stimulus being found in greater concentrations in the exhaled breath condensate of patients with COPD, a limitation to the study is that the concentrations used were not physiologically relevant, but were ones that have previously been used in the literature to induce oxidative stress. In patients with acute exacerbations of chronic obstructive pulmonary disease levels of hydrogen peroxide in exhaled breath condensate has been found to be up to 3040 nM which is less than the 400 µM we used ⁽²⁹¹⁾. However there is evidence to suggest that H₂O₂ in alveolar lining fluid can be 5 x 10⁴ times greater than that of exhaled H₂O₂ but is balanced out by antioxidants ⁽²⁹¹⁾, so it is possible that airway epithelial cells do come into contact with much higher concentrations of H₂O₂ such as those used in this study ⁽²⁹²⁾.

Vitamin D did not significantly affect ROS production alone. It could be that the effect of vitamin D alone is very small and possibly undetectable by this method. However, the assay was sensitive enough to detect differences in dose of ROS inducers and also the effects of vitamin D on ROS induction.

In this study 1 hour of 10 nM vitamin D pretreatment reduced oxidative stress induced by H₂O₂ in A549 cells, and increased ROS induced by tBHP in NCI-H292 cells. The latter fits in with literature on vitamin D sensitising breast, colon and prostate cancer cells to ROS induced injury or cell death therefore increasing ROS. However, the former was unexpected especially with effects only seen with the

lower dose of vitamin D and in the cell type most resistant to vitamin D treatment. The 1 hour time point is unlikely to be a genomic response but more a rapid response to vitamin D treatment which gives an anti-oxidant action. This effect might involve binding to the newly characterised 1,25-MARRS receptor which is known to have thioredoxin like sites and has been thought to participate in the mechanisms of cell protection against oxidative stress ⁽²⁹³⁾. Although A549 express high levels of Nrf2 in comparison to NCI-H292 cells ⁽²⁹⁴⁾ both cell types had a similar induction of ROS in response to H₂O₂ and tBHP. A recent study has shown that vitamin D reduced endogenous oxidants in lymphocytes but not in A549 cells ⁽²⁵⁴⁾. In contrast to Bo-Ying et al who reported that vitamin D protects normal prostate cells from oxidant induced damage, vitamin D synergistically increased ROS when pre-incubated, co-incubated and post incubated with H₂O₂ in the 16HBE14o- cells ⁽²⁹⁵⁾. This effect was found at both doses of H₂O₂ used. Interestingly, the effects of vitamin D pre-incubation were greater at the 100 nM of vitamin D, however the co-incubation and post-incubation was greater only at the 10 nM vitamin D. These results are difficult to explain as it might be expected that if vitamin D is having a pro-oxidant effect, then the highest concentration of vitamin D used will give the greatest effect. This again highlights the different effects of vitamin D in the different airway epithelial cells and needs further investigation.

To further determine the role of vitamin D in oxidative stress, the effect of vitamin D on the expression of anti-oxidant genes was investigated. Treatment with vitamin D had no effect on expression of antioxidant genes in the SAEC, however it induced HO-1 and ferritin H mRNA expression after 24 hours of treatment in the NCI-H292 cells. Anti-oxidants such as sulforaphane increase Nrf2 expression and thus the expression of anti-oxidant genes, however these are also increased by pro-inflammatory stimuli such as TNF α and LPS. Therefore this still does not conclusively say whether vitamin D is acting in a pro-oxidant or anti-oxidant nature in the NCI-H292 cells. Nrf2 is the main transcription factor controlling the expression of antioxidant expression and was found to be increased by vitamin D treatment at the protein level at 8 hours which would be conducive to activation of the mRNA of the other genes at 16-24 hours. Nrf2 mRNA expression was also found to be increased but at a later time point than the protein. Nrf2 has been shown to regulate its own transcription and also agents can change Nrf2 expression by

stabilisation of the Nrf2 protein rather than changing mRNA which is possibly how vitamin D is working in this case ⁽²⁹⁶⁾. Previously in Chapter 4 section 4.3.5, it was found that vitamin D also increased TNF α expression in the NCI-H292 cells but this was also at 24 hours of treatment and therefore could not be what was driving the anti-oxidant response. It is clear that vitamin D is activating a pathway that is then driving a pro-inflammatory and possibly pro-oxidant effect in this cell type.

To see which molecular pathways vitamin D was activating in this cell type in order to get the expression of HO-1 and Ferritin H, molecular inhibitors were used. Inhibiting the proteasome leads to stabilisation of Nrf2 and therefore would increase the expression of Nrf2 related genes ⁽²⁹⁷⁾. However the MAPK and PI3K phosphorylation pathways have also been implicated in the regulation of Nrf2, although this is predominantly in its activation.

Using the inhibitors no pathways were found to be responsible for vitamin D increasing the expression of HO-1 and Ferritin H mRNA expression but again this data was variable and would need further investigation to confirm this.

In this study HO-1 and ferritin H were activated by vitamin D but the other Nrf2 - regulated gene NQO-1 was not. HO-1 participates in generating the antioxidants carbon monoxide and bilirubin. The activation of ferritin sequesters iron which is the product of HO-1 catalysis and therefore the induction of both leads to an antioxidant state. Although NQO-1 is one of the most robust responders to Nrf2 activation it was not induced by vitamin D in this study. It appears vitamin D was increasing the expression of proteins which have antioxidant properties rather than those involved in xenobiotic metabolism ⁽²⁹⁷⁾.

The strengths of this study is that the ROS assay developed was robust, gave reproducible data with tight error bars and was sensitive enough to detect a dose dependent effect and also the effect of vitamin D. One limitation is that no control anti-oxidant was used such as NAC to block the induction of ROS to show that the assay was sensitive enough to detect changes in antioxidants, although significant changes were detected with 1,25(OH)₂D₃ treatment. There is also evidence in the literature to suggest that the compound H₂DCFDA is not completely stable and in some cell types has been shown to leak out of the cell ^(269, 271, 298). Therefore, with a microplate based assay where not only cellular fluorescence but also buffer fluorescence is measured then cellular leakage would affect the results. This was

controlled for as much as possible with the cells with H₂DCFDA taken as control with any change in fluorescence removed from the other values, although this effect was found to be minimal. However, one cannot rule out that H₂DCFDA could have leaked out of cells and some of the fluorescence was from the buffer. Therefore any effects seen of vitamin D could be that it increases or decreases leakage of H₂DCFDA from the cells. This could be improved in the future by using a more stable derivative of H₂DCFDA such as CM-H₂DCFDA which has an increased cellular retention. In addition, the media could be removed at the end of the experiment and then read on the microplate reader so that any fluorescence of the media could be removed and therefore only cellular fluorescence is measured. In this study the protein expression for the antioxidant genes could have been measured to strengthen the current data. Equally the protein should have been quantified before gel loading so that the western blot data could be quantified as β actin can also change over time. Although vitamin D was shown to increase Nrf2 protein expression and also activate HO-1 and Ferritin H mRNA which are genes controlled by Nrf2, one cannot rule out that these could be independent events and further study should look at silencing Nrf2 to see if these effects are still seen.

5.5 Conclusion

Vitamin D modulates oxidative stress in different ways in the different airway epithelial cells. In the NCI-H292 cell line vitamin D was found to induce the expression of Nrf2 and anti-oxidant genes. Further study is required to fully determine the mechanism of action of vitamin D and whether its action is due to being an anti-oxidant or pro-oxidant. Different effects might occur under different conditions and concentrations of vitamin D.

Chapter 6:

General discussion and future studies

6.1 Airway epithelial cells and lung inflammation

COPD is both driven and exacerbated by inflammatory responses which with oxidative stress form a vicious circle generating excessive inflammation and irreversible damage to the airways. With cigarette smoke exposure being the largest contributing factor to COPD development ⁽¹⁹⁹⁾, many studies have focussed on cigarette smoke induced damage. However, not all smokers develop COPD ⁽³⁾ and another contributing factor for disease progression is exacerbations due to infection ⁽¹²⁾. One of the main pathogens causing exacerbation is *Pseudomonas aeruginosa* whose LPS was used to initiate inflammatory responses in this study ⁽¹⁰⁻¹²⁾. TNF α is also a key cytokine contributing to this disease which is found in increasing concentrations in the sputum and BAL of patients with COPD, and which can induce both local and systemic effects ^(3, 7, 199). It is often written that the major cell type responsible for COPD pathology is the neutrophil ⁽⁷⁴⁾. However, the airway epithelium is the first contact to noxious substances and pathogens entering the lung and responds by the production and release of cytokines for the recruitment of inflammatory cells. Therefore they play a crucial role in the initiation and extension of the inflammatory response and contribute to the disease.

In this study the cell types chosen were those that have been widely used to study airway inflammation; A549, NCI-H292 and 16HBE14o- along with primary small airway epithelial cells. These were characterised by determining the basal expression of different mediators. A summary of the similarities and differences between the different cell types and conclusions is shown in table 6.1. Key mediators found to be expressed at mRNA level were IL1 β , IL-6, IL-8, TNF α , ICAM1, MMP-9 and MUC5AC and at the protein level were IL-6, IL-8, MMP9 and MUC5AC. This is the first study to compare each of these cell types which were found to express very similar mediators. LPS and TNF α were used to activate an immune response to see what mediators were induced which might indicate how the airway epithelial cells contribute to COPD pathology. Key mediators found to be induced were IL-6, IL-8, and MUC5AC. Comparisons were made of the basal expression and kinetic induction of mediators in the different cell types but further study should compare the expression of receptors TLR4 and TNFR1 to determine if this explains any differences in expression patterns found. Equally the cells in this study were

cultured in 2D and further study in 3D air liquid interface would be useful to compare cell types. For example, mucin expression is likely to be different in 3D culture especially in the homogeneous SAEC where 3D culture would allow differentiation into goblet cells and subsequent mucin expression. Other studies could look at further MMPs and also look at the expression of TIMPs in the airway epithelium.

These results emphasise that investigators should validate that their chosen cell line is appropriate by checking that it performs in a similar manner under the assay conditions used to primary cells.

Another area of potential investigation derived from this study is to look into the induction of mediators by LPS from different pathogens and in different tissues to test the hypothesis that epithelial cells from different tissues respond differently to the same variant of LPS.

Cell Similarities	Cell Differences	Conclusions
<ul style="list-style-type: none"> • All cells expressed mRNA for IL1β, IL-6, IL-8, TNFα, TGFβ, MMP1, MMP9, MMP12. • All cells did not express IL-10 and IL-12 mRNA • IL-6 and IL-8 protein expressed basally by all cell types, IL-10 protein not expressed basally by all cell types. • 1 hour maximal mRNA induction by TNFα matches in the NCI-H292, SAEC and 16HBE14o- cells for IL-6, IL-8, TNFα and IL1β • Similar kinetic pattern of MMP9 mRNA induction in NCI-H292, SAEC and 16HBE14o- cells. • 16HBE14o- and SAEC match in response to vitamin D with reduction in IL-6 mRNA and protein. 	<ul style="list-style-type: none"> • Only NCI-H292 and A549 cells expressed MUC5AC mRNA and protein. • 16HBE14o- cells do not express basal ICAM-1 mRNA • Cells differ in their response to LPS treatment • MMP1 mRNA induced by TNFα only in A549 and NCI-H292 • MMP12 mRNA induced by TNFα only in the SAEC • A549 not responsive to vitamin D treatment • NCI-H292 give a biphasic response to vitamin D treatment • Vitamin D reduces MUC5AC mRNA and protein in NCI-H292 cells • Vitamin D synergistically induces ROS in 16HBE14o- cells • Vitamin D treatment increases mRNA expression of ROS genes in NCI-H292 but not SAEC 	<p>16HBE14o- cells are a good model of the SAEC for vitamin D reduction in IL-6 expression</p>
		<p>16HBE14o- cells are not a good model of SAEC for MMP9 enzyme activity</p>
		<p>SAEC are the most sensitive of the cell types tested to vitamin D treatment</p>
		<p>Cells respond very differently to vitamin D treatment and therefore primary cells should be used for experiments.</p>
<p>Table 6.1 – Summary of the similarities and differences between the cell types used in the study and conclusions</p>		

6.2 Vitamin D and lung inflammation

Vitamin D is now widely accepted as an immunomodulatory agent that has been shown to have many functions. Vitamin D deficiency has been shown to be associated with an increased risk of respiratory tract infections, tuberculosis⁽⁸⁵⁾ and impaired lung function^(125, 127). Despite this, very little has been investigated on the effects of vitamin D in airway epithelial cells. More recently, groups have established that airway epithelial cells constitutively express CYP27 suggesting they are responsive to vitamin D treatment⁽¹²³⁾ and vitamin D has been shown to modulate inflammatory responses to respiratory syncytial virus infection⁽²⁴⁹⁾. Dysregulation of the vitamin D signalling pathway has also been implicated in lung cancer with increased expression of CYP24 in cells from these patients as well as reduced CYP27 and VDR^(178-180, 252). All of these together point to the fact that vitamin D could be a very important modulator in the lungs and requires further investigation.

This is the first study to investigate the effect of vitamin D in a wide range of airway epithelial cells looking specifically at modulation of agents which are important in the pathology of COPD. This study demonstrated vitamin D to have a number of different effects which were cell type dependent as shown in table 6.1. IL-6 was a key cytokine found to be modulated in the SAEC, 16HBE14o- and NCI-H292 cells. This cytokine is important in the initiation and extension of the immune response. MUC5AC is another important mediator involved in airway obstruction which was found to be modulated by vitamin D treatment with reduced expression at both the mRNA and protein level in the NCI-H292 cells. MMP9 was interestingly reduced in the 16HBE14o- cells but in the SAEC was reduced at the mRNA level but increased at the protein level. The 1,25-MARRS is important in packaging processes in the ER and is also involved in signalling at the cell membrane. In chondrocytes, vitamin D causes release of MMP9 from extracellular matrix vesicle organelles into the ECM by binding to the 1,25MARRs. Vitamin D is also involved in the activation of MMPs from their inactive zymogen form⁽²⁶²⁾. Therefore a possible hypothesis from this is that in the SAEC vitamin D could be causing release of 'ready made' MMP9 from vesicles by binding to 1,25MARRS or it may be causing activation of MMP9 which has already been secreted as only active MMP9 is picked up by gelatin zymography.

This area is certainly interesting and requires further investigation. One way to investigate this would be to block the 1,25-MARRS to see if it prevents the increase in MMP9 activity. However, it does not explain why a different effect was found in the 16HBE14o- cells.

The concentration of vitamin D used in this study was higher than physiological levels and therefore was more a therapeutic dose. However, as yet no-one has determined how much active vitamin D is produced within the lung given adequate serum levels of 25(OH)D₃. The *in vivo* concentrations of active vitamin D generated in tissue is currently an area of interest for a number of researchers some of which are looking at prostate tissue of patients supplemented with vitamin D to determine how much active vitamin D is produced. This area has been quite under-researched though this is mainly due to discrepancies on how to measure vitamin D levels accurately due to the assays detecting its different epimers and metabolites⁽²⁹⁹⁾. It would be very interesting to look at how much active vitamin D is produced within the lung.

The mechanistic data in this study needs further work to show what signalling pathways vitamin D is activating / modulating in airway epithelial cells. This could be done a number of ways but for a comprehensive approach would need to study changes in the level of protein expression and phosphorylation status of different signalling components at various time points after vitamin D treatment.

6.3 Vitamin D and oxidative stress

Oxidative stress is key to driving inflammation in COPD and is mainly caused by smoking. A number of different methodologies have been used to study oxidative stress but these rely on the detection of specific species whereas the H₂DCFDA assay developed in this study allowed high throughput screening and reproducible results. Future studies should further develop this assay with more stable reagents in order to screen potential oxidative stress inducers or antioxidants. Vitamin D has been shown to sensitise cancer cells to oxidative damage⁽²⁶⁶⁾. In this study vitamin D was actually found to give a synergistic induction of ROS in the normal bronchial cells whereas literature has shown vitamin D to protect normal prostate cells from

oxidative injury⁽²⁶⁸⁾. This again appears to be cell type dependent and it will be key to identify cellular characteristics which show similar vitamin D attributes so that an explanation for this can be established.

Vitamin D was found to induce antioxidant gene expression and Nrf2 protein expression in the NCI-H292 cells. It was not found to be cytotoxic to the cells or significantly induce ROS and therefore did not appear to be pro-oxidant except when pre-treated before tBHP where there was found to be a synergistic effect of vitamin D. Vitamin D was also found to increase TNF α mRNA expression at the same time point as the anti-oxidant response genes. It appears vitamin D treatment is activating a late pro-inflammatory response and therefore also increasing anti-oxidants at this time point. The reason for this still needs to be established.

6.4 New hypotheses from this study to be investigated

This study has identified novel mediators that vitamin D modulates in airway epithelial cells which may be important in disease pathology. It has also investigated the mechanisms involved in this although this requires further study. Initially the plan for this study was to include the effects of vitamin D in airway epithelial cells from patients who have COPD and this would still be important to study in the future to fully determine if vitamin D is beneficial in these diseased cell types.

Oxidative stress has been shown to be important for the induction of MUC5AC in NCI-H292 cells as ROS are required to activate latent TNF α converting enzyme (TACE) exposing the catalytic domain to cleave pro-TGF α which is the ligand for EGFR, initiating a MAPK cascade leading to mucin gene expression and protein production⁽²⁴³⁾. Nrf2 has been shown to block MUC5AC expression because it activates antioxidants blocking ROS production⁽³⁰⁰⁾. Therefore in this study with vitamin D increasing Nrf2 and blocking MUC5AC expression in the NCI-H292 cells these two events could be a potential mechanism and would be interesting to study further. Also, the VDR has been shown to directly inhibit EGFR transcriptional activity in breast cancer cells which is augmented on 1,25(OH)₂D₃ treatment⁽³⁰¹⁾

suggesting that this could also be a mechanism and therefore is a VDR dependent effect which would need further investigation.

Taking into account vitamin D metabolism and the activation of CYP24 by vitamin D, these latter effects of induction of TNF α and antioxidant genes at 24 hours in the NCI-H292 cells could be due to early activation of signalling molecules by vitamin D or it could be that vitamin D is metabolised into its epimer or other metabolites which are then driving an inflammatory response. An important study would be to look at the metabolism of vitamin D in the different cell types studied to see the half life of active vitamin D and its metabolites and then whether any of these metabolites have a biological activity as has been found in some studies ⁽³⁰²⁾.

The A549 cells appear insensitive to vitamin D treatment and this is likely to be due to over expression of CYP24, but it would be interesting to KO CYP24 to see if vitamin D can then modulate inflammatory mediators like the other cell types. As it has been shown recently that inhibiting CYP24 does increase the antiproliferative effect of vitamin D in these cells.

Another area of vitamin D research is the rapid responses vs the genomic responses and both the time frame of these responses and whether they involve the 1,25MARRs or VDR needs further investigation but these two different modes of action might be responsible for the effects we have seen at different time points.

6.5 Human Studies

As yet much of the data on the potential of vitamin D in COPD is speculative with many more molecular papers on its action showing anti-inflammatory, anti-protease and anti-bacteria properties all of which would be beneficial to COPD pathology ⁽³⁰³⁾. Higher vitamin D levels have been associated with improved lung function ⁽¹²⁵⁾, and vitamin D deficiency is found in many COPD patients and correlates with disease severity ⁽²⁴⁸⁾. However, studies have also shown that in patients with severe COPD, circulating 25(OH)D is not predictive of acute exacerbations of the disease ⁽³⁰⁴⁾ and that 25(OH)D levels are not associated with adult lung function ⁽³⁰⁵⁾. These discrepancies could be down to the genetics of the populations studied and it may be that subsets of patients with COPD are more

responsive to the effects of vitamin D, although much larger patient cohorts are required to get sufficient data in order to characterise these subsets.

With vitamin D deficiency linked to COPD disease severity it is as yet unclear whether deficiency gives you higher risk of the disease or if more vitamin D is used up when you have the disease ⁽¹⁸⁷⁾. It may be that vitamin D may play more of a preventative role than a possible therapeutic role but until clinical trials are carried out on vitamin D supplementation of patients with COPD and of control subjects and smokers to see if it reduces COPD development and / or modulates disease severity then this question will remain unanswered.

It is clear that COPD patients are at an increased risk of getting osteoporosis and therefore vitamin D supplementation to give patients sufficient levels is an important step ⁽¹⁸⁷⁾. COPD patients have a high rate of vitamin D deficiency and intervention studies are necessary to determine if vitamin D supplementation is of benefit in the prevention and treatment of osteoporosis in patients with COPD ⁽³⁰⁶⁾.

There is no doubt that having a sufficient vitamin D status should be a goal for the whole population due to its broad range of health benefits that have been and continue to be found. However, as yet recommended levels of vitamin D have only been set for its skeletal actions, despite much evidence for other beneficial effects, and as such it is likely to be a long while before recommendations are set for vitamin D in lung disease as this is a somewhat under studied area.

6.6 Conclusion

As is the nature of research, this study brought about many more questions than it answered. However, it has identified novel mediators which are modulated by vitamin D in airway epithelial cells and are important in lung disease pathology. Further studies should investigate how vitamin D is modulating IL-6 and MUC5AC expression in airway epithelial cells and establish if vitamin D is beneficial in disease pathology using human studies.

APPENDIX I

Cytotoxicity data for reagents and inhibitors

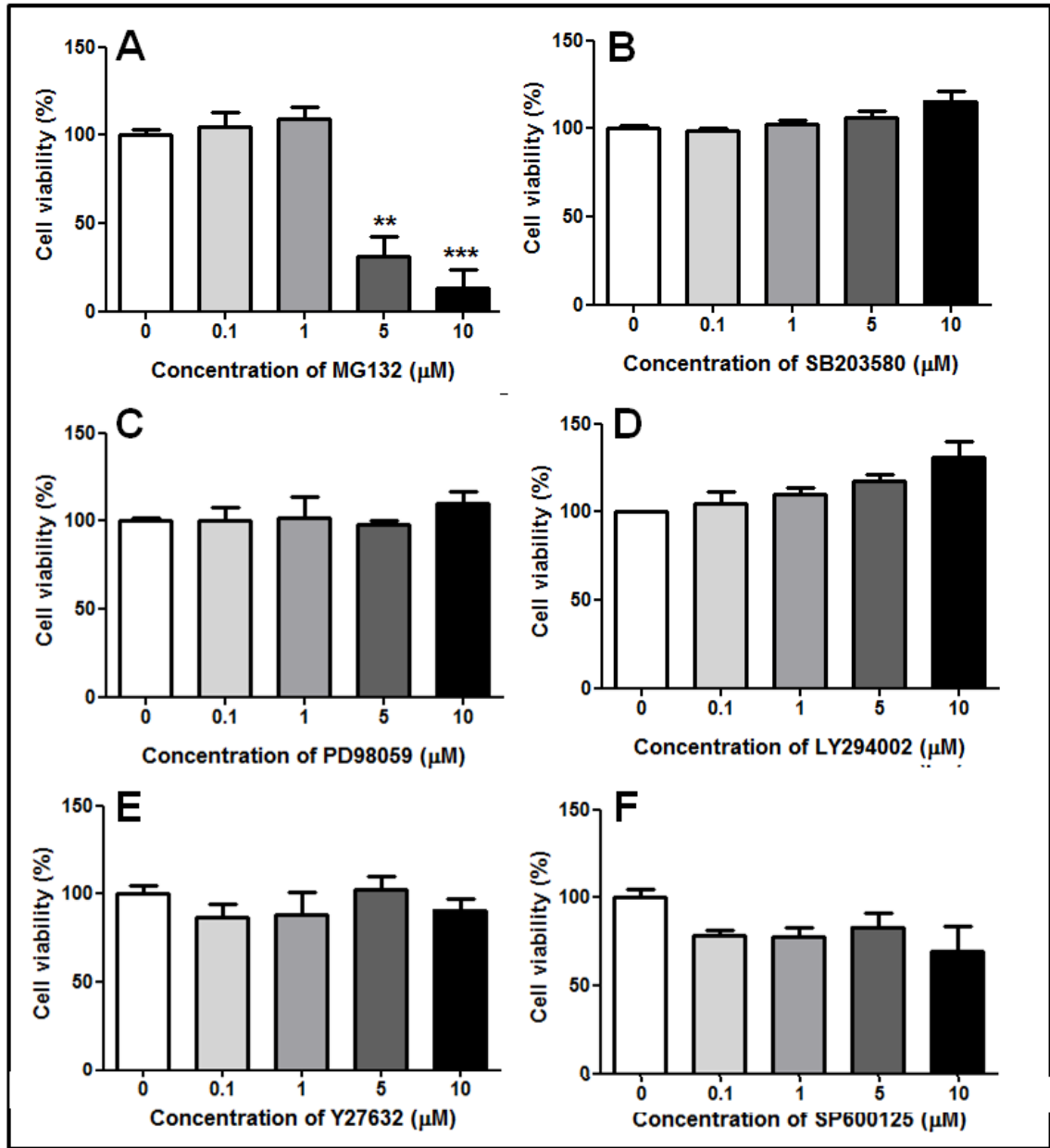
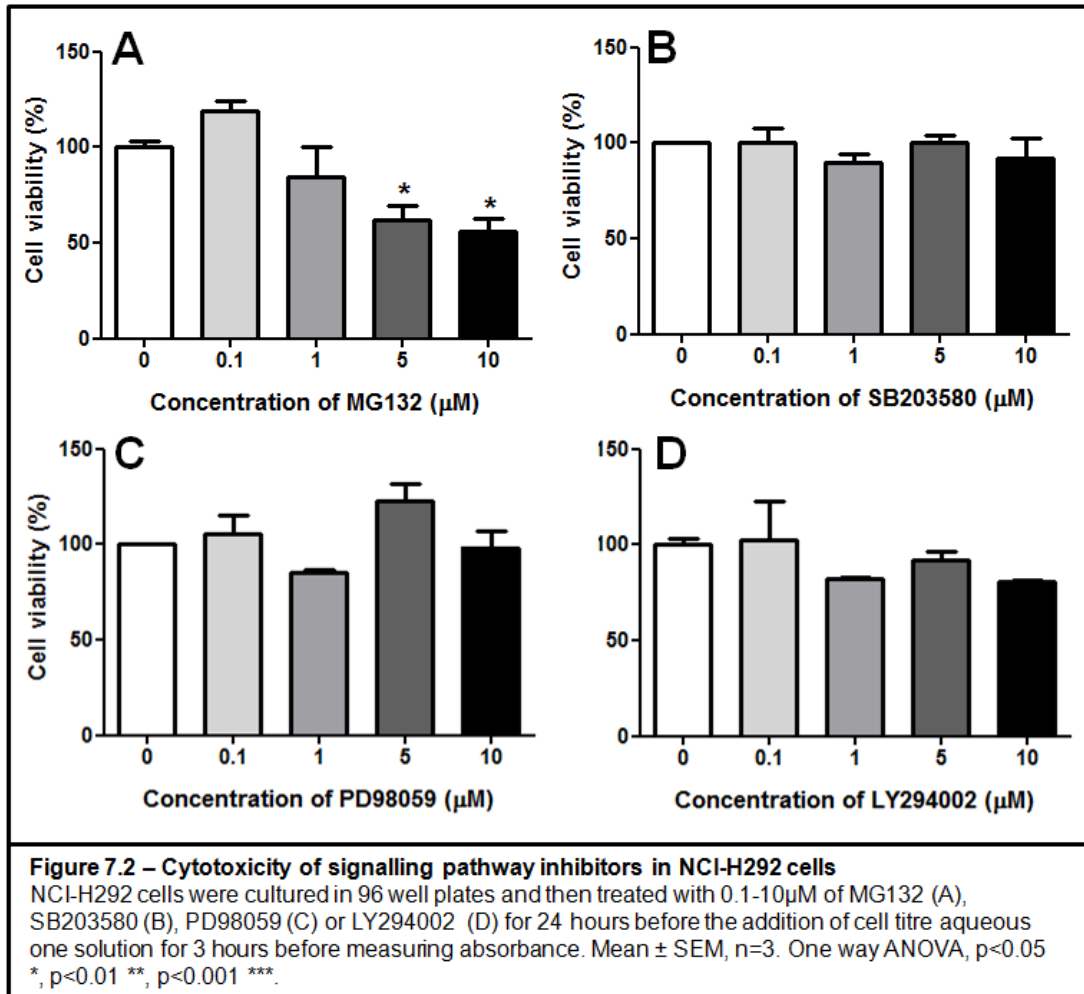
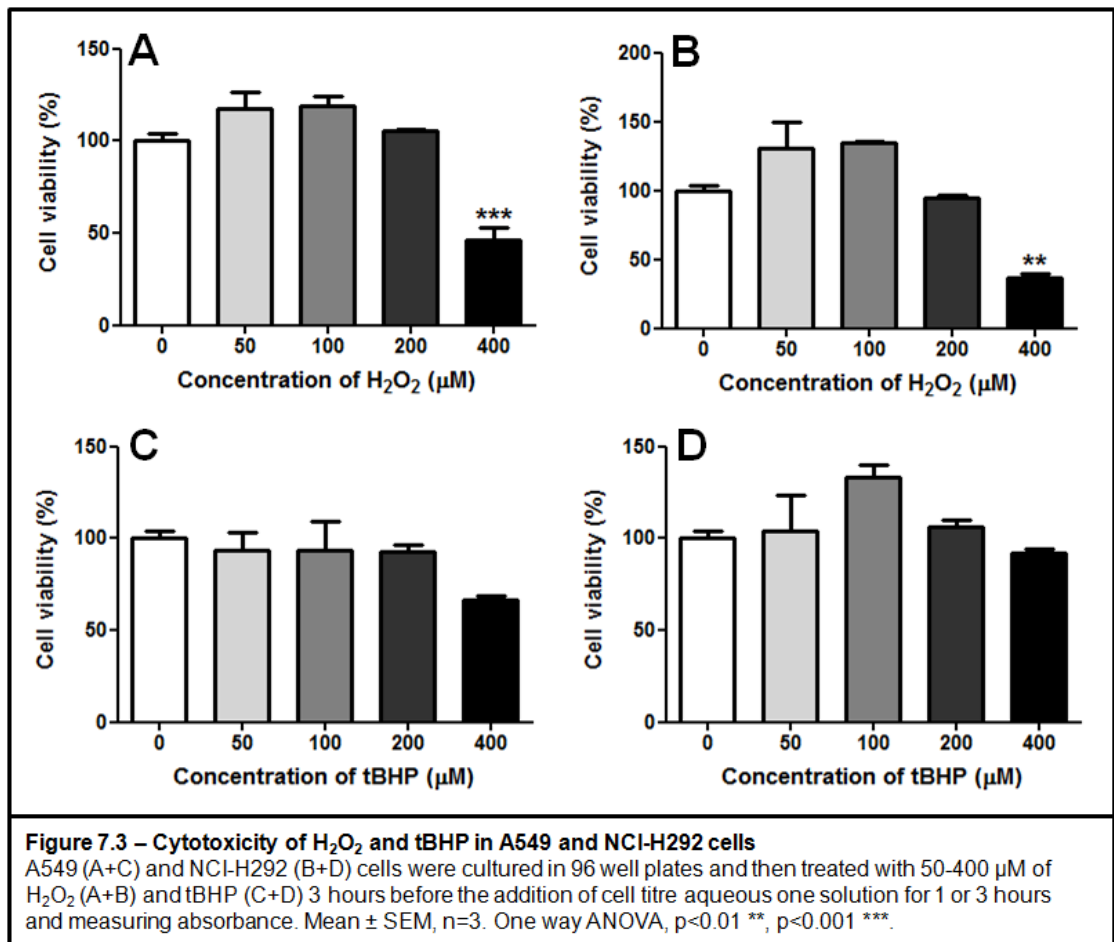


Figure 7.1 – Cytotoxicity of signalling pathway inhibitors in 16HBE14o-cells

16HBE14o-cells were cultured in 96 well plates and then treated with 0.1-10 μM of MG132 (A), SB203580 (B), PD98059 (C), LY294002 (D), Y27632 (E) and SP600125 (F) for 24 hours before the addition of cell titre aqueous one solution for 3 hours and measuring absorbance. Mean \pm SEM, n=3. One way ANOVA, $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.





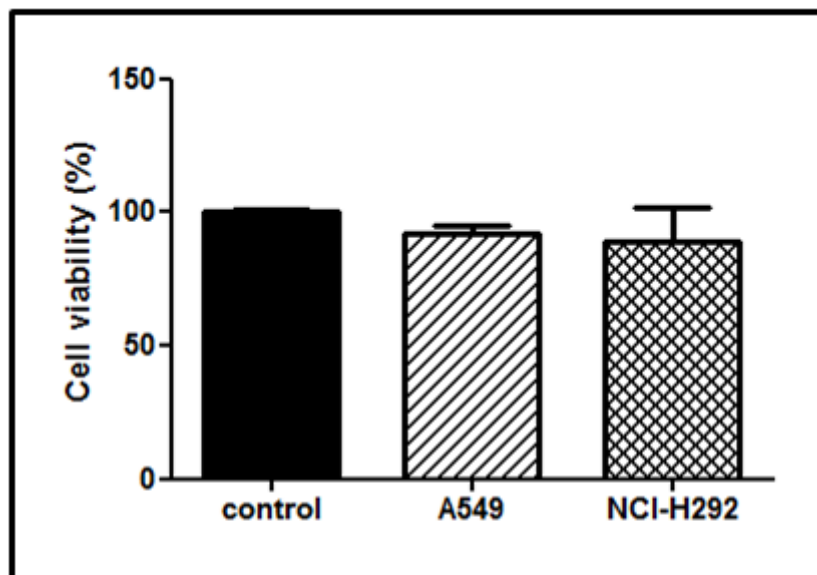


Figure 7.4 – Cytotoxicity of ROS assay

A549 and NCI-H292 cells were cultured in 96 well plates and then treated with 10 μ M H₂DCDFA for 40 minutes followed by 20 minute recovery in fresh media before the addition of cell titre aqueous one solution for 1-3 hours and measuring absorbance. Mean \pm SEM, n=3.

Appendix II

Buffers and Solutions

Cell culture

Fibronectin coating solution for 16HBE14o- cells

88 mL LHC basal medium, 10 mL 0.1% BSA, 1 mL vitrogen, 1 mL human fibronectin

cDNA synthesis

10 µl Reverse Transcription Reagents Mastermix

RNA at 50 ng/µl

10 µl reaction = 4.5 µl (RNA+H₂O) + 5.5 µl Mastermix

Number of Samples	MgCl ₂ (25 µM)	10 x RT buffer	dNTP	RT	RNase	Random Hexamers
1	x 2.5 µl	x 1 µl	x 1 µl	x 0.25 µl	x 0.25 µl	x 0.5 µl

Random hexamers were diluted ½ before use to 25 µM.

qRT-PCR

Polymerase Chain Reaction Reagent Mastermix

20 µl PCR reaction = 5 µl cDNA + 15 µl Mastermix

Number of Samples	SYBR green	H ₂ O	Primer (5 µM) forward + reverse
1	x 10 µl	x 4 µl	x 1 µl

Western blot

1 x running buffer

760 mL dH₂O and 40 mL 20 X MOPS SDS running buffer

1 x transfer buffer

680 mL dH₂O, 80 mL methanol, 40 mL 20 X transfer buffer, 1 mL anti-oxidant

Blocker

20 g Marvel and 400 mL TBST

10 X TBST

48.4 g tris base

160 g NaCl
62 mL 5M HCl
20 mL Tween-20
pH to 7.6
Dilute to 1L with distilled water

Gelatin Zymography

10% Resolving Gel (2plates)

15 mg gelatine
5.9 mL dH₂O
3.8 mL 1.5 M Tris pH 8.8
5 mL 30% acrylamide: 0.8% bis-acrylamide
150 µL 10% sodium dodecyl sulphate
150 µL 10% ammonium persulphate (0.1 g in 1 mL dH₂O)
6 µL TEMED

5% Stacking Gel (2 plates)

2.75 mL dH₂O
0.5 mL 1 M Tris pH 6.8
0.65 mL 30% acrylamide: 0.8% bis-acrylamide
40 µL 10% sodium dodecyl sulphate
40 µL 10% ammonium persulphate
4 µL TEMED

5 x running buffer (1L)

15 g tris
94 g glycine
50 mL 10% SDS (10 g in 100 mL dH₂O)
Make up to 1L with dH₂O

Rinse buffer

25 mL Triton-X100

Make up to 1 L with dH₂O

Incubation Buffer

50 mM tris pH 7.5 (50 mL of 1 M Tris pH8 (24.22 g in 200 mL))

5 mM CaCl₂ (5 mL 1 M CaCl₂ (14.7 g in 100 mL))

Make up to 1L with dH₂O

4 x Loading buffer (50 mL)

200 mM tris pH 6.8 (20 mL 1 M Tris pH 6.8)

4% SDS (2g)

0.1% bromophenol blue (50 mg)

40% glycerol (20 mL)

Coomassie Blue stain (200 mL)

30% isopropanol (60 mL)

10% acetic acid (20 mL)

2.5 mg/mL coomassie brilliant blue R (0.5 g in 120 mL dH₂O)

Destain solution (200mL)

10% isopropanol (20mL)

10% acetic acid (20mL)

160 mL dH₂O

EDTA 0.05M

Immunostaining

Gelatin quench buffer

1 g gelatin in 450 mL H₂O

50 mL 10 x PBS

Blocking buffer

15 mL normal goat serum

35 mL gelatin quench buffer

Wash buffer

500 mL PBS

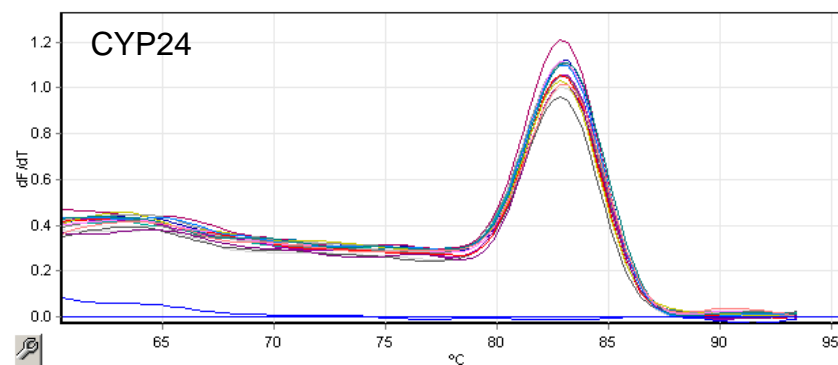
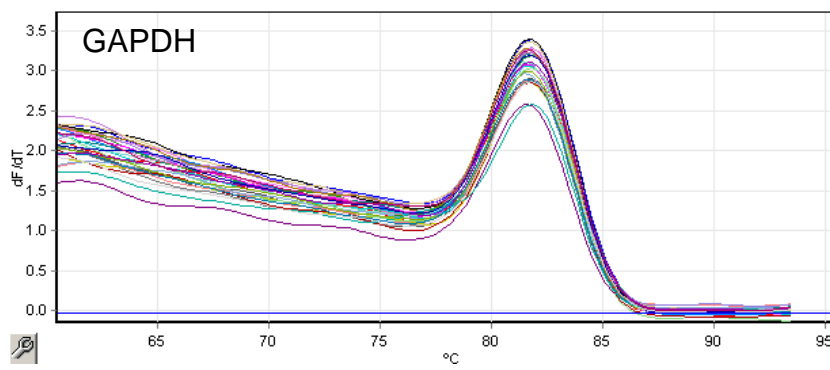
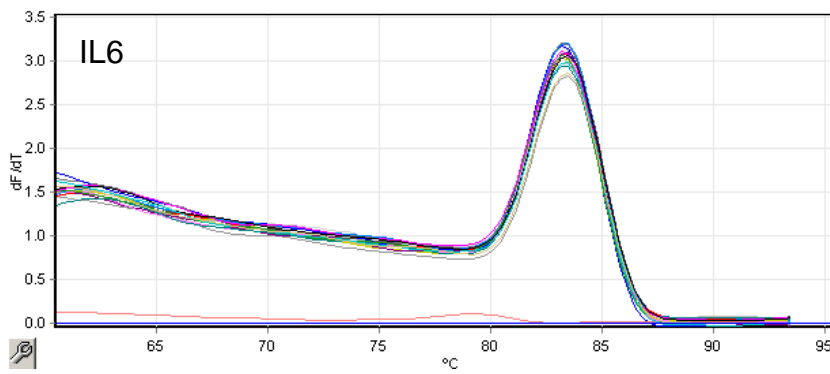
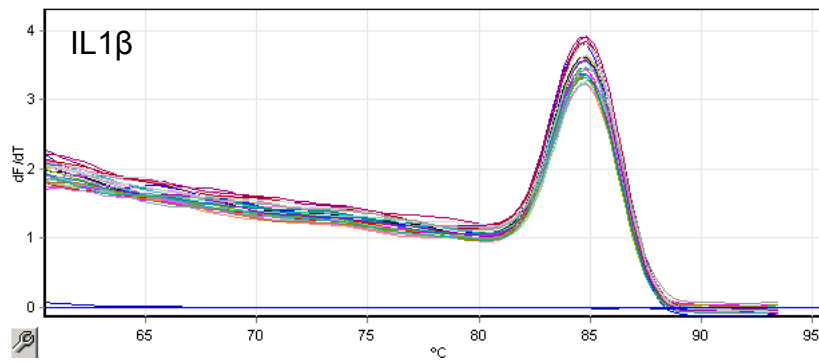
30 mL 10% goat serum/PBS

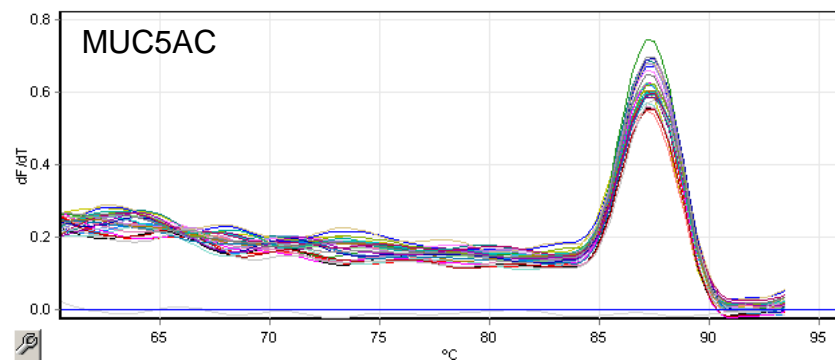
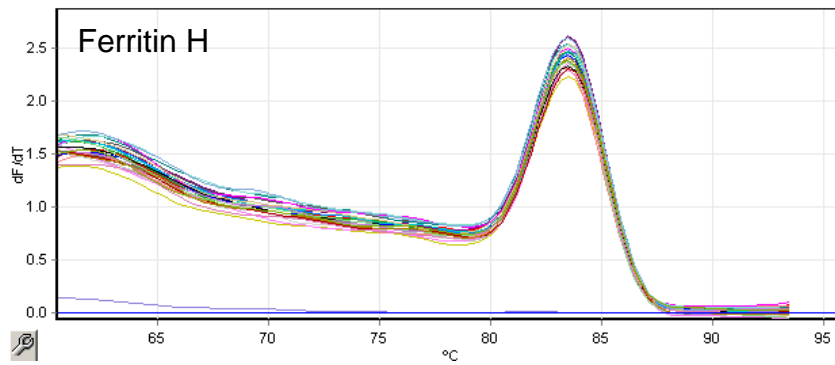
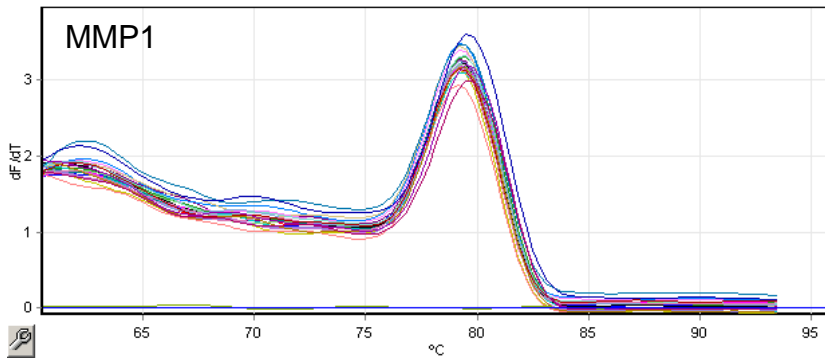
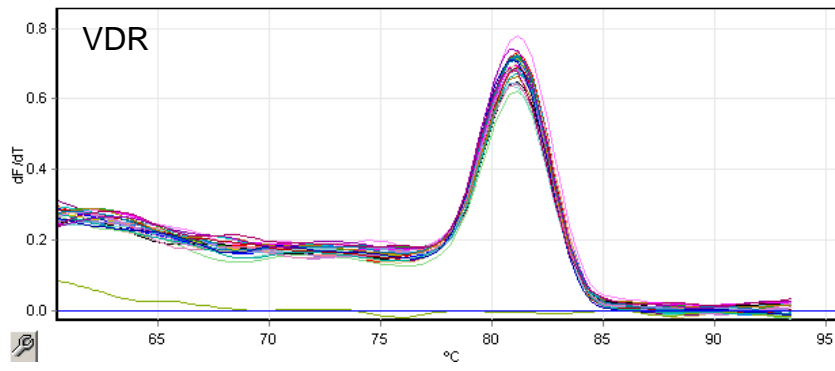
10 mL goat serum

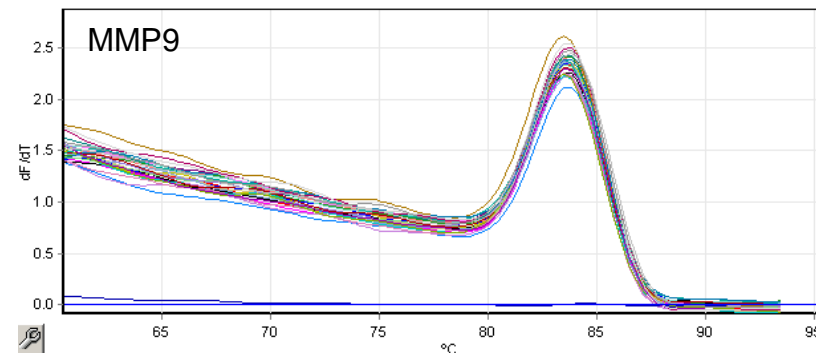
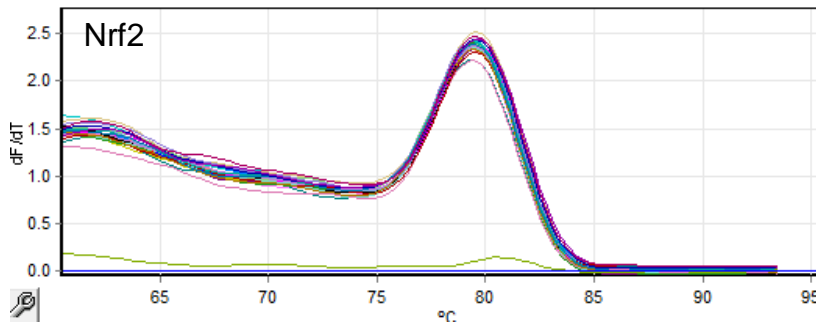
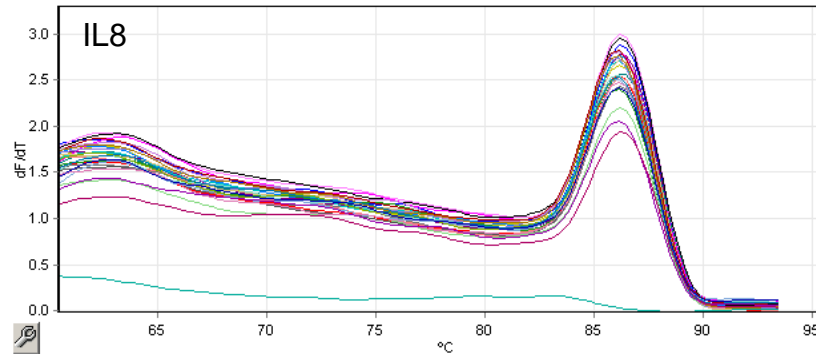
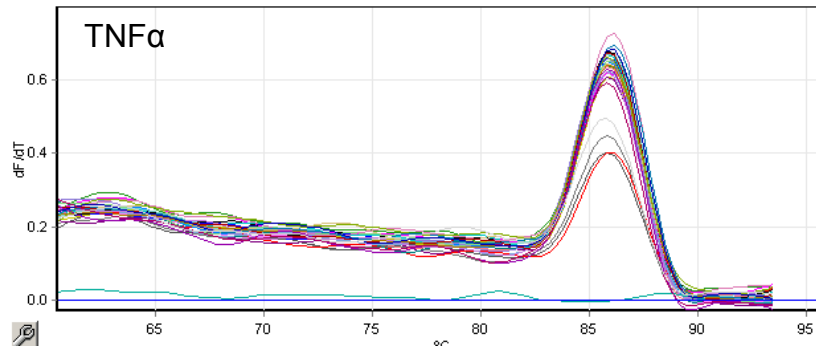
APPENDIX III

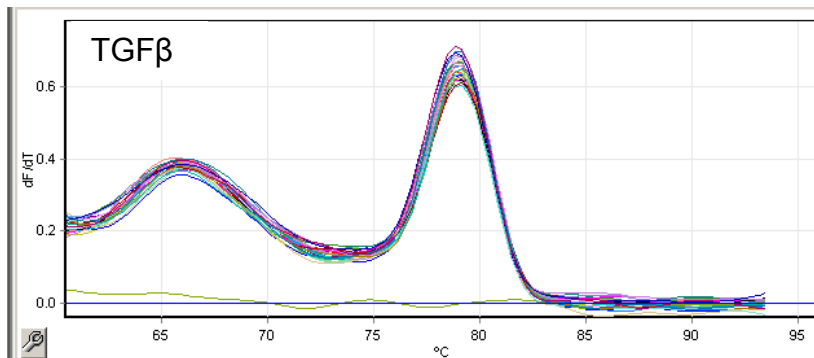
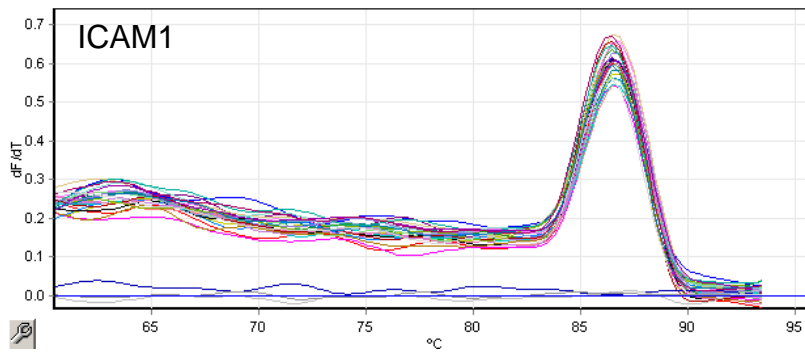
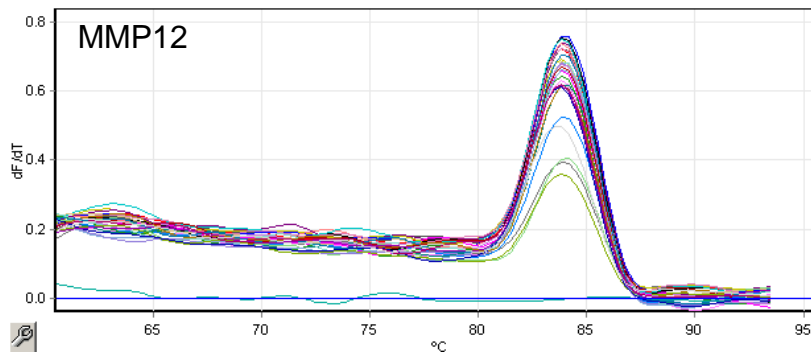
Dissociation curves and standard curves for SYBR green qRT-PCR

Dissociation curves

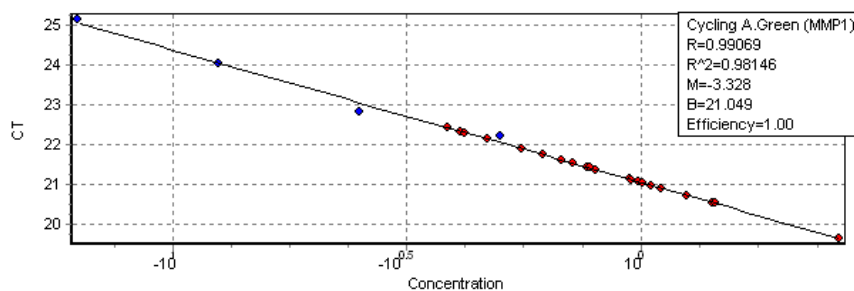
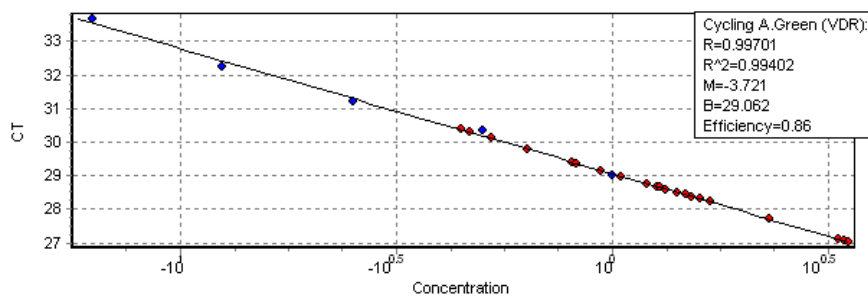
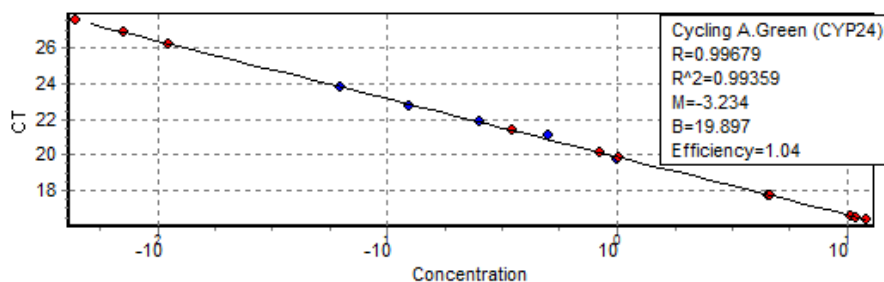
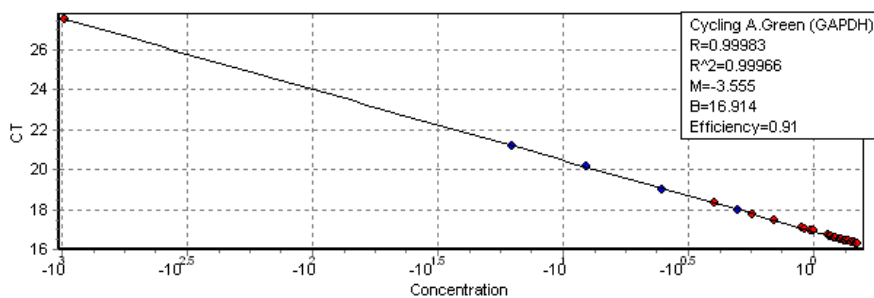
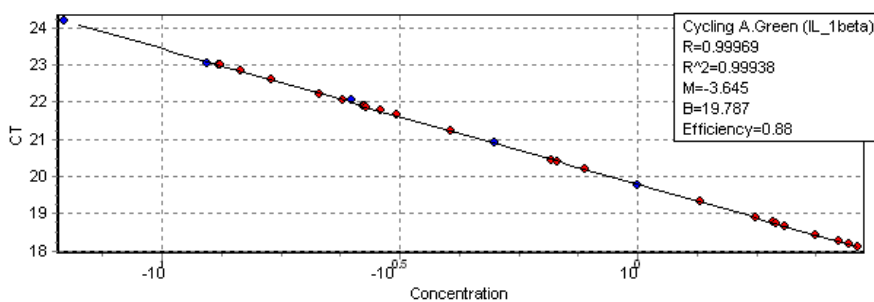


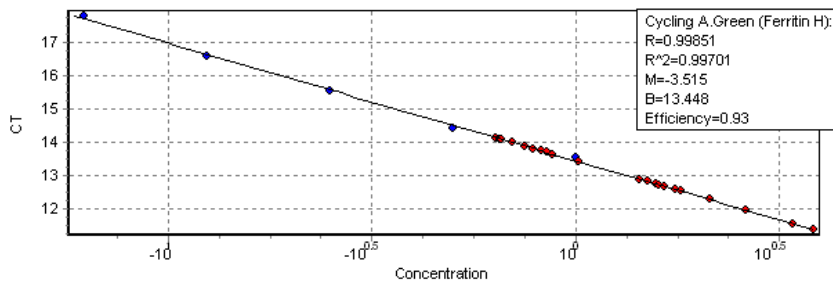
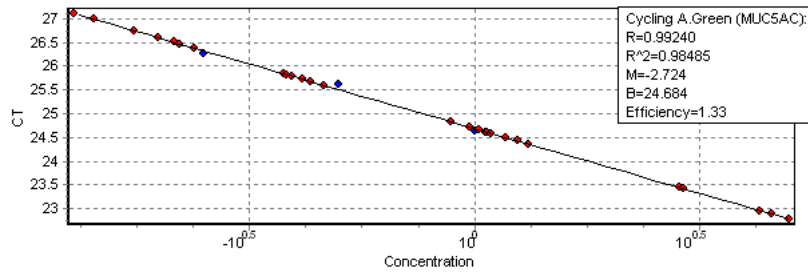
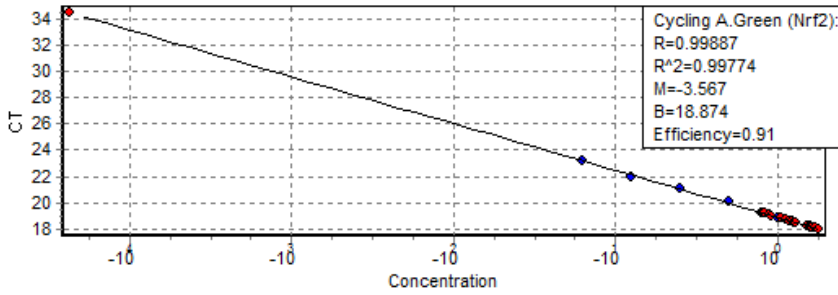
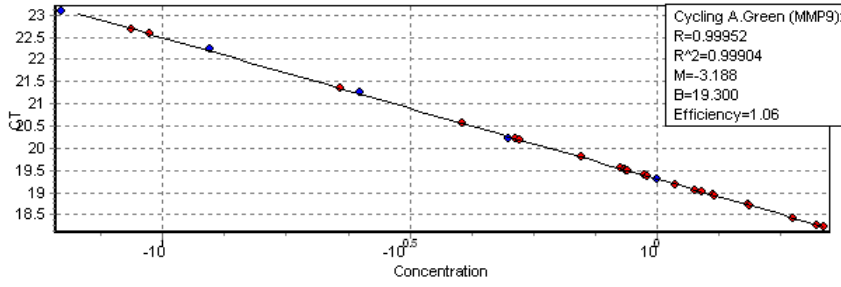
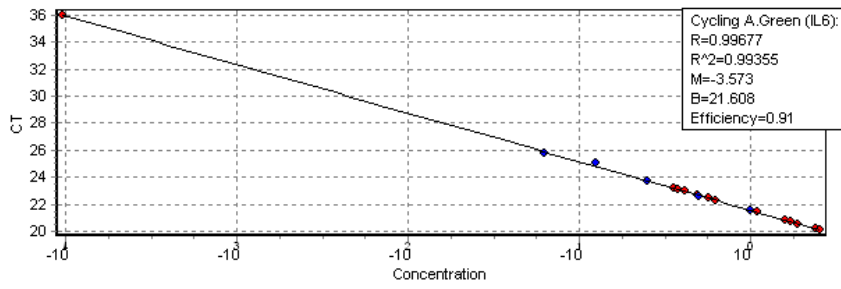


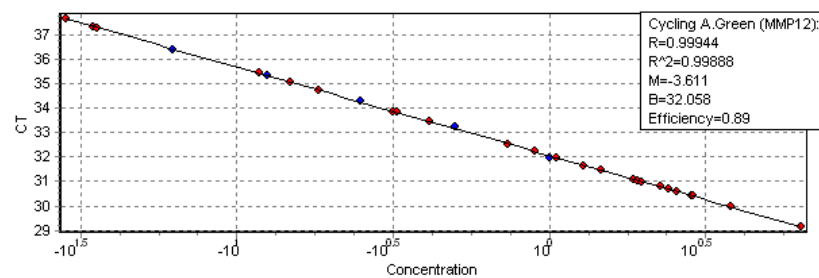
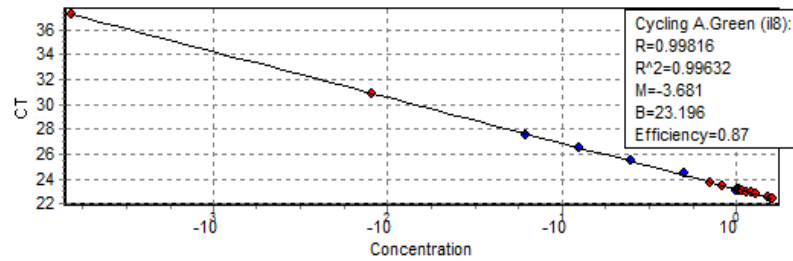
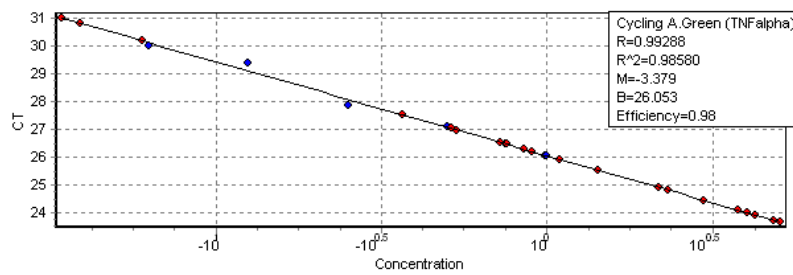
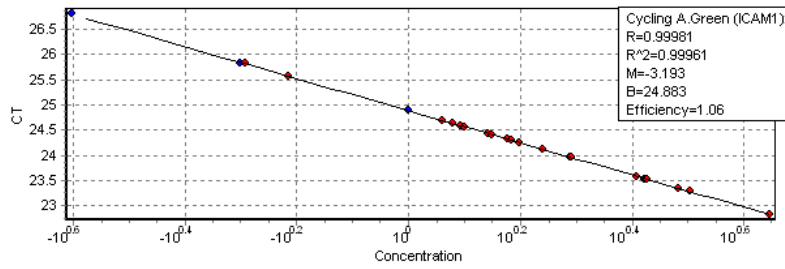
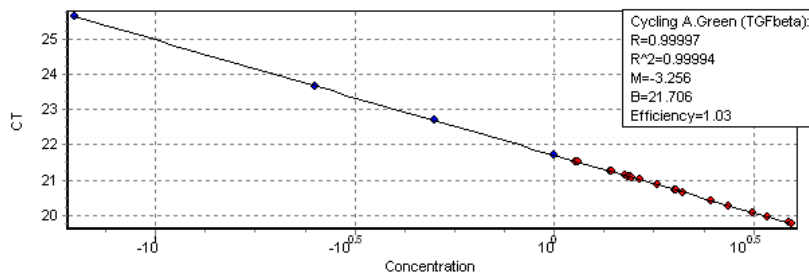




Standard curve efficiencies

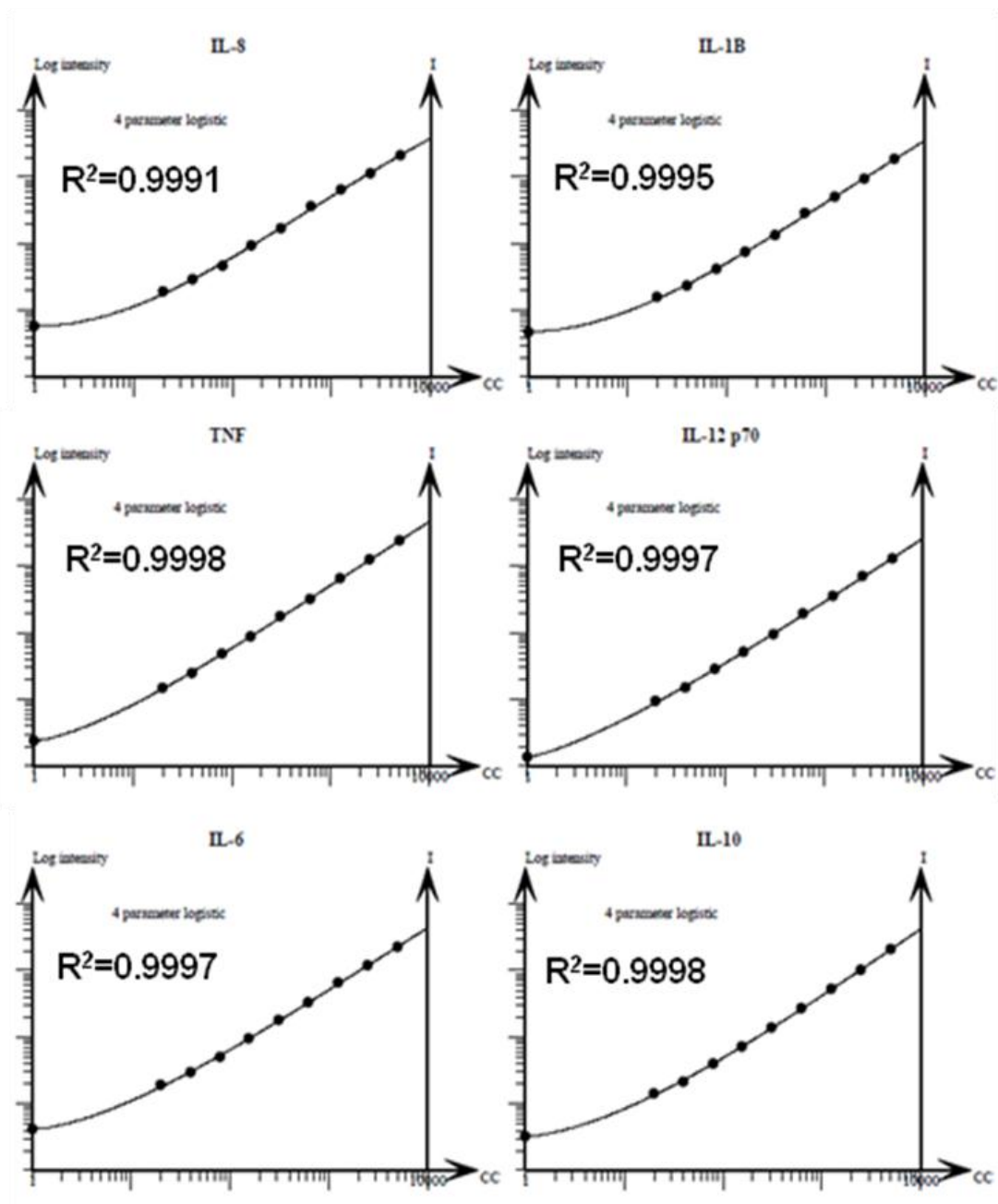






APPENDIX IV

Standard curves for flow cytometric bead array



References

1. Mroz RM HA, Chyczewska E, Braszko JJ: Chronic obstructive pulmonary disease: an update on nuclear signaling related to inflammation and anti-inflammatory treatment. *J Physiol Pharmacol* 59(6): 35-42, 2008.
2. Kuebler KK, Buchsel PC, and Balkstra CR: Differentiating chronic obstructive pulmonary disease from asthma. *J Am Acad of Nurse Pract* 20(9): 445-454, 2008.
3. Kim V, Rogers TJ, and Criner GJ: New Concepts in the Pathobiology of Chronic Obstructive Pulmonary Disease. *Proc Am Thorac Soc* 5(4): 478-485, 2008.
4. Tzortzaki EG, Tsoumakidou M, Makris D, and Siafakas NM: Laboratory markers for COPD in "susceptible" smokers. *Clin Chim Acta* 364(1-2): 124-138, 2006.
5. Rahman I: Pharmacological antioxidant strategies as therapeutic interventions for COPD. *Biochim Biophys Acta* 1822(5): 714-28, 2012.
6. Yoshida T and Tuder RM: Pathobiology of Cigarette Smoke-Induced Chronic Obstructive Pulmonary Disease. *Physiol Rev* 87: 1047-1082, 2007.
7. Decramer M, De Benedetto F, Del Ponte A, and Marinari S: Systemic effects of COPD. *Resp Med* 99(Supplement 2): S3-S10, 2005.
8. Hawkins NM, Petrie MC, Jhund PS, Chalmers GW, Dunn FG, and McMurray JJV: Heart failure and chronic obstructive pulmonary disease: diagnostic pitfalls and epidemiology. *Eur J Heart Fail* 11(2): 130-139, 2009.
9. National Institute for Health and Clinical Excellence: *CG101 Chronic obstructive pulmonary disease (update): full guideline*, 2010. Available from: <http://guidance.nice.org.uk/CG101>.
10. Miravittles M: Exacerbations of chronic obstructive pulmonary disease: when are bacteria important? *Eur Respir J* 20(36_suppl): 9S-19, 2002.
11. Sykes A, Mallia P, and Johnston SL: Diagnosis of Pathogens in Exacerbations of Chronic Obstructive Pulmonary Disease. *Proc Am Thorac Soc* 4(8): 642-646, 2007.

12. Hill AT, Campbell EJ, Hill SL, Bayley DL, and Stockley RA: Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am J Med* 109(4): 288-295, 2000.
13. Engler K, Muhlemann K, Garzoni C, Pfahler H, Geiser T, and von Garnier C: Colonisation with *Pseudomonas aeruginosa* and antibiotic resistance patterns in COPD patients. *Swiss Medical Wkly epub* 2012.
14. Hansen LA, Poulsen OM, and Würtz H: Endotoxin potency in the A549 lung epithelial cell bioassay and the limulus amoebocyte lysate assay. *J Immunol Methods* 226(1-2): 49-58, 1999.
15. Peinado VR, J, Roca J, Rodriguez-Roisin R, and Barbera J: Identification of vascular progenitor cells in pulmonary arteries of patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 34(3): 257-63, 2006.
16. Rao SP SL, Hosseinkhani MR, Pinkerton KE, Srirmarao P: Exposure to environmental tobacco smoke induces angiogenesis and leukocyte trafficking in lung microvessels. *Exp Lung Res* 35(2): 119-35, 2009.
17. Kjell L: Aspects on pathophysiological mechanisms in COPD. *J Int Med* 262(3): 311-340, 2007.
18. Mayer AK, Bartz H, Fey D, Schmidt LM, and Dalpke AH: Airway epithelial cells modify immune responses by inducing an anti-inflammatory microenvironment. *Eur J Immunol* 38(6): 1689-1699, 2008.
19. Foster KA, Oster CG, Mayer MM, Avery ML, and Audus KL: Characterization of the A549 Cell Line as a Type II Pulmonary Epithelial Cell Model for Drug Metabolism. *Exp Cell Res* 243(2): 359-366, 1998.
20. dos Santos CC, Han B, Andrade CF, Bai X, Uhlig S, Hubmayr R, Tsang M, Lodyga M, Keshavjee S, Slutsky AS, and Liu M: DNA microarray analysis of gene expression in alveolar epithelial cells in response to TNF α , LPS, and cyclic stretch. *Physiol Genomics* 19(3): 331-342, 2004.
21. Binker MG, Binker-Cosen AA, Richards D, Oliver B, and Cosen-Binker LI: LPS-stimulated MUC5AC production involves Rac1-dependent MMP-9 secretion and activation in NCI-H292 cells. *Biochem Biophys Res Commun* 386(1): 124-129, 2009.

22. Gruenert DC, Basbaum CB, Welsh MJ, Li M, Finkbeiner WE, and Nadel JA: Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc Nat Acad Sci USA* 85(16): 5951-5955, 1988.
23. Henson PM, Vandivier RW, and Douglas IS: Cell Death, Remodeling, and Repair in Chronic Obstructive Pulmonary Disease? *Proc Am Thorac Soc* 3(8): 713-717, 2006.
24. Hansel T and Barnes P, *An atlas of chronic obstructive pulmonary disease*. 2004: Parthenon, London.
25. Hansel TT and Barnes PJ: New drugs for exacerbations of chronic obstructive pulmonary disease. *Lancet* 374(9691): 744-755, 2009.
26. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, and Haegeman G: Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochem Pharmacol* 60(8): 1185-1195, 2000.
27. Matera MG, Calzetta L, and Cazzola M: TNF- α inhibitors in asthma and COPD: We must not throw the baby out with the bath water. *Pulm Pharmacol Ther* 23(2): 121-128, 2010.
28. Nydam TL ME, McIntyre RC Jr, Wright FL, Gamboni-Robertson F, Eckels PC, Banerjee A: Hypertonic Saline Attenuates TNF α induced NF κ B activation in pulmonary epithelial cells. *Shock* 31(5): 466-72, 2009.
29. Churg A, Wang RD, Tai H, Wang X, Xie C, and Wright JL: Tumor Necrosis Factor α Drives 70% of Cigarette Smoke-induced Emphysema in the Mouse. *Am J Respir Crit Care Med* 170(5): 492-498, 2004.
30. Sullivan DE, Ferris M, Pociask D, and Brody AR: Tumor Necrosis Factor- α Induces Transforming Growth Factor β 1 Expression in Lung Fibroblasts Through the Extracellular Signal-Regulated Kinase Pathway. *Am J Respir Cell Mol Biol* 32(4): 342-349, 2005.
31. Kim S and Nadel JA: Fibrinogen Binding to ICAM-1 Promotes EGFR-Dependent Mucin Production in Human Airway Epithelial Cells. *Am J Physiol Lung Cell Mol Physiol* 297(1): L174-L183, 2009.
32. Fakler C, Wu B, McMicken H, Geske R, and Welty S: Molecular mechanisms of lipopolysaccharide induced ICAM-1 expression in A549 cells. *Inflamm res* 49: 63-72, 2000.

33. Aul R, Armstrong J, Duvoix A, Lomas D, Hayes B, Miller BE, Jagger C, and Singh D: Inhaled LPS challenges in smokers: a study of pulmonary and systemic effects. *Br J Clin Pharmacol* 74(6): 1023-1032, 2012.
34. Jiang Y-Y, Xiao W, Zhu M-X, Yang Z-H, Pan X-J, Zhang Y, Sun C-C, and Xing Y: The effect of human antibacterial peptide LL-37 in the pathogenesis of chronic obstructive pulmonary disease. *Respir Med* 106(12): 1680-1689, 2012.
35. Tudor RM, Yoshida T, Fijalkowka I, Biswal S, and Petrache I: Role of Lung Maintenance Program in the Heterogeneity of Lung Destruction in Emphysema. *Proc Am Thorac Soc* 3(8): 673-679, 2006.
36. Tse AK, Zhu GY, Wan CK, Shen XL, Yu ZL, and Fong WF: $1\alpha,25$ -dihydroxyvitamin D₃ inhibits transcriptional potential of nuclear factor kappa B in breast cancer cells. *Mol Immunol* 47: 1728-1738, 2010.
37. Banerjee A, Koziol-White C, and Panettieri R: p38 MAPK inhibitors, IKK2 inhibitors, and TNF α inhibitors in COPD. *Curr Opin Pharmacol epub* 2012.
38. Rahman I and Adcock IM: Oxidative stress and redox regulation of lung inflammation in COPD. *Eur Respir J* 28(1): 219-42, 2006.
39. Mercer B and D'Armiento J: Emerging role of MAP kinase pathways as therapeutic targets in COPD. *Int J Chron Obstruct Pulmon Dis* 1(2): 137-50, 2006.
40. Mercer B, Brinckerhoff C, and D'Armiento J: Activation of the MMP-1 Promoter by Cigarette Smoke in Human Small Airway Epithelial Cells Requires ERK MAP Kinase Signaling: Differential Response of the 1G and 2G Promoter Sequences. *Proc Am Thorac Soc* 3(6): 477, 2006.
41. Bennett B: c-Jun N-terminal kinase-dependent mechanisms in respiratory disease. *Eur Respir J* 28(3): 651-61, 2006.
42. Lin CC, Tseng HW, Hsieh HL, Lee CW, Wu CY, Cheng CY, and Yang CM: Tumor necrosis factor- α induces MMP-9 expression via p42/p44 MAPK, JNK, and nuclear factor- κ B in A549 cells. *Toxicol Appl Pharmacol* 229(3): 386-398, 2008.
43. Yao H, de boeur W, and Rahman I: Targeting lung inflammation: novel therapies for the treatment of COPD. *Curr Respir Med Rev* 4(1): 57-68, 2008.

44. Adcock IM, Caramori G, and Barnes PJ: Chronic Obstructive Pulmonary Disease and Lung Cancer: New Molecular Insights. *Respiration* 81(4): 265-284, 2011.
45. Ghigo A, Damilano F, Braccini L, and Hirsch E: PI3K inhibition in inflammation: Toward tailored therapies for specific diseases. *BioEssays* 32(3): 185-196, 2010.
46. Crosby LM, and Waters CM: Epithelial Repair Mechanisms in the Lung. *Am J Physiol Lung Cell Mol Physiol* 298(6):L715-31, 2010.
47. Fernandes LB, Henry PJ, and Goldie RG: Review: Rho kinase as a therapeutic target in the treatment of asthma and chronic obstructive pulmonary disease. *Ther Adv Respir Dis* 1(1): 25-33, 2007.
48. Schaafsma D, Gosens R, Zaagsma J, Halayko A, and Meurs H: Rho kinase inhibitors: a novel therapeutical intervention in asthma? *Eur J Pharmacol* 13(585(2-3)): 398-406, 2008.
49. Abboud RT and Vimalanathan S: Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema [State of the Art Series. Chronic obstructive pulmonary disease in high- and low-income countries. Edited by G. Marks and M. Chan-Yeung. Number 3 in the series]. *Int J Tuberc Lung Dis* 12(4): 361-367, 2008.
50. Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, and Belaaouaj A: Neutrophil Elastase Contributes to Cigarette Smoke-Induced Emphysema in Mice. *Am J Pathol* 163(6): 2329-2335, 2003.
51. Sullivan A, Dafforn T, Hiemstra P, and Stockley R: Neutrophil elastase reduces secretion of secretory leukoprotease inhibitor (SLPI) by lung epithelial cells: role of charge of the proteinase-inhibitor complex. *Resp Res* 9(1): 60, 2008
52. Chaudhuri R, McSharry C, Brady J, Donnelly I, Grierson C, McGuinness S, Jolly L, Weir CJ, Messow C, Spears M, Miele G, Nocka K, Crowther D, Thompson J, Brannigan M, Lafferty J, Sproule M, MacNee W, Connell M, Murchinson J, Shepherd M, Feuerstein G, Miller DK, and Thomson N: Sputum matrix metalloproteinase-12 in patients with chronic obstructive pulmonary disease and asthma: Relationship to disease severity. *J Allergy Clin Immunol* 129(3): 655-663, 2012.

53. Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, and D'Armiento J: Human Collagenase (Matrix Metalloproteinase-1) Expression in the Lungs of Patients with Emphysema. *Am J Respir Crit Care Med* 163(3): 786-791, 2001.
54. Piesiak P, Brzecka A, Kosacka M, Passowicz-Muszynska E, Dyla T, and Jankowska R: [Concentrations of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 in serum of patients with chronic obstructive pulmonary disease. *Pol Merkur Lekarski* 31(185): 270-3, 2011.
55. Wu Y, Li J-D, Wu J, Morgan P, Xu X, Rancati F, Vallese S, Raveglia L, Hotchandani R, Fuller N, Bard J, Cunningham K, Fish S, Krykbaev R, Tam S, and Goldman SJ: Discovery of potent and selective matrix metalloproteinase 12 inhibitors for the potential treatment of chronic obstructive pulmonary disease (COPD). *Bioorg Med Chem Lett* 22(1): 138-43, 2012.
56. Dahl R, Titlestad I, Lindqvist A, Wielders P, Wray H, Wang M, Samuelsson V, Mo J, and Holt A: Effects of an oral MMP-9 and -12 inhibitor, AZD1236, on biomarkers in moderate/severe COPD: A randomised controlled trial. *Pulm Pharmacol Ther epub* 2012.
57. Lim SAM, Roche N, Oliver BG, Mattos W, Barnes PJ, and Fan Chung K: Balance of Matrix Metalloprotease-9 and Tissue Inhibitor of Metalloprotease-1 from Alveolar Macrophages in Cigarette Smokers. Regulation by Interleukin-10. *Am J Respir Crit Care Med* 162(4): 1355-1360, 2000.
58. Mercer PF, Shute JK, Bhowmik A, Donaldson GC, Wedzicha JA, and Warner JA: MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Resp Res* 6(1): 151, 2005.
59. Turner J and Jones CE: Regulation of mucin expression in respiratory diseases. *Biochem Soc Trans* 037(4): 877-881, 2009.
60. Thornton DJ, Rousseau K, and McGuckin MA: Structure and Function of the Polymeric Mucins in Airways Mucus. *Ann Rev Phy* 70(1): 459-486, 2008.
61. Horváth I, Donnelly LE, Kiss A, Balint B, Kharitonov SA, and Barnes PJ: Exhaled Nitric Oxide and Hydrogen Peroxide Concentrations in Asthmatic Smokers. *Respiration* 71(5): 463-468, 2004.

62. Rahman I and MacNee W: Antioxidant pharmacological therapies for COPD. *Current Opinion in Pharmacology epub* 2012.
63. Rahman I, Gilmour PS, Jimenez LA, and MacNee W: Oxidative stress and TNF- α induce histone acetylation and NF- κ B/AP-1 activation in alveolar epithelial cells: Potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 234-235(1): 239-248, 2002.
64. Mak J: Pathogenesis of COPD. Part II. Oxidative-antioxidative imbalance. *Int J Tuberc Lung Dis* 12(4): 368-374, 2008.
65. Boutten A, Goven D, Artaud-Macari E, Boczkowski J, and Bonay M: NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease. *Trends Mol Med* 17(7): 363-371, 2011.
66. Gerritsen WB, Asin J, Zanen P, van den Bosch JM, and Haas FJ: Markers of inflammation and oxidative stress in exacerbated chronic obstructive pulmonary disease patients. *Resp Med* 99(1): 84-90, 2005.
67. Cho H-Y and Kleeberger SR: Nrf2 protects against airway disorders. *Toxicol Appl Pharmacol* 244(1): 43-56, 2010.
68. Reddy NM, Potteti HR, Mariani TJ, Biswal S, and Reddy SP: Conditional Deletion of Nrf2 in Airway Epithelium Exacerbates Acute Lung Injury and Impairs the Resolution of Inflammation. *Am J Respir Cell Mol Biol* 45(6): 1161-8.
69. Ishii Y, Itoh K, Morishima Y, Kimura T, Kiwamoto T, Iizuka T, Hegab AE, Hosoya T, Nomura A, Sakamoto T, Yamamoto M, and Sekizawa K: Transcription Factor Nrf2 Plays a Pivotal Role in Protection against Elastase-Induced Pulmonary Inflammation and Emphysema. *J Immunol* 175(10): 6968-6975, 2005.
70. Malhotra D, Thimmulappa R, Vij N, Navas-Acien A, Sussan T, Merali S, Zhang L, Kelsen SG, Myers A, Wise R, Tudor R, and Biswal S: Heightened Endoplasmic Reticulum Stress in COPD Lungs: The Role of Nrf2-Regulated Proteasomal Activity. *Am J Respir Crit Care Med* 180(12): 1196-207, 2009.
71. Kingwell K: Lung disease: Antioxidant protection against cigarette smoke. *Nat Rev Drug Discov* 8(2): 110-110, 2009.
72. Sussan TE, Rangasamy T, Blake DJ, Malhotra D, El-Haddad H, Bedja D, Yates MS, Kombairaju P, Yamamoto M, Liby KT, Sporn MB, Gabrielson KL,

- Champion HC, Tudor RM, Kensler TW, and Biswal S: Targeting Nrf2 with the triterpenoid CDDO- imidazole attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice. *Proc Nat Acad Sci* 106(1): 250-255, 2009.
73. Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, and Barnes PJ: Decreased Histone Deacetylase Activity in Chronic Obstructive Pulmonary Disease. *N Engl J Med* 352(19): 1967-1976, 2005.
 74. Sarir H, Henricks PAJ, van Houwelingen AH, Nijkamp FP, and Folkerts G: Cells, mediators and Toll-like receptors in COPD. *Eur J Pharmacol* 585(2-3): 346-353, 2008
 75. Hanania NA, Ambrosino N, Calverley P, Cazzola M, Donner CF, and Make B: Treatments for COPD. *Resp Med* 99(Supplement 2): S28-S40, 2005.
 76. Rabe K and Wedzicha JA: Controversies in treatment of chronic obstructive pulmonary disease. *Lancet* 378(9795): 1038-1047, 2011.
 77. Edwards MR, Bartlett NW, Clarke D, Birrell M, Belvisi M, and Johnston SL: Targeting the NF- κ B pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol Ther* 121(1): 1-13, 2009.
 78. Bafadhel M, McKenna S, Terry S, Mistry V, Reid C, Haldar P, McCormick M, Haldar K, Keadze T, Duvoix A, Lindblad K, Patel H, Rugman P, Dodson P, Jenkins M, Saunders M, Newbold P, Green RH, Venge P, Lomas DA, Barer MR, Johnston SL, Pavord ID, and Brightling CE: Acute Exacerbations of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 184(6): 662-671, 2011.
 79. Bourbeau J and Johnson M: New and Controversial Therapies for Chronic Obstructive Pulmonary Disease. *Proc Am Thorac Soc* 6(6): 553-554, 2009.
 80. Barnes PJ: Cytokine-directed therapies for the treatment of chronic airway diseases. *Cytokine Growth Factor Rev* 14(6): 511-522, 2003.
 81. Hu J, Van den Steen PE, Sang Q-XA, and Opdenakker G: Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov* 6: 480-498, 2007.
 82. Malhotra D, Thimmulappa RK, Mercado N, Ito K, Kombairaju P, Kumar S, Ma J, Feller-Kopman D, Wise R, Barnes P, and Biswal S: Denitrosylation of

- HDAC2 by targeting Nrf2 restores glucocorticosteroid sensitivity in macrophages from COPD patients. *J Clin Invest* *121(11)*: 4289-302, 2011.
83. Biswal S, Thimmulappa RK, and Harvey C: Experimental Therapeutics of Nrf2 as a Target for Prevention of Bacterial Exacerbations in COPD. *Proc Am Thorac Soc* *9(2)*: 47-51, 2012.
 84. Gabriella Matera M, Calzetta L, Rinaldi B, and Cazzola M: Treatment of COPD: moving beyond the lungs. *Current Opinion in Pharmacology* *12(3)*: 315-322, 2012.
 85. Holick MF and Chen TC: Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr* *87(4)*: 1080S-1086, 2008.
 86. Adams JS, Liu PT, Chun R, Modlin RL, and Hewison M: Vitamin D in Defense of the Human Immune Response. *Ann N Y Acad Sci* *1117(Skeletal Biology and Medicine, Part B: Disease Mechanisms and Therapeutic Challenges)*: 94-105, 2007.
 87. Holick MF: McCollum Award Lecture, 1994: Vitamin D - new horizons for the 21st Century. *Am J Clin Nutr* *60*: 619-30, 1994.
 88. Etten Ev and Mathieu C: Immunoregulation by 1,25-dihydroxyvitamin D₃: Basic concepts. *J Steroid Biochem Mol Biol* *97(1-2)*: 93-101, 2005.
 89. Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, Madronich S, Garland CF, and Giovannucci E: Epidemic influenza and vitamin D. *Epidemiol Infect* *134(06)*: 1129-1140, 2006.
 90. Ebert R, Schütze N, Adamski J, and Jakob F: Vitamin D signaling is modulated on multiple levels in health and disease. *Mol Cell Endocrinol* *248(1-2)*: 149-159, 2006.
 91. Avila E, Díaz L, Barrera D, Halhali A, Méndez I, González L, Zuegel U, Steinmeyer A, and Larrea F: Regulation of Vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D₃ and cyclic AMP in cultured human syncytiotrophoblasts. *J Steroid Biochem Mol Biol* *103(1)*: 90-96, 2007.
 92. Baeke F, Etten Ev, Gysemans C, Overbergh L, and Mathieu C: Vitamin D signaling in immune-mediated disorders: Evolving insights and therapeutic opportunities. *Mol Aspect Med* *29(6)*: 376-387, 2008.
 93. Laaksi I, Ruohola J-P, Tuohimaa P, Auvinen A, Haataja R, Pihlajamaki H, and Ylikomi T: An association of serum vitamin D concentrations < 40 nmol/L

- with acute respiratory tract infection in young Finnish men. *Am J Clin Nutr* 86(3): 714-717, 2007.
94. Speeckaert M, Huang G, Delanghe JR, and Taes YEC: Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. *Clinica Chimica Acta* 372(1-2): 33-42, 2006.
 95. Koyama T, Shibakura M, Ohsawa M, Kamiyama R, and Hirosawa S: Anticoagulant Effects of 1 α ,25-Dihydroxyvitamin D₃ on Human Myelogenous Leukemia Cells and Monocytes. *Blood* 92(1): 160-167, 1998.
 96. Holick MF: Vitamin D Deficiency. *N Engl J Med* 357(3): 266-281, 2007.
 97. Haussler MR, Jurutka PW, Mizwicki M, and Norman AW: Vitamin D receptor (VDR)-mediated actions of 1 α ,25(OH)₂vitamin D₃: Genomic and non-genomic mechanisms. *Best Pract Res Clin Endocrinol Metab* 25(4): 543-559, 2011.
 98. Nemere I, Safford SE, Rohe B, DeSouza MM, and Farach-Carson MC: Identification and characterization of 1,25D₃-membrane-associated rapid response, steroid (1,25D₃-MARRS) binding protein. *J Steroid Biochem Mol Biol* 89-90(0): 281-285, 2004.
 99. Coe H, and Michalak M: ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. *Int J Biochem Cell Biol* 42(6): 796-799, 2010.
 100. Adams J S HM: Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat Clin Pract Endocrinol Metab* 4(2): 80-90, 2008.
 101. Prosser DE and Jones G: Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem Sci* 29(12): 664-673, 2004.
 102. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, and Kumar R: Dendritic cell modulation by 1 α ,25 dihydroxyvitamin D₃ and its analogs: A vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proc Nat Acad Sci USA* 98(12): 6800-6805, 2001.
 103. Embry AF, Snowdon LR, and Vieth R: Vitamin D and seasonal fluctuations of gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. *Ann Neurol* 48(2): 271-272, 2000.

104. Hyppönen E, Läärä E, Reunanen A, Järvelin M-R, and Virtanen SM: Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *Lancet* 358(9292): 1500-1503, 2001.
105. Szodoray P, Nakken B, Gaal J, Jonsson R, Szegedi A, Zold E, Szegedi G, Brun JG, Gesztelyi R, Zeher M, and Bodolay E: The Complex Role of Vitamin D in Autoimmune Diseases. *Scand J Immunol* 68(3): 261-269, 2008.
106. Matilainen JM, Räsänen A, Gynther P, and Väisänen S: The genes encoding cytokines IL-2, IL-10 and IL-12B are primary $1\alpha,25(\text{OH})_2\text{D}_3$ target genes. *The J Steroid Biochem Mol Biol In Press, Uncorrected Proof*.
107. Prehn JL, Fagan DL, Jordan SC, and Adams JS: Potentiation of lipopolysaccharide-induced tumor necrosis factor- α expression by 1,25-dihydroxyvitamin D₃. *Blood* 80(11): 2811-2816, 1992.
108. D'Ambrosio D CM, Cocciolo M G, Mazzeo D, Di Lucia P, Lang R, Sinigaglia D, Panina-Bordignon P: Inhibition of IL-12 production by 1,25-dihydroxyvitamin D₃. Involvement of NF- κ B downregulation in transcriptional repression of the p40 gene. *J Clin Invest* 101(1): 252-262, 1998.
109. Chung J, Koyama T, Ohsawa M, Shibamiya A, Hoshi A, and Hirose S: 1,25(OH)₂D₃ blocks TNF-induced monocytic tissue factor expression by inhibition of transcription factors AP-1 and NF- κ B. *Lab Invest* 87(6): 540-547, 2007.
110. Riis JL, Johansen C, Gesser B, Møller K, Larsen CG, Kragballe K, and Iversen L: $1\alpha,25(\text{OH})_2\text{D}_3$ regulates NF κ B DNA binding activity in cultured normal human keratinocytes through an increase in I κ B α expression. *Arch Dermatol Res* 296(5): 195-202, 2004.
111. Cohen-Lahav M, Shany S, Tobvin D, Chaimovitz C, and Douvdevani A: Vitamin D decreases NF κ B activity by increasing I κ B α levels. *Nephrol Dial Transplant* 21(4): 889-897, 2006.
112. Tse AK-W, Wan C-K, Shen X-L, Zhu G-Y, Cheung H-Y, Yang M, and Fong W-F: $1\alpha,25$ -Dihydroxyvitamin D₃ induces biphasic NF- κ B responses during HL-60 leukemia cells differentiation through protein induction and PI3K/Akt-dependent phosphorylation/degradation of I κ B. *Exp Cell Res* 313(8): 1722-1734, 2007.

113. Berry DM, Clark CS, and Meckling-Gill KA: $1\alpha,25$ -Dihydroxyvitamin D₃ Stimulates Phosphorylation of I κ B α and Synergizes with TPA to Induce Nuclear Translocation of NF κ B during Monocytic Differentiation of NB4 Leukemia Cells. *Exp Cell Res* 272(2): 176-184, 2002.
114. Baeke F, Gysemans C, Korf H, and Mathieu C: Vitamin D insufficiency: implications for the immune system. *Pediatr Nephrol* 25(9): 1597-606, 2010.
115. Farmer PK, He X, Schmitz ML, Rubin J, and Nanes MS: Inhibitory effect of NF- κ B on $1,25$ -dihydroxyvitamin D₃ and retinoid X receptor function. *Am J Physiol Endocrinol Metab* 279(1): E213-220, 2000.
116. Nonn L, Peng L, Feldman D, and Peehl DM: Inhibition of p38 by Vitamin D Reduces Interleukin-6 Production in Normal Prostate Cells via Mitogen-Activated Protein Kinase Phosphatase 5: Implications for Prostate Cancer Prevention by Vitamin D. *Cancer Res* 66(8): 4516-4524, 2006.
117. Wang J, Zhao Y, Kauss MA, Spindel S, and Lian H: Akt regulates vitamin D₃-induced leukemia cell functional differentiation via Raf/MEK/ERK MAPK signaling. *Eur J Cell Biol* 88(2): 103-115, 2009.
118. Bahar-Shany K, Ravid A, and Koren R: Upregulation of MMP-9 Production by TNF α in Keratinocytes and Its Attenuation by Vitamin D. *J Cell Physiol* 222: 729-737, 2010.
119. Ziv E, Rotem C, Miodovnik M, Ravid A, and Koren R: Two modes of ERK activation by TNF in keratinocytes: Different cellular outcomes and bi-directional modulation by vitamin D. *J Cell Biochem* 104(2): 606-619, 2008.
120. Ordonez-Moran P, Larriba MJ, Palmer HG, Valero RA, Barbachano A, Dunach M, de Herreros AG, Villalobos C, Berciano MT, Lafarga M, and Munoz A: RhoA-ROCK and p38MAPK-MSK1 mediate vitamin D effects on gene expression, phenotype, and Wnt pathway in colon cancer cells. *J Cell Biol* 183(4): 697-710, 2008.
121. Ordóñez-Morán P, Álvarez-Díaz S, Valle N, Larriba MJ, Bonilla F, and Muñoz A: The effects of $1,25$ -dihydroxyvitamin D₃ on colon cancer cells depend on RhoA-ROCK-p38MAPK-MSK signaling. *J Steroid Biochem Mol Biol* *In Press, Corrected Proof*.
122. Giuseppe P, Benedetta F, Susana A, Elisa C, Gilles L, Enrico S, Aravinda C, Annamaria M, Erica S, Gabriella Barbara V, Mauro G, Enrico C, Mario M, and

- Luciano A: The vitamin D receptor agonist elocalcitol inhibits IL-8-dependent benign prostatic hyperplasia stromal cell proliferation and inflammatory response by targeting the RhoA/Rho kinase and NF- κ B pathways. *Prostate* 69(5): 480-493, 2009.
123. Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, and Hunninghake GW: Respiratory Epithelial Cells Convert Inactive Vitamin D to Its Active Form: Potential Effects on Host Defense. *J Immunol* 181(10): 7090-7099, 2008.
 124. Lowery E, Bemiss B, Cascino T, Durazo-Arvizu RA, Forsythe S, Alex C, Laghi F, Love R, and Camacho P: Low vitamin D levels are associated with increased rejection and infections after lung transplantation. *J Heart Lung Transplant* epub 2012.
 125. Black PN and Scragg R: Relationship Between Serum 25-Hydroxyvitamin D and Pulmonary Function in the Third National Health and Nutrition Examination Survey. *Chest* 128(6): 3792-3798, 2005.
 126. Burns JS, Dockery DW, and Speizer FE: Low levels of dietary vitamin D intake and pulmonary function in adolescents. *Proc Am Thoracic Soc* 3 A536(abstr), 2006.
 127. Hughes DA and Norton R: Vitamin D and respiratory health. *Clin Exp Immunol* 158(1): 20-25, 2009.
 128. Liu PT and Modlin RL: Human macrophage host defense against *Mycobacterium tuberculosis*. *Curr Opin Immunol* 20(4): 371-376, 2008.
 129. Adams JS, Chen H, Chun R, Ren S, Wu S, Gacad M, Nguyen L, Ride J, Liu P, Modlin R, and Hewison M: Substrate and Enzyme Trafficking as a Means of Regulating 1,25-Dihydroxyvitamin D Synthesis and Action: The Human Innate Immune Response. *J Bone Miner Res* 22(s2): V20-V24, 2007.
 130. Nnoaham KE and Clarke A: Low serum vitamin D levels and tuberculosis: a systematic review and meta-analysis. *Int J Epidemiol* 37(1): 113-119, 2008.
 131. Nursyam EW AZ, Rumende CM: The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. *Acta Med Indones* 38(1): 3-5, 2006.
 132. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schaubert J, Wu K, Meinken C, Kamen DL, Wagner M, Bals R, Steinmeyer A, Zugel U,

- Gallo RL, Eisenberg D, Hewison M, Hollis BW, Adams JS, Bloom BR, and Modlin RL: Toll-Like Receptor Triggering of a Vitamin D-Mediated Human Antimicrobial Response. *Science* 311(5768): 1770-1773, 2006.
133. Martineau AR, Wilkinson RJ, Wilkinson KA, Newton SM, Kampmann B, Hall BM, Packe GE, Davidson RN, Eldridge SM, Maunsell ZJ, Rainbow SJ, Berry JL, and Griffiths CJ: A Single Dose of Vitamin D Enhances Immunity to Mycobacteria. *Am J Respir Crit Care Med* 176(2): 208-213, 2007.
134. Martineau AR, Timms PM, Bothamley GH, Hanifa Y, Islam K, Claxton AP, Packe GE, Moore-Gillon JC, Darmalingam M, Davidson RN, Milburn HJ, Baker LV, Barker RD, Woodward NJ, Venton TR, Barnes KE, Mullett CJ, Coussens AK, Rutterford CM, Mein CA, Davies GR, Wilkinson RJ, Nikolayevskyy V, Drobniewski FA, Eldridge SM, and Griffiths CJ: High-dose vitamin D3 during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *Lancet* 377(9761): 242-250, 2011.
135. Ralph AP, Kelly PM, and Anstey NM: L-arginine and vitamin D: novel adjunctive immunotherapies in tuberculosis. *Trend Microbiol* 16(7): 336-344, 2008.
136. Krutzik SR, Hewison M, Liu PT, Robles JA, Stenger S, Adams JS, and Modlin RL: IL-15 Links TLR2/1-Induced Macrophage Differentiation to the Vitamin D-Dependent Antimicrobial Pathway. *J Immunol* 181(10): 7115-7120, 2008.
137. Adams JS and Hewison M: Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. *Nat Clin Pract Endocrinol Metab* 4(2): 80-90, 2008.
138. Khoo A-L, Chai LYA, Koenen HJPM, Oosting M, Steinmeyer A, Zuegel U, Joosten I, Netea MG, and van der Ven AJAM: Vitamin D3 down-regulates proinflammatory cytokine response to Mycobacterium tuberculosis through pattern recognition receptors while inducing protective cathelicidin production. *Cytokine In Press, Corrected Proof*.
139. Vidyarani M, Selvaraj P, Jawahar MS, and Narayanan PR: 1, 25 Dihydroxyvitamin D3 modulated cytokine response in pulmonary tuberculosis. *Cytokine* 40(2): 128-134, 2007.
140. Anna C, Peter MT, Barbara JB, Timothy RV, Anthony TA, Keira HS, Sandra MN, Katalin AW, Robert ND, Christopher JG, Robert JW, and Adrian RM:

- 1,25-dihydroxyvitamin D₃ inhibits matrix metalloproteinases induced by *Mycobacterium tuberculosis* infection. *Immunol* 127(4): 539-548, 2009.
141. Anand SP and Selvaraj P: Effect of 1, 25 dihydroxyvitamin D₃ on matrix metalloproteinases MMP-7, MMP-9 and the inhibitor TIMP-1 in pulmonary tuberculosis. *Clin Immunol In Press, Corrected Proof*.
142. Van Reeth K: Cytokines in the pathogenesis of influenza. *Vet Microbiol* 74(1-2): 109-116, 2000.
143. Najada AS, Habashneh MS, and Khader M: The Frequency of Nutritional Rickets among Hospitalized Infants and its Relation to Respiratory Diseases. *J Trop Pediatr* 50(6): 364-368, 2004.
144. Ginde AA, Mansbach JM, and Camargo CA, Jr: Association Between Serum 25-Hydroxyvitamin D Level and Upper Respiratory Tract Infection in the Third National Health and Nutrition Examination Survey. *Arch Intern Med* 169(4): 384-390, 2009.
145. Urashima M, Segawa T, Okazaki M, Kurihara M, Wada Y, and Ida H: Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. *Am J Clin Nutr* 91(5): 1255-60, 2010.
146. Stephenson A, Brotherwood M, Robert R, Atenafu E, Corey M, and Tullis E: Cholecalciferol significantly increases 25-hydroxyvitamin D concentrations in adults with cystic fibrosis. *Am J Clin Nutr* 85(5): 1307-1311, 2007.
147. Chandra P, Wolfenden LL, Ziegler TR, Tian J, Luo M, Stecenko AA, Chen TC, Holick MF, and Tangpricha V: Treatment of vitamin D deficiency with UV light in patients with malabsorption syndromes: a case series. *Photodermatol Photoimmunol Photomed* 23(5): 179-185, 2007.
148. Speeckaert MM, Wehlou C, Vandewalle S, Taes YE, Robberecht E, and Delanghe JR: Vitamin D binding protein, a new nutritional marker in cystic fibrosis patients. *Clin Chem Lab Med* 46(3): 365-370, 2008.
149. Yim S, Dhawan P, Rangunath C, Christakos S, and Diamond G: Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D₃. *J Cyst Fibros* 6(6): 403-410, 2007.
150. McNally P, Coughlan C, Bergsson G, Doyle M, Taggart C, Adorini L, Uskokovic MR, El-Nazir B, Murphy P, Greally P, Greene CM, and McElvaney NG: Vitamin D receptor agonists inhibit pro-inflammatory cytokine production

from the respiratory epithelium in cystic fibrosis. *J Cyst Fibros* In Press, Corrected Proof.

151. Song Y, Qi H, and Wu C: Effect of 1,25-(OH)₂D₃ (a vitamin D analogue) on passively sensitized human airway smooth muscle cells. *Respirol* *12*(4): 486-494, 2007.
152. Camargo CA, Rifas-Shiman SL, Litonjua AA, Rich-Edwards JW, Weiss ST, Gold DR, and Gillman MW: Prospective Study Of Maternal Intake Of Vitamin D During Pregnancy And Risk Of Wheezing Illnesses In Children At Age 2 Years. *J Allergy Clin Immunol* *117*(3): 721-722, 2006.
153. Devereux G, Litonjua AA, Turner SW, Craig LCA, McNeill G, Martindale S, Helms PJ, Seaton A, and Weiss ST: Maternal vitamin D intake during pregnancy and early childhood wheezing. *Am J Clin Nutr* *85*(3): 853-859, 2007.
154. Nicholson KG, Kent J, and Ireland DC: Respiratory viruses and exacerbations of asthma in adults. *BMJ* *307*: 982-986, 1993.
155. Barnes PJ and Adcock IM: Glucocorticoid resistance in inflammatory diseases. *Lancet* *373*(9678): 1905-1917, 2009.
156. Xystrakis E, Boswell S, Peek E, Urry Z, Richards DF, Adikibi T, Pridgeon C, Dallman M, Tuck-Kay L, Robinson DS, Barrat FJ, O'Garra A, Lavender P, Lee TH, Corrigan C, and Hawrylowicz CM: Reversing the defective induction of IL-10 secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J Clin Investigation* *116*(1): 146-155, 2006.
157. Manso L, Reche M, Padial MA, Valbuena T, and Pascual C: Diagnostic tools assessing airway remodelling in asthma. *Allergol Immunopathol* *40*(2): 108-116, 2012.
158. Koppelman G, and Sayers I: Evidence of a genetic contribution to lung function decline in asthma. *J Allergy Clin Immunol* *128*(3): 479-484, 2011.
159. Herbst RS, Heymach JV, and Lippman SM: Lung Cancer. *NEJM* *359*(13): 1367-1380, 2008.
160. Young RP and Hopkins RJ: How the genetics of lung cancer may overlap with COPD. *Respirol* *16*(7): 1047-1055, 2011.

161. Brody, J.S. and A. Spira, State of the Art. Chronic Obstructive Pulmonary Disease, Inflammation, and Lung Cancer. *Proc Am Thorac Soc* 3(6): 535-537, 2006.
162. Rooney C and Sethi T: The Epithelial Cell and Lung Cancer: The Link between Chronic Obstructive Pulmonary Disease and Lung Cancer. *Respiration* 81(2): 89-104, 2011.
163. Yao H and Rahman I: Current concepts on the role of inflammation in COPD and lung cancer. *Curr Opin Pharmacol* 9(4): 375-383, 2009.
164. Norton R and O'Connell M: Vitamin D: Potential in the Prevention and Treatment of Lung Cancer. *Anticancer Res* 32: 211-222, 2012.
165. Higashimoto Y, Ohata M, Nishio K, Iwamoto Y, Fujimoto H, Uetani K, Suruda T, Nakamura Y, Funasako M, and Saijo N: 1 α , 25-dihydroxyvitamin D₃ and all-trans-retinoic acid inhibit the growth of a lung cancer cell line. *Anticancer Res* 16(5A): 2653-9, 1996.
166. Güzey M, Sattler C, and DeLuca HF: Combinational Effects of Vitamin D₃ and Retinoic Acid (All trans and 9 cis) on Proliferation, Differentiation, and Programmed Cell Death in Two Small Cell Lung Carcinoma Cell Lines. *Biochem Biophys Res Commun* 249(3): 735-744, 1998.
167. Saito T, Okamoto R, Haritunians T, O'Kelly J, Uskokovic M, Maehr H, Marczak S, Jankowski P, Badr R, and Koeffler HP: Novel Gemini vitamin D₃ analogs have potent antitumor activity. *J Steroid Biochem Mol Biol* 112(1-3): 151-156, 2008.
168. Maeda Y, Yamato H, Hirai T, Kobori N, Fujii T, Kobayashi Y, Saitoh K, Inoguchi E, Hakozaiki M, Iijima H, Kodaira T, Konai Y, Katoh T, Yoshikumi C, Kawai Y, and Nomoto K: Antitumor and Other Effects of 24R,25-Dihydroxycholecalciferol in Lewis Lung Carcinoma Causing Abnormal Calcium Metabolism in Tumor-Bearing Mice. *Oncol* 45(3): 202-205, 1988.
169. Rita M, Young I, Halpin J, Wang J, Wright MA, Matthews J, and Pak AS: 1 α ,25-Dihydroxyvitamin D₃ plus gamma-Interferon Blocks Lung Tumor Production of Granulocyte-Macrophage Colony-stimulating Factor and Induction of Immunosuppressor Cells. *Cancer Res* 53(24): 6006-6010, 1993.
170. Young M, Ihm J, Lozano Y, Wright M, and Prechel M: Treating tumor-bearing mice with vitamin D₃ diminishes tumor-induced myelopoiesis and

- associated immunosuppression, and reduces tumor metastasis and recurrence. *Cancer Immunol Immunother* 41(1): 37-45, 1995.
171. Young M, Lozano Y, Ihm J, Wright M, and Prechel M: Vitamin D3 treatment of tumor bearers can stimulate immune competence and reduce tumor growth when treatment coincides with a heightened presence of natural suppressor cells. *Cancer letters* 104(2): 153-61, 1996.
172. Prechel M, Lozano Y, Wright M, Ihm J, and Young M: Immune modulation by interleukin-12 in tumor-bearing mice receiving vitamin D3 treatments to block induction of immunosuppressive granulocyte/macrophage progenitor cells. *Cancer Immunol Immunother* 42(4): 213-20, 1996.
173. Nakagawa K, Sasaki Y, Kato S, Kubodera N, and Okano T: 22-Oxa-1 α ,25-dihydroxyvitamin D3 inhibits metastasis and angiogenesis in lung cancer. *Carcinogenesis* 26(6): 1044-1054, 2005.
174. Mavroiedi A, O'Neill F, Lee PA, Darling AL, Fraser WD, Berry JL, Lee WT, Reid DM, Lanham-New SA, and Macdonald HM: Seasonal 25-hydroxyvitamin D changes in British postmenopausal women at 57°N and 51°N: A longitudinal study. *J Steroid Biochem Mol Biol* 121(1-2): 459-461, 2010.
175. Hirani V, Tull K, Ali A, and Mindell J: Urgent action needed to improve vitamin D status among older people in England! *Age Ageing* 39(1): 62-68, 2010.
176. Pilz S, Tomaschitz A, Obermayer-Pietsch B, Dobnig H, and Pieber TR: Epidemiology of Vitamin D Insufficiency and Cancer Mortality. *Anticancer Rese* 29(9): 3699-3704, 2009.
177. Freedman DM, Looker AC, Chang S-C, and Graubard BI: Prospective Study of Serum Vitamin D and Cancer Mortality in the United States. *J Nat Cancer Inst* 99(21): 1594-1602, 2007.
178. Mawer EB, Hayes ME, Heys SE, Davies M, White A, Stewart MF, and Smith GN: Constitutive synthesis of 1,25-dihydroxyvitamin D3 by a human small cell lung cancer cell line. *J Clin Endocrinol Metab* 79(2): 554-60, 1994.
179. Parise RA, Egorin MJ, Kanterewicz B, Taimi M, Petkovich M, Lew AM, Chuang SS, Nichols M, El-Hefnawy T, and Hershberger PA: CYP24, the enzyme that catabolizes the antiproliferative agent vitamin D, is increased in lung cancer. *Int J Cancer* 119(8): 1819-1828, 2006.

180. Anderson M, Nakane M, Ruan X, Kroeger P, and Wu-Wong J: Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemotherapy and Pharmacology* 57(2): 234-240, 2006.
181. Kim S, Chen G, King A, Jeon C, Christensen PJ, Zhao L, Simpson RU, Thomas DG, Giordano TJ, Brenner DE, Hollis B, Beer DG, and Ramnath N: Characterization of vitamin D receptor (VDR) in lung adenocarcinoma. *Lung Cancer epub* 2012.
182. Turner A, McGowan L, Millen A, Rajesh P, Webster CL, G, Rock G, Tachibana I, Tomlinson M, Berditchevski F, and Naidu B: Circulating DBP level and prognosis in operated lung cancer: an exploration of pathophysiology. *Eur Respir J epub* 2012.
183. Lehouck A, Mathieu C, Carremans C, Baeke F, Verhaegan J, Van Eldere J, Decallonne B, Bouillon R, Decramer M, and Janssens W: High dose vitamin D to reduce exacerbations in chronic obstructive pulmonary disease: a randomized trial. *Ann Intern Med* 156(2): 105-14, 2012.
184. Zhou X, Han J, Song Y, Zhang J, and Wang Z: Serum levels of 25-hydroxyvitamin D, oral health and chronic obstructive pulmonary disease. *J Clin Periodontol epub* 2012.
185. Pfeifer M, Begerow B, and Minne HW: Vitamin D and Muscle Function. *Osteoporos Int* 13(3): 187-194, 2002.
186. Hopkinson NS, Li KW, Kehoe A, Humphries SE, Roughton M, Moxham J, Montgomery H, and Polkey MI: Vitamin D receptor genotypes influence quadriceps strength in chronic obstructive pulmonary disease. *Am J Clin Nutr* 87(2): 385-390, 2008.
187. Franco C, Paz-Filho G, Gomes P, Nascimento V, Kulak C, Boguszewski C, and Borba V: Chronic obstructive pulmonary disease is associated with osteoporosis and low levels of vitamin D. *Osteoporos Int* 20(11): 1881-7, 2009.
188. Janssens W, Lehouck A, Carremans C, Bouillon R, Mathieu C, and Decramer M: Vitamin D Beyond Bones in COPD: Time to Act. *Am J Respir Crit Care Med*. 179(8): 630-6, 2009.

189. Graat-Verboom L, Smeenk FW, van den Borne BE, Spruit MA, Jansen FH, van Enschoot JW, and Wouters EF: Progression of osteoporosis in patients with COPD: A 3-year follow up study. *Respir Medicine* 106(6): 861-70, 2012.
190. Song Y, Qi H, and Wu C: Effect of 1,25-(OH)₂D₃ (a vitamin D analogue) on passively sensitized human airway smooth muscle cells. *Respirol* 12(4): 486-494, 2007.
191. Banerjee A and Panettieri R: Vitamin D modulates airway smooth muscle function in COPD. *Cur Opin Pharmacol epub* 2012.
192. Banerjee A, Damera G, Bhandare R, Gu S, Lopez-Boado YS, Panettieri RA, and Tliba O: Vitamin D and glucocorticoids differentially modulate chemokine expression in human airway smooth muscle cells. *Br J Pharmacol* 155(1): 84-92, 2008.
193. Michael L, George T, Barry S, Andras S, and Walter N-R: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 17(1): 62-70, 1976.
194. Yoakum GH, Korba BE, Lechner JF, Tokiwa T, Gazdar AF, Seeley T, Siegel M, Leeman L, Autrup H, and Harris CC: High-frequency transfection and cytopathology of the hepatitis B virus core antigen gene in human cells. *Science* 222(4622): 385-389, 1983.
195. Cozens A, Yezzi M, Kunzelmann K, Ohrui T, Chin L, Finkbeiner W, Widdecombe J, and Gruenert DC: CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 10(1): 38-47, 1994.
196. Parker D, and Prince A: Innate Immunity in the Respiratory Epithelium. *Am J Respir Cell Mol Biol* 45(2):189-201, 2011.
197. Swain RJ, Kemp SJ, Goldstraw P, Tetley TD, and Stevens MM: Assessment of Cell Line Models of Primary Human Cells by Raman Spectral Phenotyping. *Biophys J* 98(8): 1703-1711, 2010.
198. Newland N and Richter A: Agents associated with lung inflammation induce similar responses in NCI-H292 lung epithelial cells. *Toxicol in Vitro* 22(7): 1782-1788, 2008.
199. Yoshida T and Tuder RM: Pathobiology of Cigarette Smoke-Induced Chronic Obstructive Pulmonary Disease. *Physiological Rev* 87(3): 1047-1082, 2007.

200. Wang Y, Shen Y, Li K, Zhang P, Wang G, Gao L, and Bai C: Role of matrix metalloproteinase-9 in lipopolysaccharide-induced mucin production in human airway epithelial cells. *Arch Biochem Biophys* 486(2): 111-118, 2009.
201. Ritter M, Mennerich D, Weith A, and Seither P: Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response. *J Inflamm* 2(1): 16, 2005.
202. Koff JL, Shao MXG, Kim S, Ueki IF, and Nadel JA: Pseudomonas Lipopolysaccharide Accelerates Wound Repair via Activation of a Novel Epithelial Cell Signaling Cascade. *J Immunol* 177(12): 8693-8700, 2006.
203. Chokki M, Mitsuhashi H, and Kamimura T: Metalloprotease-dependent amphiregulin release mediates tumor necrosis factor- α -induced IL-8 secretion in the human airway epithelial cell line NCI-H292. *Life Sci* 78(26): 3051-3057, 2006.
204. Chen CC, Chou CY, Sun YT, and Huang WC: Tumor necrosis factor α -induced activation of downstream NF- κ B site of the promoter mediates epithelial ICAM-1 expression and monocyte adhesion: Involvement of PKC α , tyrosine kinase, and IKK2, but not MAPKs, pathway. *Cellular Signalling* 13(8): 543-553, 2001.
205. Hewson CA, Edbrooke MR, and Johnston SL: PMA Induces the MUC5AC Respiratory Mucin in Human Bronchial Epithelial Cells, via PKC, EGF/TGF- α , Ras/Raf, MEK, ERK and Sp1-dependent Mechanisms. *J Mol Biol* 344(3): 683-695, 2004.
206. Shao MXG, Ueki IF, and Nadel JA: Tumor necrosis factor α -converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Nat Acad Sci USA* 100(20): 11618-11623, 2003.
207. MacRedmond R, Greene C, Taggart C, McElvaney N, and O'Neill S: Respiratory epithelial cells require Toll-like receptor 4 for induction of Human beta-defensin 2 by Lipopolysaccharide. *Resp Res* 6(1): 116, 2005.
208. Standiford TJ, Kunkel SL, Basha MA, Chensue SW, Lynch JP, Toews GB, Westwick J, and Strieter RM: Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J Clin Inv* 86(6): 1945-1953, 1990.

209. Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, Nagai S, and Izumi T: The potential of various lipopolysaccharides to release IL-8 and G-CSF. *Am J Physiol Lung Cell Mol Physiol* 278(4): L658-666, 2000.
210. Schulz C, Farkas L, Wolf K, Kratzel K, Eissner G, and Pfeifer M: Differences in LPS-Induced Activation of Bronchial Epithelial Cells (BEAS-2B) and Type II-Like Pneumocytes (A-549). *Scand J Immunol* 56(3): 294-302, 2002.
211. van Wissen M, Snoek M, Smids B, Jansen HM, and Lutter R: IFN- γ Amplifies IL-6 and IL-8 Responses by Airway Epithelial-Like Cells Via Indoleamine 2,3-Dioxygenase. *J Immunol* 169(12): 7039-7044, 2002.
212. Lutter R, Loman S, Snoek M, Roger T, Out TA, and Jansen HM: IL-6 protein production by airway epithelial (-like) cells disabled in IL-6 mRNA degradation. *Cytokine* 12(8): 1275-1279, 2000.
213. Roger T, Out T, Mukaida N, Matsushima K, Jansen H, and Lutter R: Enhanced AP-1 and NF- κ B activities and stability of interleukin 8 (IL-8) transcripts are implicated in IL-8 mRNA superinduction in lung epithelial H292 cells. *Biochem J* 330(1): 429-435, 1998.
214. Donnarumma G, Paoletti I, Buommino E, Iovene MR, Tudisco L, Cozza V, and Tufano MA: Anti-inflammatory effects of moxifloxacin and human β -defensin 2 association in human lung epithelial cell line (A549) stimulated with lipopolysaccharide. *Peptides* 28(12): 2286-2292, 2007.
215. Levine SJ, Adamik B, Hawari FI, Islam A, Yu Z-X, Liao D-W, Zhang J, Cui X, and Rouhani FN: Proteasome inhibition induces TNFR1 shedding from human airway epithelial (NCI-H292) cells. *Am J Physiol Lung Cell Mol Physiol* 289(2): L233-243, 2005.
216. Sachse F, Ahlers F, Stoll W, and Rudack C: Neutrophil chemokines in epithelial inflammatory processes of human tonsils. *Clin Exp Immunol* 140(2): 293-300, 2005.
217. Wang, Malmberg, Ek, Larsson, and Palmberg: Swine dust induces cytokine secretion from human epithelial cells and alveolar macrophages. *Clin Exp Immunol* 115(1): 6-12, 1999.
218. Wang JY, Zheng Z, Xu ZY, Guo J, and Su W: Effects of Feiyanning Decoction on proliferation of lung adenocarcinoma cell line and their production of

- interleukin-6 and interleukin-8 induced by tumor necrosis factor- α . *J Chinese Integrative Med* 7(8): 741-745, 2009.
219. Newton R, Holden NS, Catley MC, Oyelusi W, Leigh R, Proud D, and Barnes PJ: Repression of Inflammatory Gene Expression in Human Pulmonary Epithelial Cells by Small-Molecule I κ B Kinase Inhibitors. *J Pharmacol Exp Ther* 321(2): 734-742, 2007.
220. Radomska-Lesniewska D, Sadowska A, Van Overveld F, Demkow U, Zielinski J, and De Backer W: Influence of N-Acetylcysteine on ICAM-1 expression and IL-8 release from endothelial and epithelial cells. *J Physiol Pharmacol* 57: 325-334, 2006.
221. Roebuck K and Finnegan A: Regulation of intercellular adhesion molecule-1 (CD54) gene expression. *J Leukocyte Biol* 66(6): 876-888, 1999.
222. Oh JH and Kwon TK: Withaferin A inhibits tumor necrosis factor- α -induced expression of cell adhesion molecules by inactivation of Akt and NF κ B in human pulmonary epithelial cells. *Int Immunopharmacol* 9(5): 614-619, 2009.
223. Kim H, Hwang J, Woo C, Kim E, Cho K, Kim J, Seo J, and Lee S: TNF- α -induced up-regulation of intercellular adhesion molecule-1 is regulated by a Rac-ROS-dependent cascade in human airway epithelial cells. *Exp Mol Med* 40(2): 167-75, 2008.
224. Huang WC, Chen JJ, and Chen CC: c-Src-dependent Tyrosine Phosphorylation of IKK β Is Involved in Tumor Necrosis Factor- α induced Intercellular Adhesion Molecule-1 Expression. *J Biological Chem* 278(11): 9944-9952, 2003.
225. Kwong KYC, Literat A, Zhu NL, Huang HH, Li C, Jones CA, and Minoo P: Expression of transforming growth factor beta (TGF- β 1) in human epithelial alveolar cells: a pro-inflammatory mediator independent pathway. *Life Sciences* 74(24): 2941-2957, 2004.
226. Lagente V, Manoury B, Nénan S, Le Quément C, Martin-Chouly C, and Boichot E: Role of matrix metalloproteinases in the development of airway inflammation and remodeling. *Brazilian J Medical Biological Res* 38: 1521-1530, 2005.

227. Armstrong DA, Phelps LN, and Vincenti MP: CCAAT Enhancer Binding Protein- β Regulates Matrix Metalloproteinase-1 Expression in Interleukin-1 β Stimulated A549 Lung Carcinoma Cells. *Mol Cancer Res* 7(9): 1517-1524, 2009.
228. Planus E, Galiacy S, Matthay M, Laurent V, Gavrilovic J, Murphy G, Clerici C, Isabey D, Lafuma C, and d'Ortho MP: Role of collagenase in mediating in vitro alveolar epithelial wound repair. *J Cell Sci* 112(2): 243-252, 1999.
229. Mercer BA, Wallace AM, Brinckerhoff CE, and D'Armiento JM: Identification of a Cigarette Smoke-Responsive Region in the Distal MMP-1 Promoter. *Am J Respir Cell Mol Biol* 40(1): 4-12, 2009.
230. Vliagoftis H, Schwingshackl A, Milne CD, Duszyk M, Hollenberg MD, Wallace JL, Befus AD, and Moqbel R: Proteinase-activated receptor-2-mediated matrix metalloproteinase-9 release from airway epithelial cells. *J Allergy Clin Immunol* 106(3): 537-545, 2000.
231. Lee IT, Chih-Chung L, Yang-Chang W, and Chuen-Mao Y: TNF- α induces matrix metalloproteinase-9 expression in A549 cells: Role of TNFR1/TRAF2/PKC α -dependent signaling pathways. *J Cell Physiol* 224(2): 454-64, 2010.
232. Qu P, Du H, Wang X, and Yan C: Matrix Metalloproteinase 12 Overexpression in Lung Epithelial Cells Plays a Key Role in Emphysema to Lung Bronchioalveolar Adenocarcinoma Transition. *Cancer Res* 69(18): 7252-61, 2009.
233. Thornton DJ and Sheehan JK: From Mucins to Mucus: Toward a More Coherent Understanding of This Essential Barrier. *Proc Am Thorac Soc* 1(1): 54-61, 2004.
234. Berger JT, Voynow JA, Peters KW, and Rose MC: Respiratory Carcinoma Cell Lines . MUC Genes and Glycoconjugates. *Am J Respir Cell Mol Biol* 20(3): 500-510, 1999.
235. Yu H, Li Q, Kolosov VP, Perelman JM, and Zhou X: Regulation of Cigarette Smoke-Induced Mucin Expression by Neuregulin1 β /ErbB3 Signalling in Human Airway Epithelial Cells. *Basic Clin Pharmacol Toxicol* 109(1): 63-72, 2011.

236. Perrais MI, Pigny P, Copin M-C, Aubert J-P, and Van Seuningen I: Induction of MUC2 and MUC5AC Mucins by Factors of the Epidermal Growth Factor (EGF) Family Is Mediated by EGF Receptor/Ras/Raf/Extracellular Signal-regulated Kinase Cascade and Sp1*. *J Biol Chem* 277(35): 32258-32267, 2002.
237. Wang G, Xu Z, Wang R, Al-Hijji M, Salit J, Strulovici-Barel Y, Tilley A, Mezey J, and Crystal R: Genes associated with MUC5AC expression in small airway epithelium of human smokers and nonsmokers. *BMC Med Genomics* 5(1): 21, 2012
238. Borchers MT, Carty MP, and Leikauf GD: Regulation of human airway mucins by acrolein and inflammatory mediators. *Am J Physiol Lung Cell Mol Physiol* 276(4): L549-L555, 1999.
239. Baginski TK, Dabbagh K, Satjawatcharaphong C, and Swinney DC: Cigarette Smoke Synergistically Enhances Respiratory Mucin Induction by Proinflammatory Stimuli. *Am J Respir Cell Mol Biol* 35(2): 165-174, 2006.
240. Shimizu T, Shimizu S, Hattori R, Gabazza EC, and Majima Y: In Vivo and In Vitro Effects of Macrolide Antibiotics on Mucus Secretion in Airway Epithelial Cells. *Am J Respir Crit Care Med* 168(5): 581-587, 2003.
241. Rhee CK, Kang CM, You MB, Yoon HK, Kim YK, Kim KH, Moon HS, Park SH, and Song JS: Effect of fudosteine on mucin production. *Eur Resp J* 32(5): 1195-1202, 2008.
242. Kim J, Sikder M, Lee H, Rahman M, Kim J, Chang G, and Lee C: Phorbol Ester of Epidermal Growth-factor-induced MUC5AC Mucin Gene Expression and Production from Airway Epithelial Cells are Inhibited by Apigenin and Wogonin. *Phytotherapy Res epub* 2012.
243. Shao MXG and Nadel JA: Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Nat Acad Sci USA* 102(3): 767-772, 2005.
244. Fan X-Y, van den Berg A, Snoek M, van der Flier L, Smids B, Jansen H, Liu R-Y, and Lutter R: Arginine deficiency augments inflammatory mediator production by airway epithelial cells in vitro. *Respiratory Res* 10(1): 62, 2009.
245. Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey B-G, Crystal RG, McCray PB, and Zabner J: The air-liquid interface and use of primary cell

- cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. *Am J Physiol Lung Cell Mol Phys* 300(1): L25-L31, 2010.
246. Comer DM, Elborn JS, and Ennis M: Comparison of Nasal and Bronchial Epithelial Cells Obtained from Patients with COPD. *PLoS ONE* 7(3): e32924, 2012.
 247. Hewison M: An update on vitamin D and human immunity. *Clinical Endocrinol (Oxf)* 76(3): 315-25, 2012.
 248. Janssens W, Bouillon R, Claes B, Carremans C, Lehouck A, Buyschaert I, Coolen J, Mathieu C, Decramer M, and Lambrechts D: Vitamin D Deficiency is Highly Prevalent in COPD and Correlates with Variants in the Vitamin D Binding Gene. *Thorax* 65(3): 215-20, 2010.
 249. Hansdottir S, Monick MM, Lovan N, Powers L, Gerke A, and Hunninghake GW: Vitamin D Decreases Respiratory Syncytial Virus Induction of NF κ B-Linked Chemokines and Cytokines in Airway Epithelium While Maintaining the Antiviral State. *J Immunol* 184(2): 965-74, 2010.
 250. Tashiro K, Abe T, Oue N, Yasui W, and Ryoji M: Characterization of Vitamin D-mediated induction of the CYP 24 transcription. *Mol Cell Endocrinol* 226(1-2): 27-32, 2004
 251. Zhang Q, Kanterewicz B, Buch S, Petkovich M, Parise R, Beumer J, Lin Y, Diergaarde B, and Hershberger PA: CYP24 inhibition preserves 1 α ,25-dihydroxyvitamin D3 anti-proliferative signaling in lung cancer cells. *Mol Cell Endocrinol epub* 2012.
 252. Ramnath N, Kim S, and Christensen PJ: Vitamin D and lung cancer. *Expert Rev Res Med* 5(3): 305-309, 2011.
 253. Chen G, Kim SH, King AN, Zhao L, Simpson RU, Christensen PJ, Wang Z, Thomas DG, Giordano TJ, Lin L, Brenner DE, Beer DG, and Ramnath N: CYP24A1 Is an Independent Prognostic Marker of Survival in Patients with Lung Adenocarcinoma. *Clin Cancer Res* 17: 817-826, 2011.
 254. Halicka H, Zhao H, Li J, Traganos F, Studzinski GP, and Darzynkiewicz Z: Attenuation of constitutive DNA damage signalling by 1,25-dihydroxyvitamin D3. *Aging (Albany NY)* 4(4): 270-8, 2012.

255. Wang CB, Wong CK, Ip WK, Li MLY, Tian YP, and Lam CWK: Induction of IL-6 in co-culture of bronchial epithelial cells and eosinophils is regulated by p38 MAPK and NF- κ B. *Allergy* 60(11): 1378-1385, 2005.
256. Berube J, Roussel L, Nattagh L, and Rousseau S: Loss of Cystic Fibrosis Transmembrane Conductance Regulator Function Enhances Activation of p38 and ERK MAPKs, Increasing Interleukin-6 Synthesis in Airway Epithelial Cells Exposed to *Pseudomonas aeruginosa*. *J Biol Chem* 285(29): 22299-22307, 2010.
257. Berube J, Roussel L, Nattagh L, and Rousseau S: Loss of cystic fibrosis transmembrane conductance regulator function enhances activation of p38 and ERK MAPKs, increasing interleukin-6 synthesis in airway epithelial cells exposed to *Pseudomonas Aeruginosa*. *J Biol Chem* 285(29): 22299-307, 2010.
258. Wu S, Xia Y, Liu X, and Sun J: Vitamin D receptor deletion leads to reduced level of I κ B α protein through protein translation, protein-protein interaction, and post-translational modification. *Int J Biochem Cell Biol* 42(2): 329-36, 2010.
259. Golovko O, Nazarova N, and Tuohimaa P: Vitamin D-induced up-regulation of tumour necrosis factor alpha (TNF- α) in prostate cancer cells. *Life Sci* 77(5): 562-577, 2005.
260. Equils O, Naiki Y, Shapiro A, Michelsen K, Lu D, Adams J, and Jordan S: 1,25-Dihydroxyvitamin D₃ inhibits lipopolysaccharide-induced immune activation in human endothelial cells. *Clin Exp Immunol* 143(1): 58-64, 2006.
261. Takano Y, Mitsuhashi H, and Ueno K: 1 α ,25-Dihydroxyvitamin D₃ inhibits neutrophil recruitment in hamster model of acute lung injury. *Steroids* *In Press, Uncorrected Proof*.
262. Boyan B and Schwartz Z: 1,25-dihydroxy vitamin D₃ is an autocrine regulator of extracellular matrix turnover and growth factor release via ERp60-activated matrix vesicle matrix metalloproteinases. *Cells Tissues Organs* 189((1-4)): 70-4, 2009.
263. Koo JS, Kim Y-D, Jetten AM, Belloni P, and Nettesheim P: Overexpression of mucin genes induced by interleukin-1 β , tumor necrosis factor- α ,

- lipopolysaccharide, and neutrophil elastase is inhibited by a retinoic acid receptor- α antagonist. *Exp Lung Res* 28(4): 315-332, 2002.
264. Rahman I: Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem Pharmacol* 64(5-6): 935-42, 2002.
265. Sly L, Lopez M, Nauseef W, and Reiner M: 1α , 25-dihydroxyvitamin D₃ - induced monocyte antimycobacterial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. *J Biol Chem* 276: 35482-35493, 2001.
266. Weitsman GE, Koren R, Zuck E, Rotem C, Liberman UA, and Ravid A: Vitamin D sensitizes breast cancer cells to the action of H₂O₂: Mitochondria as a convergence point in the death pathway. *Free Rad Biol Med* 39(2): 266-278, 2005.
267. Koren R, Wacksberg S, Weitsman GE, and Ravid A: Calcitriol sensitizes colon cancer cells to H₂O₂-induced cytotoxicity while inhibiting caspase activation. *J Steroid Biochem Mol Biol* 101(2-3): 151-160, 2006.
268. Bao BY, Ting HJ, Hsu JW, and Lee YF: Protective role of 1α , 25-dihydroxyvitamin D₃ against oxidative stress in nonmalignant human prostate epithelial cells. *Int J Cancer* 122(12): 2699-2706, 2008.
269. Degli Esposti M: Measuring mitochondrial reactive oxygen species. *Methods* 26(4): 335-340, 2002.
270. Wang H and Joseph JA: Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Rad Biol Med* 27(5-6): 612-616, 1999.
271. Imrich A and Kobzik L: Fluorescence-Based Measurement of Nitric Oxide Synthase Activity in Activated Rat Macrophages Using Dichlorofluorescein. *Nitric Oxide* 1(4): 359-369, 1997.
272. Lin R, Cheng M, Huang J, Lo W, Yeh Y, Yen C, Lu C, and Chen C: Cytotoxic compounds from the stems of *Cinnamomum tenuifolium*. *J Nat Prod* 72(10): 1816-24, 2009.
273. Wei Y, Wang X, and Wang L: Presence and regulation of cannabinoid receptors in human retinal pigment epithelial cells. *J Molecular Vision* 15: 1243-1251, 2009.

274. Lee S, Heo J, Lee M, and Han H: Effect of dihydrotestosterone on hydrogen peroxide-induced apoptosis of mouse embryonic stem cells. *J Cell Physiol* 216(1): 269-75, 2008.
275. Wang Q, Zheng Y, Dong L, Ho Y, and Guo ZW, YX: Role of mitochondrial reactive oxygen species in hypoxia-dependent increase in intracellular calcium in pulmonary artery myocytes. *Free Rad Biol Med* 42(5): 642-53, 2007.
276. Drouin A, Thorin-Trescases N, Hamel E, and Falck JT, E: Endothelial nitric oxide synthase activation leads to dilatory H₂O₂ production in mouse cerebral arteries. *Cardiovasc Res* 73(1): 73-81, 2007.
277. Rajaraman G, Wang G, Yan J, Jiang P, Gong Y, and Burczynski F: Role of cytosolic liver fatty acid binding protein in hepatocellular oxidative stress: effect of dexamethasone and clofibrate treatment. *Mol Cell Biochem* 295(1-2): 27-34, 2007.
278. Labieniec M and Gabryelak T: Study of interactions between phenolic compounds and H₂O₂ or Cu(II) ions in B14 Chinese hamster cells. *Cell Biol Int* 30(10): 761-8, 2006.
279. Hannekan A, Lin F, Johnson J, and Maher P: Flavanoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Invest Ophthalmol Vis Sci* 47(7): 3164-77, 2006.
280. Huang H, Chen H, Xu H, and Gibson G: Modification of endoplasmic reticulum Ca²⁺ stores by select oxidants produces changes reminiscent of those in cells from patients with Alzheimers disease. *Free Rad Biol Med* 39(8): 979-89, 2005.
281. Wang G, Gong Y, Anderson J, Sun D, Minuk G, Roberts M, and Burczynski F: Antioxidative function of L-FABP in L-FABP stably transfected Chang liver cells. *Hepatology*. 42(4): 871-9, 2005.
282. Tchah H, Kim M, Kim T, Choi H, Kim J, Kim M, and Pak J: Regulation of 1-cys peroxiredoxin expression in the process of stromal wound healing after photoreactive keratectomy. *Invest. Ophthalmol Vis Sci* 46(7): 2396-403, 2005.
283. Benedi J, Arroyo R, Romero C, Martin-Aragon S, and Villar A: Antioxidant properties and protective effects of a standardized extract of *Hypericum*

- perforatum on hydrogen peroxide-induced oxidative damage in PC12 cells. *Life Sci* 75(10): 1263-76, 2004.
284. Amer J, Goldfarb A, and Fibach E: Flow cytometric measurement of reactive oxygen species production by normal and thalassaemic red blood cells. *Eur J Haematol* 70(2): 84-90, 2003.
285. Wang J, Wang Y, and Wong C: Oestrogen-related receptor alpha inverse agonist XCT-790 arrests A549 lung cancer cell population growth by inducing mitochondrial reactive oxygen species production. *Cell Proliferation* 43(2): 103-113, 2010.
286. Herzog E, Byrne HJ, Davoren M, Casey A, Duschl A, and Oostingh GJ: Dispersion medium modulates oxidative stress response of human lung epithelial cells upon exposure to carbon nanomaterial samples. *Toxicol App Pharmacol* 236(3): 276-281, 2009.
287. Ogura A, Oowada S, Kon Y, Hirayama A, Yasui H, Meike S, Kobayashi S, Kuwabara M, and Inanami O: Redox regulation in radiation-induced cytochrome c release from mitochondria of human lung carcinoma A549 cells. *Cancer letters* 277(1): 64-71, 2009.
288. Park J-H, Mangal D, Tacka KA, Quinn AM, Harvey RG, Blair IA, and Penning TM: Evidence for the aldo-keto reductase pathway of polycyclic aromatic trans-dihydrodiol activation in human lung A549 cells. *Proc Nat Acad Sci* 105(19): 6846-6851, 2008.
289. Grzelak A, Rychlik B, and Bartosz G: Light-dependent generation of reactive oxygen species in cell culture media. *Free Rad Biol Med* 30(12): 1418-1425, 2001.
290. Martín C, Martínez R, Navarro R, Ruiz-Sanz JI, Lacort M, and Ruiz-Larrea MB: tert-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. *Biochem Pharmacol* 62(6): 705-712, 2001.
291. Nagaraja C, Shashibhushan B, Sagar, Asif M, and Majunath P: Hydrogen peroxide in exhaled breath condensate: A clinical study. *Lung India* 29(2): 123-127, 2012.
292. Blue M and Janoff A: Possible mechanisms of emphysema in cigarette smokers. Release of elastase from human polymorphonuclear leukocytes by

- cigarette smoke condensates in vitro. *Am Rev Respir Dis* 117(2): 317-25, 1978.
293. Turano C, Gaucci E, Grillo C, and Chichiarelli S: ERp57/GRP58: A protein with multiple functions. *Cell Mol Biol Letters* 16(4): 539-563, 2011.
294. Homma S, Ishii Y, Morishima Y, Yamadori T, Matsuno Y, Haraguchi N, Kikuchi N, Satoh H, Sakamoto T, Hizawa N, Itoh K, and Yamamoto M: Nrf2 Enhances Cell Proliferation and Resistance to Anticancer Drugs in Human Lung Cancer. *Clin Cancer Res* 15(10): 3423-3432, 2009.
295. Bo-Ying B, Huei-Ju T, Jong-Wei H, and Yi-Fen L: Protective role of $1\alpha, 25$ -dihydroxyvitamin D₃ against oxidative stress in nonmalignant human prostate epithelial cells. *Int J Cancer* 122(12): 2699-2706, 2008.
296. Taguchi K, Motohashi H, and Yamamoto M: Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. *Genes Cells* 16(2): 123-140, 2011.
297. Baird L and Dinkova-Kostova A: The cytoprotective role of the Keap1–Nrf2 pathway. *Archives of Toxicology* 85(4): 241-272, 2011.
298. Zhu H, Bannenberg G, Moldéus P, and Shertzer H: Oxidation pathways for the intracellular probe 2',7'-dichlorofluorescein. *Arch Toxicol* 68(9): 582-587, 1994.
299. Barake M, Daherm RT, Salti I, Cortas N, Al-Shaar L, Habib R, and Fuleihan G-H: 25-hydroxyvitamin D assay variations and impact on clinical decision making. *J Clin Endocrinol Metab* 97(3): 835-43, 2012.
300. Qi L, Xiangdong Z, Hongmei Y, Xiaohong N, and Xiaoyan X: Regulation of Neutrophil Elastase-Induced MUC5AC Expression by Nuclear Factor Erythroid-2 Related Factor 2 in Human Airway Epithelial Cells. *J Investigative Med epub* 2012.
301. McGaffin KR and Chrysogelos SA: Identification and characterization of a response element in the EGFR promoter that mediates transcriptional repression by 1,25-dihydroxyvitamin D₃ in breast cancer cells. *J Mol Endocrinol* 35(1): 117-133, 2005.
302. Rehan VK, Torday JS, Peleg S, Gennaro L, Vouros P, Padbury J, Sunita Rao D, and Satyanarayana Reddy G: $1\alpha, 25$ -Dihydroxy-3-epi-vitamin D₃, a natural metabolite of $1\alpha, 25$ -dihydroxy vitamin D₃: production and biological activity

- studies in pulmonary alveolar type II cells. *Mol Genet Metab* 76(1): 46-56, 2002.
303. Herr C, Greulich T, Koczulla R, Meyer S, Zakharkina T, Branscheidt M, Eschmann R, and Bals R: The role of vitamin D in pulmonary disease: COPD, asthma, infection, and cancer. *Respir Res* 12(1): 31, 2011.
304. Kunisaki KM, Niewoehner DE, Connett JE, and for the COPD Clinical Research Network: Vitamin D Levels and Risk of Acute Exacerbations of Chronic Obstructive Pulmonary Disease: a Prospective Cohort Study. *Am J Respir Crit Care Med* 185(3): 286-90, 2012.
305. Shaheen SO, Jameson KA, Robinson SM, Boucher BJ, Syddall HE, Aihie Sayer A, Cooper C, Holloway JW, and Dennison EM: Relationship of vitamin D status to adult lung function and COPD. *Thorax* 66(8): 692-8, 2011.
306. Romme E, Rutten E, Smeenk F, Spruit M, Menheere P, and Wouters E: Vitamin D status is associated with bone mineral density and functional exercise capacity in patients with chronic obstructive pulmonary disease. *Ann Med epub* 2012.