AN INTRINSIC DEFECT OF NEUTROPHILS IN INDIVIDUALS WITH CYSTIC FIBROSIS LEADS TO PRIMARY GRANULE RELEASE AND AUTOANTIBODY PRODUCTION

Dr Fatma S Gargoum MB BCh BAO, MRCPI
Department of Medicine
Royal College of Surgeons in Ireland

A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Medicine

Supervisors: Professor Noel G McElvaney
Dr Emer Reeves
March 2017

Candidate Thesis Declaration

I declare that this thesis, which I submit to the Royal College of Surgeons in Ireland for examination in consideration of the award of a higher degree MD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed: ______________________________
RCSI student number: 14125781
Date: ______________________________
Table of Contents

Candidate Thesis Declaration 2
Table of Contents 3
List of Abbreviations 8
List of Figures 17
List of Tables 19
Abstract 20
Presentations and Publications 21
Acknowledgments 23
Dedication 24

Chapter 1

Introduction 25

1.1 An introduction to CF 26
1.2 Epidemiology of CF 29
1.3 Diagnosing CF 31
1.4 CFTR structure 33
1.5 Classes of CFTR mutations 35
1.6 CFTR polymorphisms 38
1.7 Disease pathogenesis in CF 39
1.8 Principles of treatment in CF 43
1.9 Mutation targeted therapy 45
1.10 The role of neutrophils in CF 49
1.10.1 Brief overview of neutrophil function 49
Chapter 1

1.10.2 Neutrophil granules 50
1.10.3 Primary granules 52
1.11 Neutrophil mediated inflammation in CF 56
1.11.1 Dysregulated neutrophil activity in CF 56
1.12 Abnormal degranulation processes by CF neutrophils 60
1.12.1 Antiproteases in CF airways 62
1.13 Neutrophil granule trafficking 63
1.13.1 The role of Rab proteins and neutrophil degranulation 63
1.13.2 Role of Rac2 and its activation 65
1.13.3 Rac2 activation and neutrophil primary granules 67
1.14 Autoantibodies in CF 69
1.15 Aim 71

Chapter 2

Materials and methods 72

2.1 Materials and Methods 73
2.1.1 Reagents 73
2.1.2 Antibodies 73
2.2 Patient samples 75
2.2.1 Patient recruitment 75
2.2.2 Neutrophil isolation 75
2.2.3 Trypan Blue Exclusion test 76
2.3 Preparation of protein samples 78
2.3.1 Preparation of whole cell lysates 78
2.4 Protein quantification
2.4.1 SDS PAGE
2.4.2 Coomassie Blue staining
2.4.3 Western blot analysis
2.5 Determination of degranulation
2.5.1 Degranulation assays
2.5.2 CFTR inhibitor treatment
2.6 Measuring enzyme kinetics
2.6.1 FRET analysis
2.7 Rac2 activation assay
2.8 ELISA
2.8.1 Anti-MPO and anti-PR3 autoantibody quantification
2.8.2 Anti-BPI autoantibody quantification
2.8.3 Measurement of BPI levels by ELISA
2.9 Statistical analysis

Chapter 3

Impaired neutrophil degranulation in cystic fibrosis

3.1 Introduction
3.1.1 Neutrophil degranulation in CF
3.1.2 Stimuli causing neutrophil degranulation
3.1.3 Release of serine proteases and bactericidal proteins by CF neutrophils
3.1.4 Rac signalling mechanisms leading to neutrophil degranulation
Chapter 4

The consequence of excessive primary granule release by neutrophils of individuals with cystic fibrosis

4.1 Introduction

4.2 BPI ANCA

4.3 Consequence of BPI ANCA formation

4.4 Clinical correlation of BPI ANCA in CF

4.5 Investigation of ANCA in CF

4.5.1 Assessment of ANCA directed against neutrophil primary granule proteins in CF

4.6 Discussion
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT</td>
<td>alpha-1 antitrypsin</td>
</tr>
<tr>
<td>AATD</td>
<td>alpha-1 antitrypsin deficiency</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Abz</td>
<td>o-aminobenzoyl</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>ANCA</td>
<td>anti-neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ASL</td>
<td>airway surface liquid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B. cepacia</td>
<td><em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BPI</td>
<td>bactericidal permeability increasing protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C. albicans</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>C3</td>
<td>complement component 3</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
</tbody>
</table>
Cath G  cathepsin G
CB  cytochalasin B
CBAVD  congenital bilateral absence of vas deferens
CCL  chemokine C ligand
CDC  Centre for Disease Control
CF  cystic fibrosis
CFTR  cystic fibrosis transmembrane conductance regulator
CFTRinh-172  CFTR inhibitor compound 172
Cl⁻  chloride anion
COPD  chronic obstructive pulmonary disease
CRIB  cdc42/Rac interactive binding domain
CT  computed tomography
CXCR  C-X-C chemokine receptor
DAG  diacylglycerol
del  deletion
DIOS  distal intestinal obstruction syndrome
DMF  dimethylformamide
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
DPBS  dulbecco’s phosphate-buffered saline
DTT  Dithiothreitol
*E. Coli*  *Escherichia coli*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDDnp</td>
<td>ethylene diamine 2,4-dinitrophenyl</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELF</td>
<td>epithelial lining fluid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>ENaC</td>
<td>epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FEV₁</td>
<td>forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-Methionyl-Leucyl-Phenylalanine</td>
</tr>
<tr>
<td>FPR</td>
<td>formyl peptide receptor</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GAP</td>
<td>GTP-ase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GGTase-1</td>
<td>geranylgeranyltransferase type I</td>
</tr>
<tr>
<td>GNB</td>
<td>gram negative bacteria</td>
</tr>
<tr>
<td>GNEF</td>
<td>guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GPA</td>
<td>granulomatosis with polyangiitis</td>
</tr>
<tr>
<td>GST-PAK-CRIB</td>
<td>glutathione sepharose-tagged-p21 activated kinase-cdc42/Rac interactive binding domain</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-0'-gamma-thio-triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
</tbody>
</table>

*H. influenzae*  *Haemophilus influenzae*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>H⁺</td>
<td>hydrogen anion</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HC</td>
<td>healthy control</td>
</tr>
<tr>
<td>hCAP-18</td>
<td>18 kDa human cathelicidin antimicrobial protein</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>bicarbonate</td>
</tr>
<tr>
<td>HCT</td>
<td>haematopoietic cell transplantation</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HL 60</td>
<td>human myeloid cell line</td>
</tr>
<tr>
<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>HRCT</td>
<td>high resolution computed tomography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICL</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
</tr>
<tr>
<td>IDC</td>
<td>immature dendritic cell</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IFRD1</td>
<td>interferon related developmental regulator 1</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin-G</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IRT</td>
<td>immunoreactive trypsinogen</td>
</tr>
<tr>
<td>IU/mL</td>
<td>international units per milliliter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LL-37</td>
<td>human cathelicidin LL-37</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTB₄</td>
<td>leukotriene B4</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMP-9</td>
<td>matrix metalloprotease-9</td>
</tr>
<tr>
<td>MP</td>
<td>microscopic polyangiitis</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSD</td>
<td>membrane-spanning domain</td>
</tr>
<tr>
<td>MSSA</td>
<td>methicillin sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
</tr>
<tr>
<td>NCMG</td>
<td>National Centre for Medical Genetics</td>
</tr>
<tr>
<td>NDPK</td>
<td>nucleoside diphosphate kinase</td>
</tr>
</tbody>
</table>
pHi: intracellular pH
PI: protease inhibitor
PKC: protein kinase C
PMA: phorbol 12-myristate 13 acetate
PMN: polymorphonuclear leukocyte
PMSF: phenylmethylsulfonyl fluoride
PR3: proteinase 3
PREX-1: phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger-1
PSC: primary sclerosing cholangitis
PTC: premature termination codon
PVDF: polyvinylidene fluoride
RA: rheumatoid arthritis
RabGDI: Rab GDP dissociation factor
Rac: Ras-related C3 botulinum toxin substrate
RBL: rat basophilic leukaemia cells
RIPA: radio-immunoprecipitation assay buffer
RNA: ribonucleic acid
ROS: reactive oxygen species
rpm: revolutions per minute
RT PCR: reverse transcription polymerase chain reaction
S. aureus: Staphylococcus aureus
S. maltophilia: Stenotrophomonas maltophilia
SB: sample buffer
SD  standard deviation
SDS  sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec  seconds
SEM  standard error of the mean
SLPI  secretory leucoprotease inhibitor
SNARE  soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SP-A  surfactant protein-A
t  time
TAP  transported for antigen presentation
TEMED  tetramethylethylenediamine
TGFβ  transforming growth factor beta
TIAM-1  T cell-lymphoma-invasion-and-metastasis-1
TLCK  Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride
TLR-4  toll like receptor 4
TMB  3,3′,5,5′-tetramethylbenzidine
TNF R1  tumour necrosis factor alpha receptor 1
TNFα  tumour necrosis factor alpha
Tris  tris (hydroxymethyl)aminomethane
u  units
V  volts
VAV-1  vav-1 guanine nucleotide exchange factor
VX 770  Ivacaftor
VX 809  Lumacaftor
w/v     weight/volume
w/w     weight/weight
WB      Western blot
x g     times gravity
ZnCl₂   zinc chloride
ΔF508   deletion of phenylalanine at position 508 of cystic fibrosis transmembrane conductance regulator gene
α       alpha
β       beta
Δ       delta
K       kappa
γ       gamma
µg      micrograms
µl      microlitre
List of Figures

Figure 1.1: Abnormalities in CF airway epithelia 28
Figure 1.2: Age distribution of people living with CF in 2015 30
Figure 1.3: Protein structure of CFTR 34
Figure 1.4: Classification of CFTR mutations 37
Figure 1.5: Characteristic CF findings of CF 42
Figure 1.6: CFTR function in CF human epithelia 48
Figure 1.7: Granule and vesicle contents of the neutrophil 51
Figure 1.8: Activation of Rab proteins 66
Figure 3.1: No significant difference in MPO release between CF and HC neutrophils using PMA stimulation determined by Western blot analysis 108
Figure 3.2: MPO release determined by Western blot analysis is increased in CF neutrophils in response to TNFα/fMLP 110
Figure 3.3: BPI release determined by Western blot analysis is increased in CF neutrophils in response to TNFα/fMLP 112
Figure 3.4: NE activity in CF extra-cellular supernatants determined by FRET analysis is increased in response to TNFα/fMLP 115
Figure 3.5: Cath G activity in CF extra-cellular supernatants determined by FRET analysis is increased in response to TNFα/fMLP 117
Figure 3.6: Active Rac2 as a percentage of total Rac2 determined by Western blot analysis is increased in CF neutrophils at baseline 120
Figure 3.7: Active Rac2 as a percentage of total Rac2 determined by Western blot analysis is increased in CF neutrophils in response to TNFα/fMLP 122
Figure 3.8: MPO release determined by Western blot analysis is increased in HC neutrophils in response to CFTRinh-172 treatment 124
Figure 3.9: BPI release determined by Western blot analysis is increased by HC neutrophils in response to CFTRinh-172 treatment 126
Figure 3.10: Active Rac2 as a percentage of total Rac2 determined by Western blot analysis is increased in HC neutrophils in response to treatment with CFTRinh-172

Figure 3.11: No difference in MPO release determined by Western blot analysis between CFTRinh-172 and DMSO treated HC cells

Figure 3.12: No difference in BPI release determined by Western blot analysis between CFTRinh-172 and DMSO treated HC cells

Figure 4.1: No increase in anti-MPO autoantibodies in CF individuals determined by ELISA

Figure 4.2: No increase in anti-PR3 autoantibodies in CF individuals determined by ELISA

Figure 4.3: Increased circulating IgG BPI antibody level in CF individuals determined by ELISA

Figure 4.4: Increased circulating BPI level in CF individuals determined by ELISA

Figure 4.5: Increased BPI level in CF BALF determined by ELISA

Figure 5.1: Neutrophils in CF
List of Tables

Table 2.1: Primary antibodies employed for Western blotting 74
Table 2.2: Secondary antibodies employed for Western blotting 74
Table 2.3: Demographics of CF patients used in Degranulation assays 77
Table 2.4: Constituents of 12.5% (w/v) running gel 80
Table 2.5: Constituents of 5% (w/v) stacking gel 80
Table 2.6: Composition of “Stop” Buffer 83
Table 2.7: Constituents of 1X Lysis Buffer 89
Table 2.8: Constituents of 1X Lysis Solution 89
Table 2.9: Reference ranges for EliA™ MPO and PR3 autoantibody quantification 91
Table 2.10: Characteristics of CF individuals receiving Ivacaftor therapy 95
Table 2.11: Population characteristics of BALF samples 96
Abstract

Cystic fibrosis (CF) is an autosomal recessive multi-system disorder which primarily affects respiratory epithelia and results in abnormal mucus secretions, chronic pulmonary infection culminating in respiratory failure and premature death. One of the hallmarks of CF is neutrophil derived inflammation. CF neutrophils release excessive amounts of primary granule proteins possibly due to sustained infection and the continuous presence of inflammatory mediators. However, the cause of increased primary granule release in CF is unknown. Neutrophil exocytosis is under the tight control of small GTP-binding proteins, including Ras-related C3 botulinum toxin substrate 2 (Rac2). The aim of this study was to investigate the cellular basis for aberrant neutrophil degranulation in CF.

In this study, we examined degranulation by CF neutrophils using Western blotting. This revealed excessive release of the primary granule proteins myeloperoxidase (MPO) (p=0.03) and bactericidal permeability increasing protein (BPI) (p=0.02). In addition, fluorescence resonance energy transfer (FRET) analysis confirmed increased extracellular activity of the neutrophil serine protease neutrophil elastase (NE) post activation with TNFα and fMLP (p=0.03 and 0.03 respectively). Similarly, circulating CF neutrophils exhibited greater levels of extracellular cathepsin G (Cath G) activity compared to control cells (p=0.02). Excessive Rac2 activity was confirmed in CF neutrophils using a Rac2 activation assay both at rest and post TNFα/fMLP activation (p=0.03 and p=0.004 respectively).

Furthermore, control neutrophils treated with the CFTR inhibitor drug- CFTRinh-172 also displayed increased primary granule degranulation with excessive MPO (p=0.01) and BPI levels recorded (p=0.03), as well as enhanced Rac2 activation (p=0.03), mirroring the defects observed in CF neutrophils. These results indicate that the observed increased primary granule release is an intrinsic defect due to impaired CFTR function.

In vivo, increased primary granule release resulted in elevated plasma (p=0.01) and bronchoalveolar lavage fluid (BALF) levels of BPI (p=0.008) and corresponding circulating autoantibodies in patients with CF.
Presentations and Publications

Oral Poster Presentations


The role of Rac2 in Increased Primary Granule Degranulation in Neutrophils of individuals with Cystic Fibrosis. **Gargoum FS**, Landers SA, White MM, Browne N, Hawkins P, Reeves EP, McElvaney NG. Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9. Irish Thoracic Society Scientific Meeting, November 2016.


**Original Manuscript**


**Book Chapter**

Acknowledgments

Completing my MD journey would not have been possible without the help of my mentors, friends and colleagues in the research laboratory.

Firstly I’d like to extend a sincere thank you to Prof. Gerry McElvaney for affording me this great research opportunity and for his guidance and ideas along the way. I am very grateful to Dr. Emer Reeves for her unwavering support and motivation as well as her strive for perfection in preparing this thesis.

Thank you also to my good friend and colleague Sarah for her help over the past two years, both on and off the lab bench, not to mention the countless coffee breaks and the late evening shop runs.

I would like to earnestly thank my lab colleagues for their encouragement, kindness, patience and assistance with all things scientific, Michelle, Noreen, Niall, Danielle, Laura, Tomás, Margaret, Paul, Gillian, Bojana, Ciara, Tania, Dave, Alex, Tom, Pádraig, Ollie and Chiara. A very special thank you to Claire for her keen eye and perseverance in formatting this work!

I’d like to acknowledge Dr. Mary Keogan, Ms. Anne Clooney and the scientists in the Department of Immunology at Beaumont Hospital for their technical advice and assistance with autoantibody quantification.

Finally, a heartfelt thank you to all of the CF patients and healthy volunteers for donating their blood samples and without whom this research would not advance.
Dedication

I dedicate this to my wonderful husband Maher, my beautiful baby girl Laila, my strong and supportive mother Nagea and my kind and loving father Salem, my achievements mean so much more because of you.
Chapter 1

Introduction
1.1 An introduction to Cystic Fibrosis

Cystic fibrosis (CF) is a multi-system disease which occurs due to mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a regulated chloride (Cl\textsuperscript{−}) channel (6). CFTR is essential for regulating salt and water movement across membranes. Impaired transport of Cl\textsuperscript{−} and other ions such as sodium (Na\textsuperscript{+}) and bicarbonate (HCO\textsubscript{3}\textsuperscript{−}) leads to accumulation of viscous secretions in the lungs, pancreas, liver, intestine and reproductive tract resulting in increased salt content in sweat gland secretions. In CF lungs, these secretions result in colonization with pathogenic bacteria, causing a heightened inflammatory response and persistent inflammatory influx of neutrophils to the airway. The intense and sustained neutrophil presence leads to excessive protease release (7, 8). This results in destruction of extracellular matrix proteins which contributes to lung damage and subsequent development of irreversible bronchiectasis and intermittent infective exacerbations.

In the pancreas, the exocrine ducts become blocked by secretions, leading to pancreatic enzyme insufficiency, pancreatic destruction, and ultimately diabetes mellitus. Other extra-pulmonary manifestations of CF include distal intestinal obstruction syndrome (DIOS) due to mucus accumulation within the intestinal tract causing delayed food transit and bowel emptying. This results in a build-up of stool behind the mucus filled area causing severe constipation and blockage. CF liver disease can occur secondary to slowly progressive cirrhosis causing portal hypertension and patients are screened for this complication annually via liver ultrasound scan. Other complications include osteopaenia, osteoporosis, chronic rhinosinusitis and infertility in males due to congenital bilateral absence of the vas deferens (CBAVD) (9).
In healthy individuals, intact CFTR function results in normal airway surface liquid (ASL) volume, facilitating the transport of Cl ions into and out of the cell, allowing for optimum ciliary movement within the airways. In healthy individuals ciliary movement enables the removal of mucus containing pro-inflammatory mediators, immune cells and pathogens. However, CF results in defective CFTR function and altered ion transport and increased epithelial sodium channel (ENaC) mediated ion absorption in the superficial airway epithelium causing inspissation of the mucus outside the cells which leads to a depletion of ASL, thus hindering ciliary movement and the removal of mucus (Figure 1.1) (10).

As mentioned, accumulation of mucus occurs within the pancreas and reproductive system leading to clinical disease manifestations, and also within the lungs producing severe chronic pathology; the most significant clinical expression in CF (11). CF pulmonary infections start very early on in life, with increased bacterial colonisation of the respiratory tract in children with CF as young as three months (12). Continued infections lead to persistent neutrophil dominated inflammation with increased activity of neutrophil granule proteins including neutrophil elastase (NE), causing pathogenic destruction of lung parenchyma (8).
Figure 1.1: Abnormalities in CF airway epithelia.

Image A depicts healthy airway epithelia; CFTR is intact and plays a crucial role in regulating hydration of the ASL which is comprised of the periciliary layer (PCL) and mucus layer. Due to defective CFTR in CF, Cl⁻ secretion is impaired and Na⁺ absorption through ENaC is upregulated resulting in dehydration of ASL with thick mucus accumulating causing the PCL to collapse as depicted in Image B. (Adapted from Reeves et al, 2012) (1).
1.2 Epidemiology of CF

There were approximately 15 new cases of CF diagnosed in Ireland in 2015; though this likely represents an underestimation of the total number of cases due to a delay in joining the CF registry. New-born screening has led to earlier diagnosis (2).

Ireland has the highest incidence of CF, affecting 1 in 1500 live births annually. Conversely, CF is rare in Afro-Caribbeans, but is seen in patients of Asian origin in the UK and USA. Almost 1 in 19 individuals are carriers of at least one mutated allele (13, 14). The Irish prevalence is approximately four times higher with 2.98 in 10,000 people suffering from the disease compared to the mean prevalence of 0.74 in Europe and 0.8 in the US (15). It is estimated that there are more than 70,000 CF sufferers globally (16).

By 2015, 1219 people were recorded by the CF Registry of Ireland (CFRI), in total 558 children and 661 adults (Figure 1.2). When the disorder was first described by Dorothy Andersen in 1938, survival beyond the second year of life was rare. Since then survival has improved considerably with advances in the treatment and nutrition of CF patients.

The impact of CF varies widely from one person to another. There are some patients who live until their teenage years only, whilst others can survive into their 40s and 50s and beyond. Ireland has some of the most severe genotypes of CF with the ΔF508 mutation affecting a significant proportion of CF patients. As a result, the Irish health service has established a network of specialist treatment centres incorporating members of the multidisciplinary team led by CF trained consultants to deliver specialised care.
Figure 1.2: Age distribution of people living with CF in 2015.

The median age of a person with CF in 2015 was 19 years. The age range is from birth to over 80 years; however the majority of individuals with CF are in the younger age groups (Adapted from the CF Registry of Ireland Annual Report, 2015) (2).
1.3 Diagnosing CF

New-born screening for CF was introduced in Ireland in 2011. A blood spot measurement of immunoreactive trypsinogen (IRT) is taken from a heel prick of new born infants. IRT is a precursor to trypsin and in infants with CF is not readily removed from the blood stream to its active form due to pancreatic dysfunction and impaired release of pancreatic enzymes. Therefore, most infants with CF have elevated blood levels of IRT. IRT levels fall rapidly during infancy. After eight weeks of age, a negative result is not informative, though a positive result still strongly supports a diagnosis of CF (17, 18). If IRT is found to be elevated during new-born screening, the sample will be forwarded for CF mutational analysis (19). Neonatal heel prick for IRT measurement is offered routinely as part of national screening programme in Ireland. In addition, prenatal second trimester foetal ultrasound may identify the presence of echogenic bowel, which can occur in CF. These babies are then screened at birth. Those with relatives with CF are advised to have pre-conception CF genotyping, as are the partners of a patient with CF.

Sweat Cl⁻ testing is used in conjunction with IRT to support the diagnosis of CF. The cholinergic agent pilocarpine is administered to induce sweating and the sweat sample is tested for its Cl⁻ content (20). A sweat Cl⁻ of greater than 60mmol/l by quantitative pilocarpine iontophoresis is diagnostic.

Defective Cl⁻ conductance across respiratory epithelia leads to a greater than normal negative potential difference across the cell membrane. This observation forms the basis for the nasal potential difference (NPD) test that is sometimes used in atypical cases with non-diagnostic sweat electrolyte concentrations. NPD measures the transport of Cl⁻ and Na⁺ across mucus membranes. CF individuals have greater Na⁺ transport and reduced Cl⁻ transport which can be detected by NPD. Healthy individuals have a NPD measurement of 20 millivolts (mV), whilst CF patients have a higher NPD of 50 mV (21). NPD testing is not undertaken in routine clinical diagnosis, and only serves as a diagnostic aid in atypical cases where CFTR dysfunction is suspected often in the presence of one CFTR mutation (16, 22).
CF mutational analysis is carried out by the National Centre for Medical Genetics (NCMG) in Ireland. Screening for a panel of 39 mutations by allele-specific oligonucleotide hybridisation provides an estimated sensitivity of 93.5% in the Irish population. The mean time for result from genotyping is approximately two weeks (23).

Medical advances in treatment and developments in early case detection by newborn screening have drastically improved life expectancy over the last number of decades with many patients living to 4th or 5th decades of life (24).

As more and more people with CF reach adult age, this creates new challenges as patients develop comorbidities such as bone disease and complications associated with changes of the airway micro-environment.
1.4 CFTR structure

In 1938, an American pathologist, Dr. Dorothy Andersen provided the first description of CF in the medical literature (25) calling it “cystic fibrosis of the pancreas” based on post-mortem findings of children that died of malnutrition. It was not until 1989 that the CFTR gene was identified and isolated from epithelial cells found within the pancreas, lungs, colon, sweat glands and nasal polyps of healthy control (HC) individuals (6, 26).

We now know that CF is an autosomal recessive condition, with the gene found on the long arm of chromosome 7.

CFTR belongs to the ATP-binding cassette (ABC) family of proteins, a large group of related proteins that possess similar transmembrane transport functions. CFTR functions as a regulated Cl⁻ channel, which in turn controls the activity of other Na⁺ and Cl⁻ channels at the cell surface (27).

The CFTR gene reaches over 250 kilobases on chromosome 7, encoding 1480 amino acids in the mature form. The protein is comprised of two groups of six-membrane-spanning domains (MSDs), two intracellular nucleotide binding domains (NBDs), and a highly charged “R domain” which contains a number of phosphorylation sites (Figure 1.3). Activation of the Cl⁻ channel is dependent upon phosphokinase A-mediated phosphorylation of the R domain as well as the continuous presence of ATP in the NBDs (28, 29).
Figure 1.3: Protein structure of CFTR.

The CFTR comprises of two membrane-spanning domains (MSD), two cytosolic nucleotide binding domains (NBD) for interacting with ATP and a regulatory domain (RD). Intracellular loops (ICL) are located within each MSD and interactions between ICL and NBD are critical for correct and functional protein folding (Adapted from Moran et al, 2014) (5).
1.5 Classes of CFTR mutations

Understanding of the underlying mechanisms of mutant CFTR dysfunction has particular relevance when devising new therapeutic strategies (30). Different mutations may be associated with particular clinical subgroups. In excess of 1900 CF mutations have been identified thus far, with the ΔF508 mutation being the most common cause of CF with an incidence of 67% worldwide (31). This is a deletion of 3 nucleotides resulting in the omission of a single amino acid-phenylalanine at the 508 residue. In Ireland, the ΔF508 mutation has a 76.2% allele frequency, with 56% of patients living with CF in 2015 having two copies of ΔF508 (ΔF508 homozygous) (2). This compares to 46.1% in the United States and 50.3% in the United Kingdom. The second and third most common are the missense mutations G551D (8.3%) and R117H (2.8%), respectively.

Mutations are commonly grouped into classes according to their effect on either the synthesis or function of the CFTR protein (32) (Figure 1.4). The phenotypic expression of disease varies widely, primarily as a function of the specific mutation present (33, 34).

Class I mutations arise as a result of nonsense, frameshift mutations and large deletions causing premature termination codons (PTCs) and defective protein production. As a result no CFTR is expressed (e.g. G542X mutation and W1282X mutation) and such mutations result in severe disease and account for 2-5% of CF cases worldwide (35).

Class II mutations arise from missense mutations and in-frame deletions disturbing CFTR protein configuration and trafficking to the cell surface, resulting in mis-folded CFTR which is not transported to the surface or only transported in residual amounts (e.g. mutations ΔF508, N1303K) resulting in deficiency of cell surface expression leading to alterations in ion and fluid homeostasis (36).
Class III mutations are a result of an amino acid substitution, disrupting normal CFTR channel regulation, which causes diminished channel activity in response to ATP, leading to reduced CFTR channel opening and a “gating defect” (e.g. G551D, G551S, G1349D mutations). Individuals possessing at least one G551D allele have the advantage of using Ivacaftor treatment, a Cl⁻ channel potentiator.

Class IV mutations arise from missense mutations causing a change to CFTR protein structure that forms the pore of the channel. A mis-shaped CFTR pore can restrict the passage of Cl⁻ ions through the channel causing a “conductance defect” (e.g. R117H, R334W mutations) (9).

Class V mutations result from missense mutations leading to alternative splicing that disturbs mRNA processing. This results in extremely reduced amounts of normal CFTR protein production, and resultant reduced protein levels reaching the cell surface (e.g. A455E mutation) (9).

Finally, class VI mutations are missense mutations which result in altered stability of the mature CFTR protein. It causes increased turnover due to a shortened half-life of the quantity of functional CFTR present at the cell surface (e.g. Q1412X mutation) (37).
Figure 1.4: Classification of CFTR mutations.

CFTR mutations are classified into six groups depending on their effect on CFTR function. Class I mutations affect biosynthesis, whilst class II mutations affect protein processing. Milder mutations such as class III to class VI disturb CFTR channel function. (Adapted from White et al (4)).
1.6 CFTR polymorphisms

Research in the area of CFTR has attempted to establish an association between impaired immune responses and CF lung disease independent of CFTR genotype including the detection of susceptibility genes associated with neutrophil dysfunction.

In support of genetic defects causing dysregulated CF neutrophil activity, the influence of the -436GA myeloperoxidase (MPO) promoter polymorphism has been reported. This particular polymorphism is associated with severe clinical disease in CF, and is thought to regulate inflammation and infection (38). This precise polymorphism is not specific to CF however and has been described in other disorders such as lung cancer, vasculitis (39), heart failure (40) and multiple sclerosis (MS) (41).

Subsequently, a genome-wide single nucleotide polymorphism examination revealed interferon-related developmental regulator 1 (IFRD 1) as a regulator of CF pathogenesis via the control of neutrophil function (42). IFRD 1 is a histone-deacetylase-dependent transcriptional co-regulator expressed during neutrophil differentiation. Neutrophils from IFRD1 knock-out mice demonstrated substantial impairment of oxidative burst, bacterial killing and reduced tumour necrosis factor α (TNFα) and leukotriene B4 (LTB4 ) production (42). In addition, the authors examined the role of IFRD1 in modulating airway infection with P. aeruginosa and determined that IFRD1 deficient neutrophils had significantly slower bacterial clearance. Furthermore, macro-array evaluation determined that CF neutrophils exhibit a specific gene expression profile compared with HC neutrophils. Upregulation of genes encoding for both chemokines – (chemokine ligand 17 (CCL 17) and chemokine ligand 18 (CCL 18)), interleukin receptors (IL-3, -8, -10 and -12) and colony stimulating factors (43) were reported. Within this particular study the authors compared blood and airway neutrophils with few differences with regards to gene expression observed. These results are consistent with an abnormal inflammatory profile in CF neutrophils which they concluded was genetic in origin as opposed to inflammatory neutrophil conditioning.
1.7 Disease pathogenesis in CF

The underlying pathogenesis and organ dysfunction seen in CF has been studied in both humans and CFTR-knockout mice, but remains incompletely understood (44, 45). Altered physical and chemical properties of CF airway secretions promote chronic infection with phenotypically exclusive bacteria, especially *Pseudomonas* species. However, other genetic factors, including polymorphisms of the TNFα gene, may compound susceptibility to *P. aeruginosa* infection and add to the clinical expression of CF (46).

Pulmonary inflammation in CF has been observed as early as infancy even in the absence of any demonstrable clinical infection. Airway inflammation in CF has been described with markers of airway inflammation detected in BALF of infants from as early as 4 weeks of age (47). Much of our comprehension of CF pathogenesis is derived from the AREST CF (Australian Respiratory Surveillance Team for CF) study which also established that inflammation begins in early life, prior to the onset of significant clinical infection. Impaired CFTR function was associated with enhanced pulmonary inflammation and infants could have significant lung destruction and infection in the absence of any clinical symptoms. The authors demonstrated that structural lung disease including bronchial dilatation (a precursor to bronchiectasis) could be detected in up to 20% of infants between 2-5 months of age and in excess of 80% of this cohort had evidence of pulmonary disease manifest by infection, inflammation or radiological changes (48). Further paediatric studies have suggested that there is a decline in lung function in the first 2 years of life (49). Infants with free NE detected in BALF had lower forced expiratory flows and forced vital capacities than children with undetectable NE (49). This inflammatory process is predominantly neutrophilic in nature, characterised by elevated neutrophil counts, IL-8 levels and free NE. With increasing patient age, we see increased numbers of, and potentially defective neutrophils, and increased levels of IL-8 and NE (12). Along with this, elevated levels of TNFα, IL-6, IL-1β and LTB₄ are found which propagate the chronic neutrophil presence in the pulmonary circulation (50, 51).
Neutrophil necrosis results in release of large amounts of proteases that destroy the lung architecture, promote oxidative stress and alter the viscosity of endobronchial material, impairing mucociliary clearance and further worsening airway obstruction and infection (52), a relentless vicious circle culminating in worsening progressive respiratory failure and death.

As CFTR gene mutations result in reduced Cl⁻ secretion into the airway lumen, Na⁺ and water shifts occur which tend to dehydrate the ASL and create an altered airway micro-environment. Other contributing factors to this abnormal airway micro-environment include: abnormal airway mucin and a dysregulated inflammatory response (11, 53). The net result of these anomalies is an alteration in the viscosity of airway secretions, which become thick and sticky and difficult to clear (54), culminating in bronchiectasis (Figure 1.5) and irreversible airflow obstruction.

Thickened secretions resulting from CFTR dysfunction also cause gastrointestinal complications in CF. Diminished flow of bile and pancreatic secretions cause maldigestion and malabsorption as well as progressive hepatic and pancreatic disease eventually leading to CF-related diabetes mellitus. DIOS occurs from thickened intestinal secretions and can be a major cause of morbidity in this cohort. In addition, abnormalities in fatty acid breakdown have been described in biopsies of CFTR-expressing tissue from CF sufferers (55). These alterations result in increased tissue levels of arachidonic acid. Thus increased tissue expression of arachidonic acid and its metabolites may promote the abnormal inflammation cycle that is characteristic of CF.

Despite the multi-system nature of this condition, pulmonary disease is responsible for the majority of morbidity and mortality worldwide (56). Chronic airway obstruction by viscid secretions results in gradual pulmonary colonisation with pathogenic bacteria including Haemophilus influenza (H. influenza), Staphylococcus aureus (S. aureus) and eventually P. aeruginosa and/or Burkholdeia cepacia (B. cepacia) complex.
Once infection is established, neutrophils are overwhelmed and unable to control the bacteria even though there is massive infiltration of these inflammatory cells into the airways (57). Recurrent pulmonary bacterial sepsis results in large numbers of circulating neutrophils releasing NE which overcomes the natural anti-proteases of the lung promoting tissue damage. Furthermore, copious amounts of deoxyribonucleic acid (DNA) and cytosol matrix proteins are released by degranulating and necrotic neutrophils, further enhancing the increased viscosity of the airway mucus (58).

Chronic infection with *P. aeruginosa* occurs in part due to increased oxygen consumption by CF epithelial cells, which creates an abnormally reduced oxygen tension within the hyper-viscous mucus layer (59). This local hypoxia promotes the characteristic phenotypic changes in *P. aeruginosa* such as alginate formation and loss of motility. As a consequence, bacterial macro-colonies or “biofilms” arise within the hypoxic regions of the airway mucus layer, and once this happens, complete eradication of the organism is virtually impossible (60). *P. aeruginosa* has the ability to change in order to survive in the environment of the CF lung. One study described that up to 36% of CF individuals were colonised with a hypermutable strain of *P. aeruginosa* that could persist for years and such mutant strains were specific to the CF group only (61).
Figure 1.5: Characteristic computed tomography (CT) findings of CF.

Axial images (lung windows) of normal lung parenchyma (image 1) and CF bronchiectasis (image 2). In image 2 there is evidence of abnormal bronchial dilatation, mucus plugging and cystic change. (Source www.radiopaedia.org).
1.8 Principles of treatment in CF

CF care encompasses a multidisciplinary holistic management strategy consisting of respiratory physician, CF specialist nurse, chest physiotherapist, pharmacist, dietician and clinical psychologist, with additional input from gastroenterology, endocrine and otolaryngology teams as necessary (62).

Management strategies are complex and comprehensive as follows:

Efforts at preserving lung function include fastidious attempts at airway clearance. Airway clearance techniques are taught initially to parents and later to children as they grow older. Various physical techniques are used to mobilise mucus either by percussion and postural drainage, positive expiratory pressure (PEP) devices and autogenic drainage. Chest physiotherapy is performed in conjunction with nebulised bronchodilators and mucolytics. Nebulised bronchodilators are employed to relieve bronchospasm and to ameliorate mucociliary clearance. Aerosolised recombinant dornase alfa (DNase) (Pulmozyme®) cleaves polymeric extracellular DNA in sputum and renders it less tenacious further promoting airway clearance and is the most widely used mucolytic. Nebulised hypertonic (7%) saline has also been proven in various studies to ameliorate mucociliary clearance by hydrating airways and mucus and disrupting bonds between charged macromolecules including DNA and actin (63) as well as reducing IL-8 levels in the sputum of CF patients (64) and increasing the antimicrobial activity of CF airway samples (65). Expectedly, combination use of DNase and hypertonic saline has a greater effect on mucus viscosity and clearance than each used alone (66).

Moreover, timely treatment with appropriate antibiotics in the event of an infective exacerbation and monitoring of lung bacteriology including screening for multi-drug resistant and transmissible organisms are crucial. Another important aspect of the management of lung disease is consideration for referral for lung transplantation if and when necessary (67).
All pancreatic insufficient individuals require pancreatic enzyme replacement and fat soluble vitamin supplementation. An aggressive nutrition treatment strategy employing a high calorie and high fat diet has been proven to advance survival. CF patients may require up to 120-140% of the normal recommended daily allowance for calories. Caloric supplementation and percutaneous gastrostomy feeding are indicated in cases where oral intake is poor or inadequate to maintain a healthy body mass index (BMI) (68).

All CF patients undergo annual ultra-sonographic screening for biliary cirrhosis and portal hypertension (69) as well as annual screening for diabetes mellitus. Bone densitometry is monitored every 2 to 3 years with a DEXA (dual energy x-ray absorptiometry) scan to screen for osteoporosis.

CF patients wishing to start a family are offered fertility advice and pre-conception genetic counselling (70, 71). Amongst treating the physical symptoms of this condition, it is critical to promote good psychological health and well-being with accessibility to psychosocial supports imperative (72).
1.9 Mutation targeted therapy

Class I mutations have a complete absence of stable CFTR protein (due to nonsense mutations from premature stop codons) and therefore may be treated by replacement of the defective CFTR gene or by targeting how the protein is made. Studies revolving around gene addition therapy have been developed to replace the mutant CFTR gene. The UK Gene Therapy Consortium, a phase II trial of CF with non-viral lipid vector for DNA instillation has commenced. The study investigators have recommended monthly-inhaled therapy for one-year duration (NCT 01621867). Further developments involve a lentiviral vector for gene therapy in this patient population (73). The excitement and focus relating to the research surrounding gene addition therapy is its potential use in all class mutations in CF. Ultimately the aim of this is functional gene insertion and normalizing CFTR expression.

Aminoglycosides (e.g. gentamycin) can “read through” PTCs thereby allowing translation to continue to the end of transcription (74, 75). As well as this, Ataluren (PTC 124) has been investigated for its use and role in targeting premature stop codons. This compound allows processing of premature stop codons in Gly 542X and thereby correct processing by ribosomes that will result in production of normal length and functional CFTR protein, insertion at the cell surface and key restoration of CFTR function. Its beneficial effects have been proven with analysis of nasal Cl$^-$ transport. The phase III trial in 238 patients with CF with these stop mutations, failed to achieve its primary end point (improvement in FEV$_1$) at 48 weeks, except in a small sub group of patients not on concomitant aminoglycoside treatment. Class I mutations are thought to affect only 5% of the CF population in Western society.

By far the greatest advances in CFTR mutation targeted therapy have involved class III mutations and the use of Ivacaftor (VX 770). This compound allows longer duration of CFTR presence at the cell surface, increasing Cl$^-$ transport through the CFTR channel (Figure 1.6). VX 770 was originally identified as a potentiator of CFTR function in cell culture with respiratory epithelial cells. Phase III clinical trials demonstrated very promising improvements across all health parameters in the treatment group (76).
Ivacaftor was originally developed to augment the activity and efficiency of the abnormal CFTR protein. This was a well-designed randomized, double blind, placebo-controlled trial. The study subjects had at least one G551D CFTR mutation and were randomly assigned to Ivacaftor 150 mg twice daily or placebo for 48 weeks. The treatment group showed a sustained improvement from baseline in FEV₁ by 10% compared with the placebo group, they were also 55% less likely to suffer a pulmonary exacerbation compared with their placebo counterparts, had higher health scores, gained weight and normalization of their sweat Cl⁻ levels. These benefits were sustained for the duration of the trial and the frequency of adverse events in the 2 groups was equivocal (76). However, only 2-3% of CF individuals in Western society have this G551D mutation and there is on-going research in vitro to ascertain whether there may be a role for Ivacaftor in other nonsense mutations.

Unfortunately, VX 770 has not been efficacious in the ΔF508 homozygous cohort because the abnormal protein configuration prevents CFTR from making it to the cell surface. Corrector agents that allow correct protein folding can protect the mutant protein from removal by the ER and reaching the cell membrane (77). The corrector lumacaftor (VX 809) showed good results in vitro (78) and its use in combination with Ivacaftor has been associated with a greater increase in Cl⁻ transport than has either agent alone. The phase 3 randomised controlled trials were subsequently carried out to evaluate the efficacy and safety of CFTR combination corrector and potentiator therapy in the ΔF508 homozygous CF population. The results demonstrated a modest improvement in FEV₁ as well as a reduction in frequency of infective exacerbations coupled with an acceptable side effect profile. These data confirm that combination of a CFTR corrector and potentiator addresses the underlying cause of CF by targeting the CFTR protein and provides potential benefits for patients who are homozygous for ΔF508 mutation.

Class V mutations result in reduced protein levels by an effect on splicing and causing aberrant and normal transcripts with varying levels of these in patients. Again it is postulated that a combination of potentiators and correctors may be beneficial and this hypothesis is worth testing.
Class VI mutations cause reduced anchoring at the cell membrane and instability of the protein on cell membranes. Agents that augment CFTR anchoring at the cell membrane e.g. activators of Rac1 signalling (79) have been put forward as potential targeted therapies. The authors demonstrated that surface anchoring and retention can be targeted to achieve maximal restoration of ΔF508-CFTR in patients in combination with correctors.

On the whole, this is an exciting time for individuals with CF as the repertoire for effective treatments is growing with expanding knowledge of all aspects of this disease entity.
Figure 1.6: CFTR function in CF human epithelia.

Image (A) denotes dysfunctional CFTR chloride (Cl\(^-\)) transport secondary to the G551D mutation. Image (B) displays corrected CFTR function with restoration in Cl\(^-\) transport post Ivacaftor (VX 770) therapy (Adapted from White et al, 2015) (4).
1.10 The role of neutrophils in CF

1.10.1 Brief overview of neutrophil function

Neutrophils are the most abundant white blood cell in the body, approximately $10^{11}$ produced daily. Neutrophils form the first line of host defence against bacterial and fungal infections (80). They are also the first cells to be recruited to sites of infection or inflammation. Neutrophils migrate through the bloodstream to the site of tissue injury in response to cytokines and chemokines released from these sites (81). Once they reach the site of infection, neutrophils engulf invading microorganisms via receptor recognition. Invading pathogens are opsonised by innate and acquired immune processes such as fixation of complement C3 fragments and immunoglobulin G (IgG) (82). To help with bacterial killing, neutrophils contain potent antimicrobial weaponry within four types of intracellular granules called primary, secondary and tertiary granules and secretory vesicles. In a process called degranulation, these neutrophil granules progressively release their proteases (83). The non-oxidative (oxygen independent) method of bacterial killing therefore involves fusion of neutrophil granules with the phagosome, killing bacteria within minutes of ingestion (84).

In contrast, the oxidative pathway (oxygen dependent) of killing involves activation of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) system on the phagosomal wall (85). NADPH is responsible for the respiratory burst and the formation of the oxygen free radical superoxide ($O_2^-$) in the phagosome. $O_2^-$ dismutates to hydrogen peroxide ($H_2O_2$), which in turn is converted to hypochlorous acid (HOCl), which facilitates bacterial killing by oxidation (81).

Though these two modalities can function independently, synergy is required for a fully bactericidal and destructive response.
1.10.2 Neutrophil granules

This translational research project aims to fully characterise neutrophil primary granule degranulation patterns in CF and to elucidate the underlying molecular mechanism for this. As mentioned, four types of neutrophil granules exist which contain potent antimicrobial mediators produced sequentially during their maturation (Figure 1.7). These include primary (azurophilic) granules containing the neutrophil serine proteases (NE, Cath G, PR 3), as well as MPO and bactericidal permeability increasing protein (BPI); secondary (specific) granules which contain lactoferrin and human cathelicidin antimicrobial protein (hCAP-18), and lastly tertiary (gelatinase) granules, which contain matrix metalloprotease 9 (MMP-9) (86). In addition to these three granule types, neutrophils possess secretory vesicles which contain crucial receptors including for example integrins and the fMLP receptor (FPR) (87).
Figure 1.7: Granule and vesicle contents of the neutrophil.

Granules contain specific proteolytic enzymes and receptors which are released to the plasma membrane or into the phagocytic vacuole during bacterial killing. The propensity for degranulation increases from azurophil (primary) granules to secretory vesicles. Secretory vesicles are endocytic in nature and act as an internal reservoir of membrane/cytokine receptors (Adapted from White et al, 2015) (4).
1.10.3 Primary granules

MPO is the most abundant peroxidase in the neutrophil, accounting for about 25% of primary granule content, reaching a concentration of 100mg/mL in the vacuole (81). MPO is critical for oxidative microbial killing by catalysing the conversion of H$_2$O$_2$ and Cl$^-$ ion to cytotoxic HOCl during the neutrophil respiratory burst (88). Indeed in murine studies, MPO deficient neutrophils have demonstrated ineffective killing of *Candida albicans* (89) and *Aspergillus fumigatus* (90) compared to normal neutrophils. It has also been demonstrated that alterations in sputum colour directly correlated with sputum MPO concentration and this has been proposed as a useful clinical tool for monitoring chronic airways disease and response to treatment (91). Excessive MPO levels have positively correlated with airflow obstruction and sputum production in ΔF508 homozygous CF patients (92), in addition to being associated with high oxidative stress in atherosclerosis (93).

MPO released into the extracellular milieu is capable of augmenting oxidative damage to epithelial cells as a result of HOCl formation (94, 95). It is generally believed that HOCl is the most bactericidal oxidant known to be produced by the neutrophil. In keeping with this theory, a greater level of MPO-dependent activity and chloramine production by CF homozygote and asymptomatic heterozygote peripheral blood neutrophils was found on the outside of the cell compared with HC cells, suggesting that the increased MPO activity was intrinsic, unrelated to infection and mirrored abnormal pH regulation in CF neutrophils (96).

BPI is a ~50 kilodalton (kDa) cationic protein comprised of 456-residues and stored within neutrophil primary granules. BPI is a major protein of neutrophils (from 0.5-1% of total protein) (97). The potent cytotoxicity of BPI is confined to gram negative bacteria (GNB), reflecting its high affinity for bacterial lipopolysaccharide (LPS). Binding of BPI to a live bacterium causes; i) a distinct increase in the permeability of the bacterial outer membrane (98), ii) Release of bacterial phospholipases causing hydrolysis of bacterial phospholipids (99, 100) and iii) disruption of cell division. Irreversible growth inhibition and bacterial killing occur when there is damage to the inner membrane of the LPS envelope.
In addition, recombinant BPI (rBPI21), both alone or in combination with conventional antibiotics has proven protective in experimental animals against lethal inoculations of bacteria and isolated LPS (101). Subsequent phase I and II human trials in BPI have confirmed the compound to be non-toxic and non-immunogenic in HC individuals as well as critically ill patients with meningococcal meningitis (102). Phase II trials have been or are being conducted in a range of conditions including cases of haemorrhagic trauma, partial hepatectomy, with complex peritoneal infections, and in CF, all conditions whereby GNB and their endotoxin cause septicaemia and contribute to morbidity and mortality (103). Moreover, an open label trial has been completed in fulminant paediatric meningococccemia, a life-threatening infection with a mortality ranging from 15-50%. The results of this trial demonstrated a reduction in mortality from 20% in a comparable historical control group to 4%, as well as other benefits in the clinical course (102).

Given the importance of these proteins in degranulation and in bacterial killing, the present study largely focused on patterns of MPO and BPI release from neutrophils upon activation.

Also stored within primary granules is the serine protease NE which can be released from the neutrophil to the extracellular matrix in response to infection or inflammation and has the capability to destroy invading bacteria as well as host tissue (104). NE has a critical role in protecting the lung by initiating the breakdown of pathogens which can lead to infection in the lung and in the removal of necrotic lung tissue (105). The positive surface charge of NE facilitates strong binding to bacterial membranes, which can inhibit bacterial protein production in conjunction with inducing membrane depolarisation and disturbance (106). NE possesses the ability to destroy the outer membrane protein A (ompA) of *Escherichia coli* (*E. coli*) and cleaving virulence factors of *Shigella, Salmonella* and *Yersinia* (107). Data from Reeves et al (2002) established that NE deficient mice were more susceptible to infection by gram negative and gram positive bacteria compared to wild type mice (108).
Besides this, NE also has a modulatory role in immune processes that propagate inflammation. The natural balance between proteases and antiproteases is critical in maintenance of healthy lung architecture, and an imbalance in favour of proteases gives rise to lung injury (109).

Excessive levels of serine proteases are found in airway secretions of patients with inflammatory conditions (110). Serine proteases can damage a wide range of extracellular matrix proteins if left unrestricted, including elastin (111), collagen (112), fibronectin (113), and laminin (114). Ultimately, this can propagate pro-inflammatory peptides (e.g. N-acetyl PGP) (115) and result in destruction of lung alveolar matrix and emphysema development (116). The highly reactive peptide, Pro-Gly-Pro (PGP), a by-product of collagen breakdown by NE also activates neutrophils via chemokine CXCR receptors (115). Furthermore, in CF airways NE has the ability to up-regulate expression of IL-8, a potent chemokine via toll-like receptor 4 (TLR 4) (117). Other tissue destructive enzymes which are released by neutrophils are also up-regulated by NE including MMPs and cathepsins (118). Notably levels of MMP 2 and MMP 9 are present in excess concentrations in BALF from patients with emphysema and these MMPs are elastinolytic (119). As well as this, excessive NE levels in CF BALF can cleave and inactivate the complement 5a (C5a) receptor, exacerbating ineffective local neutrophil priming and bacterial clearance (120).

Cath G and PR3 are additional serine proteases stored within neutrophil primary granules. Akin to NE, Cath G can cleave alveolar matrix proteins including elastin (121), collagen (122) and fibronectin (123) in addition to inactivating TNFα (124) and IL-8 (125). Extracellular Cath G has been proven to destroy leukotoxin, a virulence factor of *Actinobacillus actinomycetemcomitans* (126) and has also been shown to cleave flagellin, a pro-inflammatory bacterial virulence factor (127). In support of this, Cath G deficient mice were more susceptible to staphylococcal and candidal infections (108).
PR3 release from primary granules also has the capacity to destroy extracellular matrix proteins including elastin, collagen and laminin (128) and to cleave and inactivate TNFα (129) and IL-8 (125). PR3 is the chief antigenic target of antineutrophil cytoplasmic antibodies (ANCA), indeed individuals who are anti-PR3 ANCA positive are more prone to prolonged staphylococcal and streptococcal infections (130).
1.11 Neutrophil mediated inflammation in CF

1.11.1 Dysregulated neutrophil activity in CF

Sustained neutrophil recruitment and neutrophil dominated inflammation are hallmarks of CF disease progression (131, 132). However, our understanding of the pathophysiology of CF is undergoing a reassessment and focus has turned to defective immune cell function and in particular, dysregulated neutrophil activity. Within the CF lung, neutrophils constitute approximately 60-70% of the total immune cell population (133, 134). Nevertheless, these neutrophils fail to clear pathogens, leading to persistent infection within the lungs. It is as yet unclear why CF neutrophils fail to eliminate bacteria (135). The fundamental question remains as to whether these defects are primary to CF or secondary to chronic bacterial infection and inflammation.

A significant factor in this debate was to establish if neutrophils expressed CFTR and a loss thereof could confound efficient neutrophil function. In addition to this, the development of CFTR specific pharmacotherapies has spurred research into identifying the expression of CFTR mRNA transcripts and protein levels in both epithelial and non-epithelial cells. As CF is a multi-system disease with most pronounced pathology originating in the airways, initial studies aimed at identifying CFTR expression in bronchial epithelial cells. Yoshimura et al (1991) first demonstrated the expression of CFTR mRNA transcripts in human bronchial epithelial cells (26). Trapnall et al also demonstrated equal expression of CFTR mRNA transcripts from respiratory tract epithelial cells isolated from healthy controls as well as individuals’ hetero- and homozygous for ΔF508 mutation (136).

At this point in the literature, it was thought that expression of CFTR was specific only to epithelial cells. However, follow on studies by Yoshimura’s group suggested that the CFTR gene may have regulatory properties and that it could well be present in other cells types (137). The authors demonstrated expression of low levels of CFTR mRNA transcripts in non-epithelial cells including human T lymphocytes, neutrophils, monocytes and alveolar macrophages (137) and emphasised the important role of CFTR in Cl⁻ transport.
In support of this, it was determined that alveolar macrophages from CFTR deficient mice whilst exhibiting intact phagocytosis and generation of oxidative burst, showed defective killing of phagocytosed bacteria due to impaired acidification of the phagosome (138). Of interest in this study by Di et al, CFTR protein was not detectable in either murine or human neutrophils (138) and ensuing studies have demonstrated CFTR independent phagosomal acidification (139, 140). Nonetheless, CFTR mRNA expression in human macrophages was confirmed by Del Porto et al (2011), who reported that the bactericidal activities of macrophages was CFTR dependent, confirming an important operational role for CFTR protein in these immune cells (141).

Thus far, no connection had been made between abnormal neutrophil function and the expression of CFTR protein in human neutrophils, with research in this field thriving quickly with the advent of CFTR targeted pharmacotherapy. Indeed, there is still immense debate as to the true cause of impaired neutrophil activity in CF. For instance, McKeon et al (2010) detected low levels of CFTR mRNA transcripts in neutrophils using reverse transcriptase polymerase chain reaction (RT-PCR) or real time PCR amplification (142). The investigators here could not detect CFTR protein expression in membrane or cytosolic fractions nor cell lysates from human neutrophils by Western blot analysis, suggesting that human neutrophils did not express CFTR protein and that dysregulated neutrophil function in CF was due to the inflammatory status of the patient. Equally, Morris et al (2005) examined altered phagocytosis of neutrophils in CF due to cell priming, and did not detect CFTR protein in human neutrophils by Western blot analysis (143).

Over time, several reasons have been proposed in the literature for not detecting CFTR protein in neutrophil cell fractions by Western blot: susceptibility of CFTR protein to degradation, lack of reliable anti-CFTR antibodies available and the boiling of cell fractions prior to electrophoresis. As these issues were addressed, researchers have subsequently been able to detect CFTR protein in human neutrophils and have established functional roles for membrane associated CFTR.
CFTR protein expression has been described on neutrophil membranes and in secondary granules (144). In the same study, the authors confirmed the expression of CFTR protein by Western blot analysis in a human myeloid cell line (HL 60 cells) differentiated into neutrophil like cells. The authors also demonstrated that CFTR channel expression in neutrophils and its dysfunction affected neutrophil chlorination of phagocytosed bacteria leading to impaired bacterial killing (144). This is in contrast to the earlier Yoshimura study which failed to detect CFTR mRNA transcripts in differentiated HL 60 cells. However, further studies have established CFTR mRNA transcripts in differentiated HL 60 cells and demonstrated CFTR protein localisation to the phagocytic vacuole, reinforcing the similarities between HL 60 cells and human neutrophils (145). Moreover, research carried out by Pohl et al (2014) determined that neutrophils from patients with mutations in CFTR exhibit impaired neutrophil degranulation, disturbed ion homeostasis, which confers inadequate bacterial killing abilities. These deficiencies were corrected by Ivacaftor treatment as an ion channel potentiator (146). In this study, activated CF neutrophils (using TNFα or fMLP) in patients with nonsense mutations showed less secondary and tertiary granule release and altered cellular ion regulation compared with healthy controls and in subjects with non-CF bronchiectasis. The defective neutrophil degranulation and abnormal cell ion concentration were confirmed to result from dysregulated CFTR channel activity or absence as similar results were replicated with HC cells exposed to the CFTR pharmacologic inhibitor CFTRinh-172 (146).
In line with this work, use of CFTRinh-172 results in inhibition of Cl⁻ secretion to mimic CFTR dysfunction of CF. This compound works by increasing the close time and reducing the mean open time of the Cl⁻ channel resulting in reversible inhibition of CFTR within minutes (147). Thus an artificial CF environment is replicated within a HC cell. In the present study we also employed CFTRinh-172 in order to ascertain if defective degranulation of primary granules is a primary defect related to lack of CFTR function or whether it is secondary to chronic infection and inflammation that is characteristic of CF.

Whilst increased levels of neutrophils are generally needed to clear an infection, apoptosis is essential for resolution of inflammation and to safeguard against possible tissue destruction (148). As well as in CF, delayed apoptosis of neutrophils has been implicated in a number of other inflammatory conditions such as rheumatoid arthritis (RA) (149), inflammatory bowel disease (IBD) (150) and sepsis (151). The bacterial endotoxin- pyocyanin produced by \textit{P.aeruginosa} has been proven to prevent apoptosis (152). Indeed, CF patients infected with this bacterial species display high concentrations of this peptide within the lung which can further exacerbate the dysregulated apoptosis in CF neutrophils (153). Additionally, prolonged neutrophil survival has been described regardless of infection status or mutation type (142). In support of this finding, Moriceau et al (2010) demonstrated that neutrophils from heterozygous asymptomatic parents of individuals with CF exhibit delayed apoptosis (154).
1.12 Abnormal degranulation processes by CF neutrophils

CF neutrophils have been shown to have impaired degranulation processes, as greater levels of primary granule components NE (155) and MPO (156) were identified in the extracellular environment following stimulation of circulating CF cells with either CF BALF, TNFα and IL-8 or serum opsonised particles (155).

NE and the other serine proteases PR 3 and Cath G exist in similar quantities within neutrophil primary granules (157), though the majority of research revolves around NE. As well as cleaving bacterial proteins within the phagosome, NE released upon dysregulated degranulation or from necrotic neutrophils is the major destructive protease in the CF lung causing break down of structural proteins elastin, collagen and proteoglycans (158, 159). NE can also up-regulate further expression of other proteases including MMPs and cathepsins exacerbating the destruction of airway proteins further. It is therefore not surprising that counteracting NE activity has been suggested to reduce the overall protease burden (160). Moreover, NE has been confirmed to play an important role in activation of MMPs which are formed in an inactive precursor form (161). MMP-9 is a 92 kDa precursor which is cleaved by NE into an active 72 kDa protein (162). Increased levels of MMP-9 have been demonstrated in exhaled breath condensate of clinically stable CF children (163). In addition, higher levels of MMP-9 have been linked to NE levels, showing a significant inverse relationship between MMP-9 and FEV1 (164). Consequently, enhanced levels of active neutrophil released MMP-9 can cause significant airway remodelling and inflammation (165, 166) and formation of chemotactic peptides (167). A host of other enzymes can be activated by serine proteases including meprin-alpha (168), MMP-2 (169), MMP-3 (169) and pro-cathepsin B (170).

Recently, a study carried out by Pohl et al (2014) identified that CF neutrophils exhibited impaired secondary and tertiary granule degranulation in response to TNFα stimulation, with significantly reduced levels of hCAP 18, lactoferrin and MMP-9 released compared to HC cells. Decreased levels of the GTP-bound Rab27a protein were found as a cause of this aberrant pattern of degranulation by CF neutrophils (146).
This is of particular importance when we consider that lactoferrin released from neutrophil secondary granules can inhibit growth and biofilm production of \textit{P. aeruginosa} (171, 172), but within the CF lung is cleaved by excess NE, \textit{Pseudomonas} elastase and Cath G (173, 174).
1.12.1 Antiproteases in CF airways

In normal airways, the quantities of proteases and antiproteases- alpha one antitrypsin (AAT), elafin, secretory leukocyte protease inhibitor (SLPI) are meticulously balanced to allow maximal bacterial killing with minimal tissue destruction. This balance is disturbed by proteolytic degradation in the CF lung (131, 175-177). Taggart et al (2001) demonstrated the degradation and inactivation of SLPI by cysteinyl cathepsins and NE (178). In the clinical context, SLPI levels quantified in CF BALF were inversely related to neutrophil counts and *P. aeruginosa* colonisation. The oxidation and inactivation of the antiprotease elafin by NE has been shown to result in the loss of antiprotease activity *in vivo* (179) and *in vitro* (180) further compounding this critical imbalance. In addition, aerosolised AAT was found to positively impact upon neutrophil mediated killing of *P. aeruginosa* (181), perhaps by inhibiting cleavage of neutrophil complement receptors by NE (182) or by preventing cleavage of CXCR1 (183). Subsequent studies by Griese et al (2007) (184) demonstrated decreased activity of NE in sputum as well as reduced levels of TNF-α and IL-8 after 4 weeks of aerosolised AAT. A phase II clinical trial assessing the efficacy of recombinant aerosolised AAT showed a trend towards an improvement in time to first acute CF exacerbation compared with placebo (185).

To conclude, restoration of the protease-antiprotease balance is an essential strategy in modulating airway inflammation and potentially preventing progressive lung disease in CF.
1.13 Neutrophil granule trafficking

1.13.1 The role of Rab proteins and neutrophil degranulation

Exocytosis, also referred to as degranulation in neutrophils is the release of pre-formed mediators from granules. Granule subsets display vast differences in their ability for mobilisation upon stimulation (186, 187). Granules produced during the later stages of granulopoiesis are more likely to undergo exocytosis than granules produced in the earlier stages. Exocytosis levels of 100% for secretory vesicles, 38% for tertiary granules, 22% for secondary granules and only 7% for primary granules have been described post neutrophil stimulation (188). The exact stages involved in exocytosis involve granule translocation toward a target membrane by way of actin remodelling and microtubule assembly, and subsequently tethering and docking through the sequential action of core fusion machinery of Rab and SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) proteins (187, 189).

Degranulation is dependent upon the binding of guanosine triphosphate (GTP) to intracellular effector molecules as confirmed by the addition of the non-hydrolyzable analogue of GTPγS to permeabilised neutrophils leading to secretion of granule-derived mediators (190). Ras-related guanosine triphosphatases (GTPases) reside in the cytoplasm or on membranes in the cell and carry out regulation of cell degranulation. Ras–related GTPases are critical switches for turning on and off a signalling event. Activation of these proteins is mediated by binding to high energy GTP which is cleaved to form guanosine diphosphate (GDP) which in turn activates the next effector molecule in the signalling pathway. Binding to GTP generates the association of cytosolic GTPases to membrane or cytoskeletal sites in the cell.

Ras-related GTPases can be subdivided into many subfamilies depending on their homology at the amino acid level. A specific subgroup of ras-related GTPases is the Rho subfamily of GTPases; these proteins are responsible for regulating actin cytoskeletal rearrangement and the release of ROS (191).
Actin cytoskeletal rearrangements are key to allowing a range of cellular activities to occur including cell motility, phagocytosis, and degranulation. There are at least 20 Rho subfamily members identified. Ras-related C3 botulinum toxin substrate (Rac) is present in 3 isoforms: Rac1, Rac2 and Rac3. Whilst the role of Rac3 in granulocytes has not been elucidated, the functions of Rac1 and Rac2 are well defined (192). Rac1 and Rac2 display homology at the amino acid level (193) and were thought to be functionally interchangeable as regards their ability to activate cytoskeletal remodelling required for chemotaxis as well as $\text{O}_2^-$ generation through the NADPH oxidase complex (194).
1.13.2 Role of Rac2 and its activation

As the main focus of the present study is degranulation of primary granules in CF, we focused on Rac2 and examined its role in enhanced degranulation in CF. Rac2 is a small (21 kDa) signalling G protein and component of the rho family of GTPases that is localised to haematopoietic cells. Rac2 regulates a wide range of cellular events such as the regulation of vesicle transport, cell division, oxidase activity and nuclear assembly (195). It is encoded by the RAC2 gene (196).

Activation of Rac proteins is brought about by GDP/GTP nucleotide exchange factors (GNEFs) (Figure 1.8). The primary GNEFs for Rac2 are T-cell-lymphoma-invasion-and-metastasis-1 (TIAM-1) (197), phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger-1 (PREX-1) (198) as well as vav-1 guanine nucleotide exchange factor (VAV-1) (199), which is most efficacious in generating oxidase activity of Rac2 (200).

Rac2 activation necessitates isoprenylation at the C-terminus by geranylgeranyltransferase type I (GGTase-1) to anchor the protein to the vesicle membrane (199). In turn, activated Rac2 interacts with many effectors which promote activation of vital cellular pathways, incorporating Rac2 interaction with nitric oxide synthase 2A that stimulates nitric oxide (NO) production (201). Conversely, Rac2 activity is inhibited by GTPase activating proteins (GAPs) such as Rac GTPase activating protein-1 (RacGTP-1) (202).
Figure 1.8: Activation of Rab proteins.

Small GTPases act as molecular switches and regulate many aspects of intracellular signalling. Activation of small G proteins is mediated via GDP/GTP nucleotide exchange factors (GNEFs) and deactivated by GTPase activating proteins (GAPs). GTP-bound proteins bind to effector molecules and a signal is generated. Small G proteins are brought into the cytosolic form when inactive by binding to GDP dissociation inhibitors (GDIs). (Adapted from Cherfils et al, 2013) (3).
1.13.3 Rac2 activation and neutrophil primary granules

Neutrophils contain primary, secondary and tertiary granules that display a hierarchy of release in response to intracellular Ca\(^{2+}\) spikes (112). The last step of granule fusion with the neutrophil membrane is dependent upon GTP and Ca\(^{2+}\) (203). The degranulation of primary granules is tightly regulated as they contain highly reactive and cytotoxic proteolytic enzymes and the peroxidase MPO. Clostridium difficile toxin B, a Rac2 inhibitor, has been proven to prevent degranulation from rat basophilic leukaemia (RBL) cells (204).

Neutrophils are vitally dependent on Rac2 for degranulation of primary granules. Neutrophils isolated from the bone marrow or peritoneum of Rac2 knock-out mice displayed an extreme loss of primary granule release, as determined by a lack of MPO and NE release in response to secretagogues cytochalasin B (CB)/fMLP or CB/LTB\(_4\). Addition of the potent priming cytokine TNF\(\alpha\) did not correct the degranulation defect in this study. Despite the loss of primary granule exocytosis, these mice retained the ability to release secondary and tertiary granules as measured by release of lactoferrin and MMP-9 respectively, in response to the same stimuli. Alongside this finding, human neutrophils which were dominant negative for Rac2 D57N failed to release the primary granule protein MPO but had intact release of lactoferrin after fMLP exposure (205). These findings are in support of preceding research which suggested that different signalling pathways controlled the degranulation of each specific granule type in neutrophils (206, 207). Thus Rac2 plays a fundamental role in the degranulation of primary granules in neutrophils.

The exact mechanism by which Rac2 controls primary granule translocation and docking is not fully understood. The evaluation of the primary granule marker CD63 by confocal microscopy has demonstrated that Rac2 knock-out neutrophils lacked the function of translocation of primary granules to the cell membrane (208), indicating that Rac2 could control the translocation of granules to the cell membrane during degranulation. Therefore it would seem that granule translocation is likely to depend on Rac2-mediated actin cytoskeletal remodelling and/or microtubule rearrangement.
Rac2 is required for normal neutrophil chemotaxis as well as actin generation in response to chemo-attractants. It is also a critical regulator of the neutrophil actin cytoskeleton and neutrophil endothelium interactions require Rac2.

Eitzen et al (2011) carried out proteomic analysis on CB/fMLP stimulated bone marrow neutrophils that were isolated from wild type and Rac2 knockout mice and recognised an accumulation of CD 63\(^+\) staining (marker for primary granules) at the cell periphery as well as reduction in the abundance of many granule proteins in wild type neutrophils which did not occur in Rac2 deficient neutrophils therefore confirming the need for Rac2 in degranulation of neutrophil primary granules (209). Moreover, the authors described increased levels of several isoforms of the actin remodelling protein, coronin-1A in wild type but not Rac2 knockout mice post CB/fMLP stimulation suggesting that the control of Rac2 mediated degranulation in neutrophils is attributable to actin remodelling via activation of several actin-binding proteins. The cellular mechanism underlying dysregulated degranulation of neutrophil primary granules in CF has not been clarified to date.
1.14 Autoantibodies in CF

Autoimmune disease frequently involves elevated neutrophil activity in the absence of infectious agents. ANCA are autoantibodies directed against neutrophil antigens, predominantly PR3 and MPO, which have been implicated in the pathogenesis of small vessel vasculitis.

ANCA have been observed in patients with CF, especially those with chronic bacterial airway infections (210, 211). The ANCA are directed against BPI and have been found to correlate with severity of airflow obstruction (210). As a consequence these ANCA can cause inhibition of BPI mediated bacterial phagocytosis contributing to the persistence of P. aeruginosa in the CF lung and exacerbating CF lung destruction (212). Similarly, in another example of chronic airways disease alpha one antitrypsin deficiency (AATD) syndrome, excessive neutrophil degranulation of secondary and tertiary granules was demonstrated to give rise to the development of anti-lactoferrin autoantibodies (213). Treatment of AATD patients homozygous for the Z allele with AAT augmentation therapy resulted in reduced membrane TNFα expression and plasma levels of granule antigenic proteins and immunoglobulin class G (IgG) autoantibodies.

It is not yet known if ANCA are pathogenic or are simply serological markers of disease. Anti-MPO and anti-PR3 ANCA can activate neutrophils primed by TNFα (214) by interacting with FcγIIIR (215, 216), instigating degranulation and release of oxygen free radicals and active enzymes which potentially cause tissue injury in vivo (217).

Knowledge of the exact mechanism of autoimmunity in CF and the clinical significance of this autoimmunity is limited. Thus it may be a non-specific sequel of chronic infection through the inflammatory response and hypergammaglobulinaemia.

Autoimmunity may also be a marker of pro-inflammatory conditions which could propagate severe infections and lung destruction. Indeed therapies aimed at directly reversing or controlling the inflammatory response including corticosteroids, anti-inflammatories, montelukast, fish oil, macrolides have been investigated in preceding studies with varying degrees of success (218, 219).
As with all medical therapies, the risk benefit analysis is crucial. Whilst the clinical advantages of corticosteroids have been well described, so too have their accompanying side effects (220).

The exploration for autoantibodies and other biomarkers of systemic inflammation may have a role in identifying potential paediatric CF patients who may benefit from early anti-inflammatory therapies. In addition, the investigation of some modifier genes such as TNFα, transforming growth factor (TGF) β1 and mannose-binding lectin 2 may prove useful in selecting such patients with a hyper-immune response (221, 222).
1.15 Aim

This translational research project aims to establish if defective degranulation of primary granules by CF circulating neutrophils is intrinsic or inflammatory.

To fulfil this aim, the following objectives were set:

1. To investigate neutrophil degranulation patterns in CF

2. To determine the molecular mechanism underlying aberrant degranulation of neutrophil primary granules in CF

3. To establish if neutrophil degranulation and excessive Rac2 activity in CF is an intrinsic defect

4. To investigate if there are increased autoantibodies detected against neutrophil primary granule components in CF

5. To determine the effects of Ivacaftor therapy upon dysregulated degranulation and autoantibody production

By achieving these aims we will test the hypothesis that the CF neutrophil is intrinsically abnormal and leads to dysregulated neutrophil activity in CF and the generation of autoantibodies against neutrophil primary granule contents.
Chapter 2

Materials and Methods
2.1 Materials and Methods

2.1.1 Reagents

All chemicals and reagents were of the highest purity and were purchased from Sigma-Aldrich Ireland Ltd. (Dublin Ireland), unless otherwise specified.

2.1.2 Antibodies

The primary and secondary antibodies employed for Western blot analysis are listed in Tables 2.1 and 2.2 respectively.
Table 2.1: Primary antibodies employed for Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Stock</th>
<th>Concentration</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit PAb anti-MPO</td>
<td>Novus Biologicals</td>
<td>2ml</td>
<td>1:1000 (WB)</td>
<td>53</td>
</tr>
<tr>
<td>Mouse MAb anti-BPI</td>
<td>Santa Cruz Biotechnology</td>
<td>200 µg in 1ml</td>
<td>1:1000 (WB)</td>
<td>50</td>
</tr>
<tr>
<td>Rabbit PAb anti-Rac 2</td>
<td>Cell Signalling Technology</td>
<td>10 mM</td>
<td>1:1000 (WB)</td>
<td>21</td>
</tr>
</tbody>
</table>

Note: PAb = Polyclonal antibody, MAb = Monoclonal antibody, WB = Western blot analysis

Table 2.2: Secondary antibodies employed for Western blotting

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Stock</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit PAb anti-goat</td>
<td>Cell Signalling Technology</td>
<td>10 mM</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>Horse PAb HRP anti-</td>
<td>Cell Signalling Technology</td>
<td>10 mM</td>
<td>1:1000 (WB)</td>
</tr>
</tbody>
</table>

Note: PAb = Polyclonal antibody, HRP= Horseradish Peroxidase, WB = Western blot analysis
2.2 Patient samples

2.2.1 Patient Recruitment

Ethical approval was applied for and granted from the Beaumont Hospital Ethics Committee (REC reference # 14/98). Blood was collected from 22 clinically stable CF patients attending the Beaumont Hospital CF Unit (Table 2.3: Demographics of CF patients) used in degranulation assay experiments. Healthy control (HC) volunteers (n=12, mean age 30.41 years) had neither known comorbidities, nor any respiratory symptoms and were not taking any medications.

There are approximately 150 patients attending the CF service in Beaumont hospital currently. Table 2.10 displays the CF patient characteristics for those receiving Ivacaftor treatment which were recruited for auto-antibody analysis. This represents 10 out of the 14 individuals attending the CF service in Beaumont hospital on Ivacaftor. (Please see Appendix 1 and Appendix 2 for patient information leaflet and patient consent form respectively).

2.2.2 Neutrophil Isolation

Peripheral blood neutrophils were isolated from heparinised venous blood as described by Reeves et al (2002) (108). Neutrophils were isolated by mixing 5ml phosphate buffered saline (PBS) with 15 ml of blood collected in heparinised S-monovette tubes (10 units/ml; Starstedt, Germany) and 2.5 ml of 10% (w/v) dextran in PBS and allowed to sediment for 15 min. The resultant top layer was pipetted into a sterile tube and layered onto 5ml Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) so as to allow separation of the various types of blood cells by density gradient centrifugation at 836 x g for 10 min (Heraeus Megafuge 1.0 centrifuge, Kendro Laboratory products, Germany).

Neutrophils were purified by re-suspending the resultant cell pellet in 20ml ultra-pure water for hypotonic lysis of any remaining red blood cells. Tonicity was corrected by addition of 20 ml 2X saline (300 mM NaCl in PBS) and neutrophils were collected by centrifugation at 470 x g for 5 min. The resulting neutrophils were re-suspended in 1ml PBS containing 5mM glucose (PBSG) and used immediately.
2.2.3 Trypan Blue Exclusion Test

The neutrophil cell count was obtained by adding 10 µl cell suspension to 90 µl Trypan Blue on a haemocytometer (Superior, Marienfeld, Germany) with an average number of 1.0-3.0 x 10^7 cells/ml blood isolated. The proportion of viable (unstained) cells in the final cell number was calculated with the resultant cell viability being greater than 95% in all samples used.
### Table 2.3: Demographics of CF Patients used in Degranulation Assays

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cystic Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.36 (± 1.66)</td>
</tr>
<tr>
<td>Gender</td>
<td>Female (9)</td>
</tr>
<tr>
<td></td>
<td>Male (13)</td>
</tr>
<tr>
<td>Genotype</td>
<td>ΔF508/F508 (13)</td>
</tr>
<tr>
<td></td>
<td>ΔF508/G551D (2)</td>
</tr>
<tr>
<td></td>
<td>ΔF508/R117H (3)</td>
</tr>
<tr>
<td></td>
<td>ΔF508/Unknown (1)</td>
</tr>
<tr>
<td></td>
<td>ΔF508/E60X (1)</td>
</tr>
<tr>
<td></td>
<td>G551D/G542X (1)</td>
</tr>
<tr>
<td></td>
<td>ΔF508/R560T/K (1)</td>
</tr>
<tr>
<td>FEV(_1) (% predicted)</td>
<td>60.55 (± 6.06)</td>
</tr>
<tr>
<td>Neutrophil number used per</td>
<td>2 x 10(^7) cells/ml</td>
</tr>
<tr>
<td>experiment</td>
<td></td>
</tr>
</tbody>
</table>

**FEV\(_1\)** = Forced expiratory volume in one second

Values are represented as mean +/- SEM
2.3 Preparation of protein samples

2.3.1 Preparation of whole cell lysates

For preparation of whole cell lysates, 2 x 10^7 cells were lysed in 75 µl ice cold-RIPA buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 1% (v/v) TritonX-100, 1% (v/v) nonylphenoxy polyethoxylethanol-40 (NP-40), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS)) containing additional protease inhibitors (10 µg Na-Tosyl-L-lysine chloromethylketone hydrochloride (TLCK), 1µg/ml phenylmethan-sulfonyl fluoride (PMSF), 10µg/ml pepstatin A, 10 µg/ml leupeptin) and incubated on ice for 10 min. The suspension was centrifuged at 20,817 x g for 10 min at 4°C in order to remove insoluble cell debris and the supernatant was harvested. 10X Sample Buffer (SB) (2% (w/v) SDS, 1mM 1,4 dithiothreitol (DTT), 1% (w/v) sucrose, 0.004% (w/v) bromophenol blue, 5mM ethylenediaminetetraacetic acid (EDTA), 60 mM Tris HCl pH 6.7) was added to give a final concentration of 1X. Samples were boiled at 99°C for 3 min and stored frozen at -20°C for subsequent visualisation of proteins by Coomassie blue staining of SDS-polyacrylamide gel electrophoresis (PAGE) or Western blot analysis.
2.4 Protein quantification

2.4.1 Sodium dodecyl sulphate polyamide gel electrophoresis (SDS PAGE)

SDS-PAGE was performed by standard Laemmli methods (223).

Initially, two clean glass plates (AE-6450 Dual mini Slab Kit (Atto, Japan) were placed with a plastic gasket between them and clamped together. Running gel (12.5% (w/v)) was prepared and poured between the plates to fill approximately 80% of the plates capacity and allowed to set at room temperature (~30 min) (Table 2.4 composition of running gel). Isopropanol 1ml was poured directly above the 12.5% w/v gel to even out the underlying layer of gel. The isopropanol was then rinsed off with deionised water (H₂O). Stacking gel (5% (w/v)) was prepared and poured above the resolving gel and a loading comb was positioned and the gel was allowed to set at room temperature (Table 2.5 composition of stacking gel).

Cast gels were positioned in a tank containing 1X Running Buffer (6g Tris, 2.88g glycine, 2g SDS). Prepared protein samples were mixed with 10X SB. These samples were then boiled at 99°C for 3 min and centrifuged at 4°C for 1 min to ensure homogeneity of the sample. Samples (15µl) with 10X SB (2.5µl) were loaded and run alongside 4µl of molecular weight ladder SeeBlue® plus 2 pre-stained standard (Invitrogen, Bio Sciences Ltd, Ireland) for approximately 90 min at 132 volts (V). The gel was further analysed by Western blotting to quantify protein content and profile.
### Table 2.4: Constituents of 12.5% (w/v) running gel

<table>
<thead>
<tr>
<th>Volume</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ml</td>
<td>1.5 M Trizma base pH 8.9</td>
</tr>
<tr>
<td>7.6 ml</td>
<td>Deionised H$_2$O</td>
</tr>
<tr>
<td>10 ml</td>
<td>30% (w/v) Protogel (acrylamide) (National Diagnostics, Atlanta, USA)</td>
</tr>
<tr>
<td>240 µl</td>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>150 µl</td>
<td>10% (w/v) ammonium persulfate (APS) (0.1g in 1 ml H$_2$O)</td>
</tr>
<tr>
<td>6 µl</td>
<td>Tetramethylethylenenediamine (TEMED)</td>
</tr>
</tbody>
</table>

### Table 2.5: Constituents of 5% (w/v) stacking gel

<table>
<thead>
<tr>
<th>Volume</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>630 µL</td>
<td>0.5 M Trizma base pH 6.8</td>
</tr>
<tr>
<td>3.4 ml</td>
<td>Deionised H$_2$O</td>
</tr>
<tr>
<td>830 µl</td>
<td>30% (w/v) Protogel (acrylamide), National Diagnostics, Atlanta, USA</td>
</tr>
<tr>
<td>50 µl</td>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>50 µl</td>
<td>10% (w/v) APS</td>
</tr>
<tr>
<td>5 µl</td>
<td>TEMED</td>
</tr>
</tbody>
</table>
2.4.2 Coomassie Blue Staining

Gels containing protein bands were stained with Coomassie Brilliant Blue staining solution (0.2% (w/v) Brilliant Blue R, 45% (v/v) methanol, 10% (v/v) acetic acid, 45% (v/v) deionised H$_2$O) in order to visualise protein bands. Gels were stained for 1 h or overnight and destained with a solution that contained 10% (v/v) acetic acid and 25% (v/v) methanol and 65% (v/v) deionised H$_2$O until desired dye density was reached. Images were taken using the G:Box SynGene machine (Synoptics, UK) or Epson Perfection Photo scanner (V330).

2.4.3 Western Blot Analysis

Western blotting was carried out with 1X Western blot wet Transfer Buffer (3 g Trizma base, 14.4 g glycine, 800 ml deionised H$_2$O and 200 ml methanol) using the XCell II Surelock Mini-Cell wet Western Blotter device™ (Invitrogen, Bio Sciences Ltd., Ireland). Methanol submersion was used to activate polyvinylidene fluoride (PVDF) membrane (Roche) whilst the transfer sponges and Whatman™ filter paper were pre-soaked in 1X Western blot wet Transfer Buffer. Following electrophoresis, gels were sandwiched between activated PVDF, pre-soaked Whatman™ filter paper and sponges to facilitate wet protein transfer. Transfer was carried out at 30 V for 120 min.

For immunoblotting, PVDF membranes were blocked with blocking solution which contained 3% (w/v) dried skimmed milk (Marvel, Chivers Ireland Ltd, Ireland) and 1% (w/v) bovine serum albumin (BSA) in PBS solution containing 0.1% (v/v) Tween® 20 (PBST) for 1 h at room temperature. The blots were incubated overnight at 4°C with relevant antibodies and individual concentrations as specified in Table 2.1.

Blots were subsequently washed in PBS-Tween® (0.01 M DPBS, 0.5% Tween) every 10 min for 30 min and then probed with HRP-linked secondary antibodies against the primary antibody for 1 h at room temperature. Blots were washed again of secondary antibody every 10 min for 30 min in PBS-Tween®. The blots were incubated with Immobilon Western Chemiluminescent HRP-substrate solution (Millipore, Billerica, MA, USA) and bands were visualised with the G-Box Chemie XL (Syngene, Cambridge, UK) using the GeneSnap software and quantified with the GeneTools software.
2.5 Determination of degranulation

2.5.1 Degranulation Assays

Circulating neutrophils (2 x 10⁷ cell/ml) from patients with CF and HC were either unstimulated (time 0) or stimulated with either 1ng/ml TNF α (R&D Systems) (224) and 100 ng/ml formyl-methionyl-leucyl phenylalanine (Palmer et al, 1983) (225) or 1 µg/ml phorbol-12-myristate-13-acetate (PMA) (226) at 37°C. Cross et al, (2008) and Bergin et al (2014) have reported previously that TNFα at concentrations of less than 10 ng/ml does not cause neutrophil apoptosis (213, 224).

Samples of 100µl aliquots were collected after each time point (0, 5, 10 or 20 min) and added to eppendorf tubes containing ice-cold “Stop” Buffer (protease inhibitor cocktail) in DPBS to counteract any proteolysis of released proteins. “Stop” Buffer contained 10 µg/ml TLCK, 10 µg/ml Pepstatin A, 10 µg/ml Leupeptin and 1µg/ml PMSF in 5ml of PBS (Table 2.6 composition of stop buffer).

The collected cells were then centrifuged at 425 x g for 5 min at 4°C. 10X SB (20 µl) was added to 100µl of supernatant containing degranulated proteins. Samples were then boiled at 99°C for 3 min and stored frozen at -80°C. Remaining cells were processed for preparation of whole cell lysates harvesting as described previously for loading controls.
Table 2.6: Composition of “Stop” buffer

<table>
<thead>
<tr>
<th></th>
<th>Protease Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml DPBS</td>
<td></td>
</tr>
<tr>
<td>10 μl TLCK</td>
<td>Trypsin inhibitor</td>
</tr>
<tr>
<td>10 μl Leupeptin</td>
<td>Serine and cysteine inhibitor</td>
</tr>
<tr>
<td>20 μl Pepstatin A</td>
<td>Aspartic acid inhibitor</td>
</tr>
<tr>
<td>25 μl PMSF</td>
<td>Serine protease inhibitor</td>
</tr>
</tbody>
</table>
2.5.2 CFTR inhibitor treatment

To inhibit Cl\(^-\) channel function of the CFTR, neutrophils (2x10\(^7\)) from HC individuals were pre-treated for 15 min with either 10 µM CFTRinh-172 or the corresponding vehicle control (0.1% (v/v) DMSO) at room temperature (227). Following this incubation period, 100 µl aliquot was collected from resting cells (time 0) and placed into an eppendorf containing 100 µl Stop Buffer on ice. Samples were centrifuged at 425 x g for 5 min 4\(^\circ\)C to harvest the extracellular supernatant for examination of degranulated proteins. The remaining neutrophil pellets were subjected to whole cell lysis for use as loading controls.

Degranulation assay was carried out subsequent to collection of time 0 min (unstimulated) supernatants. The neutrophil pellets were then re-suspended in TNF\(\alpha\) (1 ng/10\(^7\) cells) and fMLP (100 ng/10\(^7\) cells) and were free of CFTRinh-172 or DMSO for 20 min. Extracellular supernatants were collected at 5, 10 and 20 min post TNF\(\alpha\) and fMLP stimulation for examination of degranulated proteins.
2.6 Measuring enzyme kinetics

2.6.1 Fluorescence resonance energy transfer (FRET) analysis

The following protocol was adapted from Kormaz et al, (2008) (228). Neutrophils (2 x 10^7 cells/ml) from HC and CF individuals were isolated and either unstimulated or stimulated with TNFα (1ng/10^7 cells) and fMLP (100 ng/10^7 cells) at 37°C for 0, 5, 10 or 20 min. Extracellular supernatant was removed after centrifugation at 425 x g for 5 min at 4°C.

In advance, 30% N,N-Dimethylformamide (DMF) was prepared with ultra-pure water in the fume hood. FRET assay buffer was prepared and carefully set to pH 7.5. The standards for NE and Cath G (TS563, Elastin products, Athens Research and Technology) were prepared in their required concentrations and loaded into the appropriate wells of a 96 well plate (Nunc® Microwell ™ 96 well polypropylene plates, P6866, Sigma Aldrich). The nM range of concentrations used for NE was: 20, 10, 5, 4, 2.5, 1.12, 0.62 and 0nM. The nM range of concentrations used for Cath G was 60, 40, 30, 15, 7.5, 3.75, 1.875 and 0nM.

FRET substrate for human NE was prepared by adding 152µl of 30% (w/v) DMF into a vial of Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp (3230-v, Peptide Institute, Inc) and followed by 608 µl DPBS to make up a final concentration of 1mM. FRET substrate for Cath G was prepared by adding 174 µl of 30% (w/v) DMF into a vial of Abz-Glu-Pro-Phe-Trp-Glu-Asp-Gln-EDDnp (3231-v, Peptide Institute) followed by a further 698 µl DPBS to achieve a final concentration of 1 mM. The relevant FRET substrate master mix was prepared on ice and kept covered from light.

Extracellular supernatants were thawed on ice and centrifuged at 479 x g for 1 min (to sediment the proteins that have fallen out of solution). Once the samples have thawed, proteases were thawed and dilutions were prepared. The 96 well plate was kept on ice during well loading in duplicate fashion. DMF controls were employed for every FRET substrate used. 50µl of standards and 50µl of samples are loaded into each well.
DMF master mix (50 µl) was loaded into the relevant wells and 50µl of FRET master mix added to the appropriate wells using a multi-channel pipette. The 96 well plate was then loaded onto the spectrofluorometer (Spectramax M3, Molecular Devices, USA) and read for 1 h at 28°C. The excitation emission wavelengths were 320-420 nanometres (nm).

Samples were analysed in duplicate and concentrations were expressed in nM. The slope of the standard curve was used to extrapolate unknown concentrations of extracellular NE and Cath G. The data was analysed using Graph Pad Prism 5.0 Software (La Jolla, CA, USA).
2.7 Rac2 activation assay

Neutrophils (2x10^7 cells / ml) from HC and CF individuals were isolated and either unstimulated (resting cells-time 0) or stimulated with TNF α (1 ng/10^7 cells) and fMLP (100 ng/10^7 cells) at 37°C for 40 sec. This time point was chosen in keeping with Benard et al, (1999) who previously reported that peaks in Rac2 activity are observed between 30 sec and 1 min post neutrophil stimulation (229). Aliquots of 100 µl were harvested at 0 and 40 sec post stimulation and added to an eppendorf tube containing ice cold 100 µl protease inhibitor cocktail mix (2 ml 5X lysis buffer (Abcam, catalogue # ab 139586), 8 ml deionised H2O, 5 µl PMSF, 2 µl pepstatin A, 1 µl leupeptin and 1 µl TLCK). Cell free supernatants were collected by centrifugation at 425 x g for 5 min at 4°C and the remaining neutrophil pellet retained.

Diisopropylfluorophosphate (DFP) 1 µl and 200 µl of 1 X Lysis Buffer were both added to each neutrophil pellet on ice (Table 2.7 composition of 1X Lysis Buffer). Each pellet was then lysed by passing through a 25 gauge needle x 3 times. Neutrophil lysates were subsequently cleared by centrifugation at 12,000 x g for 10 min at 4°C. Lysis Solution was added to the neutrophil lysates of each time point and lysates were cleared again by centrifugation at 12,000 x g for 15 min at 4°C (Table 2.8 composition 1X Lysis Solution). Neutrophil lysate (75 µl from each time point) was then removed for total Rac2 analysis. The Abcam Rac activation kit (catalogue # ab 139586) was employed to immuno-precipitate active GTP-bound Rac2 from neutrophil lysate samples. This kit exploits the selective interaction of the Cdc42/Rac interactive binding domain (CRIB) of the effector p21 activated kinase-1 (PAK-1) with the active Rac-GTP structure. The remaining neutrophil lysate 120 µl from each time point was then added to 40 µl glutathione sepharose slurry and incubated on a rotating wheel for 30 min at 4°C. Glutathione sepharose slurry binds to the glutathione sepharose-tagged –p21 activated kinase-Cdc42/Rac interactive binding domain (GST-PAK-CRIB) complex containing active GTP-bound Rac. The beads were then washed with 1ml ice-cold 1X Lysis Buffer x 3 times.
SB (2X) was then added to each total and active GTP-bound Rac sample and boiled for 3 min at 99°C followed by centrifugation at 425 x g x 2min at 4°C for ensuing SDS PAGE and Western blot analysis.

Anti-Rac2 antibody (Cell signalling Technology Rac 2 antibody, 1:1000) was used to detect total and active Rac2 in neutrophil lysates. Immunobands were visualised using the Molecular Imager® Gel Doc™ XR system (BioRad Laboratories, Inc). Quantification of resultant immunoband intensity was carried out using ImageLab® 5.2.1 software (BioRad Laboratories, Inc).
### Table 2.7: Constituents of 1X Lysis Buffer

<table>
<thead>
<tr>
<th>Volume</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 ml</td>
<td>Deionised H₂O</td>
</tr>
<tr>
<td>2 ml</td>
<td>5X Lysis Buffer stock supplied in Abcam Rac activation kit (ab # 139586)</td>
</tr>
<tr>
<td></td>
<td>Tris buffer pH 7.5, NaCl, MgCl₂, Nonyl phenoxypolyethoxyethanol (NP-40), PMSF, Pefabloc, Aprotinin, Pepstatin A.</td>
</tr>
<tr>
<td></td>
<td><strong>Note:</strong> Exact concentrations not disclosed by company</td>
</tr>
</tbody>
</table>

### Table 2.8: Constituents of Lysis Solution

<table>
<thead>
<tr>
<th>Volume</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 ml</td>
<td>5X Lysis Buffer (Rac activation kit #ab 139586)</td>
</tr>
<tr>
<td>4.8 ml</td>
<td>Deionised H₂O</td>
</tr>
<tr>
<td>60 µl</td>
<td>100X protease inhibitor mix (Rac activation kit #ab 139586)</td>
</tr>
<tr>
<td>6 µl</td>
<td>GDP 100 mM (Rac activation kit #ab 139586)</td>
</tr>
<tr>
<td>150 µg</td>
<td>GST-PAK-CRIB (Rac activation kit #ab 139586)</td>
</tr>
</tbody>
</table>
2.8 ELISA

2.8.1 Anti-MPO and anti-PR3 autoantibody quantification

Enzyme linked immunosorbent assay (ELISA) was employed for the evaluation of autoantibodies against neutrophil primary granule proteins, MPO and PR3, as per the “Routine use of the Phadia ® 250 for anti-MPO and anti-PR3 antibody testing” protocol set out by the Clinical Directorate of Laboratory Medicine, Beaumont Hospital, Dublin (Doc no. LP-IMM-ICAP0004, revision 3.6, active date 09/09/14).

The PHADIA® 250 EliA™ system (Phadia GmbH, Freiburg, Germany) is a fully automated test that measures the concentrations of antigen specific IgG antibodies in human plasma or serum. The EliA™ MPO wells (article no. 14-5537-01) were coated with human MPO protein, whilst the EliA™ PR3 (article no. 14-5536-01) wells were coated with human PR3 protein. The plates were incubated with plasma samples from HC (n=35), CF (n=29) and CF patients on Ivacaftor (CF+IVA) (n=8). (Table 2.10 displays the CF patients receiving Ivacaftor therapy characteristics that were used in this ELISA analysis).

If positive in plasma samples, antibodies to MPO or PR3 bound to their specific antigen. After washing away unbound plasma antibodies, enzyme-labelled antibodies against the human IgG antibodies, EliA™ IgG conjugate (article no. 83-1017-01), were added to form an antibody-conjugate complex. Post incubation, unbound conjugate was washed away with washing solution (article no. 10-9202-01) and the bound complex was incubated with developing solution (article no. 10-9441-01). The reaction was halted using stop solution (article no. 10-9442-01) and the fluorescence of the elute was measured. The test results were calculated from a calibration curve specific for the anti-MPO and anti-PR3 antibodies being measured. The fluorescence intensity was directly proportional to the concentration of relevant antibody in the plasma sample. Both EliA™ MPO and EliA™ PR3 were calibrated against the new Centre for Disease Control (CDC) ANCA references: CDC PR3 ANCA Human reference serum #16 and CDC MPO ANCA human Reference serum # 15 (CDC 2008). Results were recorded in international units per millilitre (IU/ml). The reference ranges are listed in Table 2.9.
**Table 2.9: Reference ranges for EliA™ MPO and EliA PR3 autoantibody quantification**

<table>
<thead>
<tr>
<th></th>
<th>Negative (IU/mL)</th>
<th>Equivocal (IU/mL)</th>
<th>Positive (IU/mL)</th>
<th>Measuring range (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EliA™ MPO</strong></td>
<td>&lt; 3.5</td>
<td>3.5-5</td>
<td>&gt; 5</td>
<td>0.2-134</td>
</tr>
<tr>
<td><strong>EliA™ PR 3</strong></td>
<td>&lt; 2</td>
<td>2-3</td>
<td>&gt; 3</td>
<td>0.2-177</td>
</tr>
</tbody>
</table>
2.8.2 Anti-BPI autoantibody quantification

Anti-BPI autoantibodies in CF and HC plasma were quantified by ELISA according to the manufacturer’s instructions (Orgentec Diagnostika, Mainz, Germany). This assay was carried out in the research laboratory as anti-BPI testing is not performed routinely in clinical practice.

For plasma anti-BPI quantification, aliquots from CF samples were diluted 1 in 1000, whilst HC plasma was diluted 1 in 500, following a pilot run to ascertain optimal dilutions and to ensure results were within the standard range.

For this experiment, we used plasma samples from HC (n=38), CF (n=28) and CF patients receiving Ivacaftor therapy (n=5) (Characteristics of CV individuals receiving Ivacaftor therapy used in this ELISA are depicted in Table 2.10).

Subsequently, 100 µl of plasma samples (post dilution) or standards (ranging from 0 to 100 U/ml) were placed in wells of a 96-well plate (pre-coated by the manufacturer) and incubated for 30 min at room temperature. Plates were washed 3 times with specific wash solution (Tris, detergent, sodium azide 0.09%), followed by incubation with 100 µl enzyme conjugate (containing anti-human IgG antibodies, HRP labelled) for 15 min at room temperature. Following this, plates are washed a further 3 times with specific wash solution as above. Plates were then incubated with 100 µl of 3’,3’,5’,5’,Tetramethylbenzidin (TMB) substrate solution for 15 min at room temperature. Stop solution (100 µl) was added to each of the wells before reading optical density at 450 nm using a spectrophotometer (Spectramax M3, Molecular Devices, USA).

To calculate antibody concentration of samples, absorbance values of standards were plotted against their antibody concentration. As the standards did not follow a linear pattern, a standard curve was fitted transforming antibody concentrations into their respective logarithms and by employing a sigmoidal dose-response model, sample concentrations were calculated using GraphPad Prism 5.0 software (La Jolla, USA).
2.8.3 Measurement of BPI levels by ELISA

Plasma and BALF levels of BPI from CF and HC were quantified by ELISA according to the manufacturer’s instructions (Hycult biotech-HK 314). This assay was performed in the research laboratory as routine BPI measurement is not carried out in clinical practice. Once again, 100 µl of plasma samples from HC (n=32), CF (n=28) and CF patients on Ivacaftor (n=6) (post 1 in 4 dilution) or standard concentrations (ranging from 0 to 10,000 pg/ml) were placed in wells of a 96-well plate (pre-coated by the manufacturer) and incubated for 2 h at room temperature.

For BPI quantification in BALF (pre-existing samples in storage), aliquots from CF samples were diluted 1 in 500, whilst HC, non CF bronchiectasis (NCFB) and COPD (as the inflammatory controls) samples were aliquoted neat onto ELISA plates following a pilot run to ascertain optimal dilutions and to ensure results were within the standard range. (Table 2.11 outlines the patient characteristics used in BPI BALF quantification). Following this, plates were washed 3 times with specific wash solution, followed by incubation with 100 µl biotinylated tracer antibody (which will bind to captured human BPI) for 1 h at room temperature. Following this, plates were washed a further 3 times with wash solution (composition not disclosed by manufacturer). Plates were then incubated with 100 µl of streptavidin-peroxidase solution for 1 h at room temperature in the dark, followed by 3 washes. TMB substrate (100 µl) was added to each well and covered for 30 min at room temperature. The reaction was stopped by the addition of oxalic acid stop solution to each well and subsequently reading optical density at 450 nm (Spectramax M3, Molecular Devices, USA).

Raw data was exported using elisaanalysis.com and statistical analysis was carried out using GraphPad Prism 5.0 software (La Jolla, USA).
2.9 Statistical analysis

All data investigated in this project was analysed using GraphPad Prism 5.0 Software (La Jolla, CA, USA). Unless stated otherwise, data are expressed as mean +/- SEM and p values were determined by Student’s t-test. One-way ANOVA was used to determine statistical significance when comparing three or more groups. A p value of ≤ 0.05 was deemed statistically significant.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Genotype</th>
<th>FEV₁, pre (% pred)</th>
<th>FEV₁, post (% pred)</th>
<th>Sputum culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>M</td>
<td>F508/G551D</td>
<td>45</td>
<td>56</td>
<td>B. cepacia H. influenza</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>M</td>
<td>F508/G551D</td>
<td>25</td>
<td>31</td>
<td>P. aeruginosa MRSA</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>M</td>
<td>F508/G551D</td>
<td>53</td>
<td>71</td>
<td>P. aeruginosa H. influenza</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>M</td>
<td>F508/G551D</td>
<td>45</td>
<td>64</td>
<td>P. aeruginosa Candida</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>M</td>
<td>F508/G551D</td>
<td>93</td>
<td>86</td>
<td>Candida</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>M</td>
<td>F508/G551D</td>
<td>56</td>
<td>27</td>
<td>P. aeruginosa Candida</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>M</td>
<td>F508/G551D</td>
<td>57</td>
<td>95</td>
<td>P. aeruginosa MSSA</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>M</td>
<td>G551D/G542X</td>
<td>24</td>
<td>24</td>
<td>S. maltophilia Candida</td>
</tr>
<tr>
<td>9</td>
<td>29</td>
<td>F</td>
<td>G551D/G542X</td>
<td>45</td>
<td>40</td>
<td>P. aeruginosa S. maltophilia</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>F</td>
<td>G551D/G551D</td>
<td>30</td>
<td>36</td>
<td>P. aeruginosa MRSA</td>
</tr>
</tbody>
</table>

Table 2.10: Characteristics of CF individuals receiving Ivacaftor therapy.

Patient demographics depicting age in years, sex- male (M) / female (F), genotype, forced expiratory volume in first second (FEV₁) expressed as percentage predicted (pre and post Ivacaftor treatment) and sputum microbiological isolates.

Note: *Burkholderia cenocepacia (B. cenocepacia), Haemophilus influenza (H. influenza), Pseudomonas aeruginosa (P.aeruginosa), methicillin resistant Staphylococcus aureus (MRSA), methicillin sensitive Staphylococcus aureus (MSSA), Candida albicans (Candida), Stenotrophomonas maltophilia (S. maltophilia).*

Note: None of the patients in this cohort were enrolled in a clinical trial at the time of participation in this study.
<table>
<thead>
<tr>
<th>Identifier</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Genotype</th>
<th>FEV&lt;sub&gt;1&lt;/sub&gt; (% pred)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF 1</td>
<td>31</td>
<td>M</td>
<td>F508/F508</td>
<td>21</td>
</tr>
<tr>
<td>CF 2</td>
<td>21</td>
<td>M</td>
<td>F508/F508</td>
<td>19</td>
</tr>
<tr>
<td>CF 3</td>
<td>32</td>
<td>M</td>
<td>F508/F508</td>
<td>52</td>
</tr>
<tr>
<td>CF 4</td>
<td>26</td>
<td>F</td>
<td>F508/F508</td>
<td>78</td>
</tr>
<tr>
<td>CF 5</td>
<td>25</td>
<td>M</td>
<td>G551D/R117H</td>
<td>80</td>
</tr>
<tr>
<td>CF 6</td>
<td>48</td>
<td>F</td>
<td>Unknown</td>
<td>67</td>
</tr>
<tr>
<td>CF 7</td>
<td>31</td>
<td>M</td>
<td>F508/G551D</td>
<td>50</td>
</tr>
<tr>
<td>CF 8</td>
<td>29</td>
<td>F</td>
<td>F508/F508</td>
<td>54</td>
</tr>
<tr>
<td>CF 9</td>
<td>32</td>
<td>M</td>
<td>F508/F508</td>
<td>42</td>
</tr>
<tr>
<td>HC 1</td>
<td>66</td>
<td>M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HC 2</td>
<td>73</td>
<td>M</td>
<td>-</td>
<td>90</td>
</tr>
<tr>
<td>HC 3</td>
<td>73</td>
<td>F</td>
<td>-</td>
<td>92</td>
</tr>
<tr>
<td>NCFB 1</td>
<td>37</td>
<td>M</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td>NCFB 2</td>
<td>66</td>
<td>F</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>NCFB 3</td>
<td>49</td>
<td>F</td>
<td>-</td>
<td>72</td>
</tr>
<tr>
<td>COPD 1</td>
<td>59</td>
<td>M</td>
<td>-</td>
<td>54</td>
</tr>
<tr>
<td>COPD 2</td>
<td>38</td>
<td>F</td>
<td>-</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 2.11: Population characteristics of BALF samples

Sample identifier: Cystic fibrosis (CF), healthy control (HC), non-CF bronchiectasis (NCFB), chronic obstructive pulmonary disease (COPD). Age is denoted in years, sex- male (M), female (F), CF genotype and forced expiratory volume in first second (FEV<sub>1</sub>) expressed as percentage predicted at the time of bronchoscopy and BALF sample retrieval.
Chapter 3

Impaired Neutrophil Degranulation in Cystic Fibrosis
3.1 Introduction

3.1.1 Neutrophil degranulation in CF

Neutrophils play an integral role in the body’s defence against bacteria and are key players in the innate immune system. Neutrophils dominate the CF inflammatory airways. Paradoxically, although present in high abundance, CF neutrophils fail to eradicate bacteria effectively. As a consequence there is an unrelenting cycle of lung parenchymal injury from airway infection and inflammation. This vicious circle of airways infection and inflammation is evident even in early life (230). Parenchymal lung injury is the major cause of morbidity and mortality in CF.

Research carried out by the Australian Respiratory Surveillance Team for CF (AREST CF) study confirmed that inflammation began in early life, preceding the onset of significant and detectable clinical infection. It was also established that structural lung disease could be detected in infants between 2-5 months of age, with the majority of this cohort displaying features of pulmonary disease including infection, inflammation or radiological changes (48). As a consequence of progressive bronchiectatic damage, CF lungs are overwhelmed by *P. aeruginosa* colonisation, resulting in progressive architectural damage culminating in respiratory failure.

Circulating neutrophils need to be activated in order to release their potent bactericidal capacity involving degranulation of cytosolic granules. When neutrophils migrate to the CF airway, they are primed and activated releasing oxidants and proteases (83).
Neutrophils contain at least four different types of granules; 1-primary granules (also known as azurophilic granules), 2- secondary granules (also known as specific granules), 3- tertiary granules and 4- secretory vesicles. Primary granules are subdivided by their content of hydrolytic and bactericidal proteins and enzymes including NE, BPI, defensins, cathepsins and MPO (231-233). Secondary and tertiary granules contain lactoferrin and MMP-9 respectively, amongst other substances. Neutrophil derived proteases have a physiological purpose and are involved in many processes such as tissue remodelling, chemotaxis and bacterial killing. To prevent perpetual deleterious proteolytic activities all serine proteases are tightly controlled by protease inhibitors typically AAT (234).

Upon neutrophil stimulation, granules translocate to either the phagosomal or plasma membrane, where they anchor and fuse with the cell membrane and release their contents.

Elevated levels of MPO have been shown to correlate with reduction in pulmonary function and indeed disease severity (235, 236), whilst the MPO promoter polymorphism -463G is associated with more severe clinical expression of CF lung disease (38). MPO is also the most abundant peroxidase in neutrophil primary granules and excess MPO levels are linked to high oxidative stress (93). Similarly elevation in serum MPO levels have shown a positive correlation to elevated MPO sputum levels and a positive correlation with pulmonary exacerbation status (237). Given the wealth of data surrounding MPO in CF, our first set of experiments centred on release of this peroxidase by CF neutrophils.
3.1.2 Stimuli causing neutrophil degranulation

This project is primarily concerned with the dysregulated primary granule degranulation in individuals with CF and its underlying mechanism. Dysregulated neutrophil function has been shown to induce widespread endothelial cell injury after priming (238). Similarly, excessive neutrophil degranulation has been described in a number of inflammatory disorders such as severe asthma (239), acute lung injury (ALI) (240), RA and septic shock (241).

Neutrophils are stimulated to release their granule contents by a variety of triggers and mediators. The priming role of TNFα and IL-8 in BALF of CF has been described (155). NE release from CF neutrophils was markedly reduced when TNFα and IL-8 were removed from BALF. Similarly, when TNFα and IL-8 were used as activation stimuli, CF neutrophils released considerably larger amounts of NE when compared to HC cells and cells from individuals with idiopathic bronchiectasis (242).

Pohl et al (2014) (146) demonstrated reduced neutrophil degranulation of secondary and tertiary granules in CF compared with HC cells in response to TNFα. This abnormality was found to be caused by defective activation of Rab27a, a low-molecular-mass GTP binding protein involved in the organisation of granule movement. In contrast, in a second airways disease example, Bergin et al, described increased plasma concentrations of neutrophil derived secondary and tertiary granule proteins from AATD neutrophils following TNFα stimulation (213).

Within this chapter the potent inflammatory cytokine- TNFα was primarily chosen as a neutrophil stimulus. Increased levels of TNFα are found in CF (243) and it has been proven to cause degranulation of only secondary and tertiary granules but not primary granules (244). The addition of the bacterial chemotactic peptide fMLP was therefore used to selectively promote primary granule exocytosis. Therefore our experiments used the synergistic TNFα and fMLP combination to promote and assess primary granule degranulation patterns by CF neutrophils compared to HC cells.
TNFα is an adipokine involved in systemic inflammation and is part of a cytokine group that stimulates acute phase reactions; it is chiefly secreted by macrophages. It should be noted that the use of large concentrations of this can lead to cellular apoptosis, so we used a low concentration of TNFα, previously shown not to induce apoptosis (213) in combination with fMLP for neutrophil activation. The concentration of TNFα used as a neutrophil activator in our experiments is much lower than that of concentrations utilised previously and proven not to induce apoptosis or cell death in the length of time to which the cells were exposed; as verified by annexin V and propidium iodide staining of neutrophils.

fMLP is a potent chemotactic peptide and formyl peptide receptor agonist that induces a metabolic burst in macrophages leading to secretion of lysosomal enzymes and superoxide anion formation. It binds to the fMLP receptor on the cell surface and activates a G protein dependent pathway (245).
3.1.3 Release of serine proteases and bactericidal proteins by CF neutrophils

In addition to MPO experiments in this chapter we also investigated the serine protease, NE as an additional marker of primary granule release. NE and other proteases including PR 3 and Cath G are seen in almost equal amounts within primary granules of neutrophils (157). Dysregulated release of NE has been described in CF and it is the main harmful protease in the CF lung causing damage to structural proteins such as elastin, collagen and proteoglycans (158).

Of note, CF neutrophils spontaneously released greater levels of NE even after resolution of clinical exacerbation. In addition, NE release was not significantly altered following aggressive CF patient treatment and recovery from pulmonary exacerbation, again demonstrating continuous dysregulation of neutrophil primary granule release despite clinical resolution of infection (246). In the present study we employed specific fluorescence resonance energy transfer (FRET) substrates for the analysis of extracellular NE activity.

FRET analysis is extremely sensitive and allows enzymatic activity measurements to be made on very small quantities of material in small volumes. It also provides kinetic information over a wide range of time scales from seconds to hours (247).

Neutrophil derived serine proteases other than NE are also recognised as playing an important role in the pathogenesis of chronic suppurative airways diseases. Mature Cath G is 28.5 kDa in size and is made up of 235 amino acid residues (248). Cath G has been shown to interfere with clearance of *P. aeruginosa* from mouse lungs (249) based on a 1-log reduction in bacteria recovered from Cath G deficient mice.

High Cath G activity has been demonstrated in CF sputum and was shown to stimulate hyper-secretion of mucus by airway submucosal glands (250). It too has the ability to degrade structural components of the extracellular matrix at high concentrations (228), hence we chose to investigate it as yet another marker of primary granule release in the ensuing experiments.
BPI is a 50 kDa protein which is also stored in the azurophilic granules in neutrophils. It has also been identified at lower levels in the specific granules of eosinophils (251). BPI constitutes part of the innate immune system. BPI forms an endogenous antibiotic protein with potent bacterial killing capacity specifically against gram negative bacteria. BPI has potent antimicrobial activity against gram negative bacteria in particular, by neutralising endotoxin (252) and facilitating opsonisation of the bacteria (253). It has also been demonstrated that BPI can induce apoptosis in human vascular endothelial cells in vitro and to inhibit cell migration in wound assays (254). Aside from its antimicrobial effects, BPI is also an important antigen for anti-neutrophil cytoplasmic antibodies (ANCA) in vasculitis and this forms the basis of our experiments in results chapter 2. In the present study we sought to determine BPI degranulation patterns from CF neutrophils compared with HC cells.
3.1.4 Rac signalling mechanisms leading to neutrophil degranulation

The cellular mechanisms underlying dysregulated neutrophil primary granule degranulation in CF have not yet been elucidated and this study aims to understand this.

The release of granule contents by granulocytes is tightly regulated by receptor coupled mechanisms leading to exocytosis. A number of factors affect neutrophil translocation and exocytosis including increases in intracellular calcium concentrations, and the hydrolysis of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) to name a few.

G proteins of the Ras superfamily have been implicated in the control of neutrophil degranulation. These small GTPases continually cycle between an inactive GDP-bound form and an active GTP-bound form. Such G proteins regulate the control of cell movement, cell division and gene transcription (255). Rac2 has been established as selectively controlling neutrophil degranulation of primary granules. Both NE and MPO release has been shown to be absent in Rac2 knock-out mice as well as the lack of mobilisation of CD63⁺, also a primary granule marker, following neutrophil stimulation with cytochalasin B (CB) and fMLP (208). Rac2 has also been associated with the propagation of NADPH oxidase (256), in turn leading to ROS production in neutrophils.

Much of the existing literature is concerned with understanding whether neutrophil dysfunction in CF is secondary to the intrinsic genetic defect or due to chronic sustained bacterial infection and inflammation. Artificial manipulation of the CFTR channel with the thiazolidinone compound- CFTR inh-172 is a commonly used reliable model of CF (147). CFTRinh-172 works by blockade of the Cl⁻ channel opening leading to rapid and reversible and voltage independent inhibition (257). Continuous inhibition of CFTR Cl⁻ channel in human tracheal epithelial cells resulted in significant elevations in IL-8 secretion at baseline and in response to *P. aeruginosa* exposure (147).
We sought to determine if pharmacological CFTR inhibition \textit{in vitro} resulted in excessive primary granule release and increased Rac2 activation. In the final experiment of this chapter we utilised CFTRinh-172 in order to continuously inhibit CFTR in HC cells and we employed its own control (cells suspended in the vehicle control) as a comparator.
**Aim of this chapter**

In this chapter we hypothesised that increased Rac2 activation was the underlying mechanism causing neutrophils of CF individuals to exhibit dysregulated primary granule degranulation processes.

Our aim was to determine if defective degranulation of primary granules by CF neutrophils was intrinsic or inflammatory in nature.

To fulfil the aim of this chapter, the following objectives were outlined:

1. To explore neutrophil primary degranulation activity patterns of CF patients compared to healthy control cells.

2. To investigate if the cellular basis for the enhanced degranulation of primary granules in CF involved increased Rac2 activation.

3. To establish if increased primary granule degranulation is an intrinsic defect caused by CFTR dysfunction.
3.2 Impaired neutrophil degranulation by CF

3.2.1 Increased degranulation of primary granules by CF neutrophils analysed by Western blot

In order to assess degranulation of primary granules, healthy control (HC) and CF circulating neutrophils were isolated and then stimulated with either phorbol-myristate-acetate (PMA) (1µg/10⁷ cells) or a combination of TNFα (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) at 37°C. The combination of TNFα (1ng/ml) and fMLP (1µM) was employed as these are relevant physiological stimuli for promoting the release of primary granules from both CF and HC neutrophils. PMA (1µg/ml) stimulation was employed as a positive control. PMA is an analog of diacylglycerol (DAG) and is a small molecular compound which is an activator of the signal transduction enzyme protein kinase C (PKC).

Neutrophils were exposed to PMA for a total of 5 min and the extracellular supernatants were collected per sample of HC and CF neutrophils. The duration of exposure to PMA is shorter (5 min) than that of TNFα/fMLP (20 min) as PMA is a stronger stimulant which can induce not only degranulation but also ROS production, and affect cell viability in a dose and time dependent manner. Extracellular supernatants were analysed for degranulated proteins. The haem protein MPO was detected by Western blot analysis using a rabbit polyclonal anti-MPO primary antibody (Novus Biologicals, 1:1000 dilution).

Figure 3.1 (A) displays a representative Coomassie blue stained SDS PAGE of whole cell lysates of unstimulated cells and those post 5 min stimulation with PMA depicting equal protein loading denoting equal cell numbers per reaction. Figure 3.1 (B) displays a representative Western blot showing no significant difference in MPO release from CF and HC neutrophils at 5 min post PMA stimulation. Densitometry values of immunoblots of 3 separate experiments (Figure 3.1 (C), n=3 individuals per group) revealed no difference in degranulation of primary granules by CF neutrophils compared with HC cells (p=0.36 and p=0.23 respectively) using PMA as a stimulant. Analysing the relative change between HC at time 0 and 5 min and CF at time 0 and 5 min did not reveal any significant differences.
Figure 3.1: No significant difference in MPO release between CF and HC neutrophils using PMA stimulation determined by Western blot analysis.

HC or CF circulating neutrophils were either unstimulated (time 0) or stimulated with PMA (1 µg/10^7 cells) at 37°C. Extracellular supernatants were collected at 0 and 5 min. (A) Coomassie stained gel of unstimulated and 5 min post PMA stimulated neutrophil whole cell lysates depicting equal protein loading confirming equal cell numbers used per reaction. (B) Representative Western blot of extracellular supernatants probed for MPO using a rabbit polyclonal anti-MPO primary antibody (Novus Biological, 1:1000 concentration). Note: supernatants were loaded on a contiguous gel. The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was performed using the GeneTools software® (HC t=0 min set as 1 and other time point data normalised to HC t=0 min). Statistical significance was calculated by Student’s t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ±SEM, (n=3 individuals per group).
In subsequent experiments we only employed the more reliable physiological neutrophil activators TNFα (1 ng/10^7 cells) and fMLP (100ng/10^7 cells) to assess neutrophil degranulation. Neutrophils were exposed to TNFα and fMLP for 20 min at 37⁰C and subsequent extracellular supernatants were harvested.

Figure 3.2 (A) displays a representative Coomassie blue stained SDS PAGE of unstimulated whole cell lysates and those post 20 min stimulation with TNFα and fMLP depicting equal protein loading, once more confirming equal cell numbers per reaction. Figure 3.2 (B) displays a representative Western blot showing increased release of MPO from CF primary granules at 20 min post TNFα and fMLP stimulation. Densitometry values of immunoblots from four separate experiments (Figure 3.2 (D), n=4 individuals per group) revealed significantly elevated levels of MPO release from CF neutrophils when compared with HC cells (p=0.03). The relative change between HC at time 0 and 20 as well as the relative change between CF at time 0 and 20 min stimulation was also determined. The relative change between unstimulated CF (0 min) neutrophils and 20 min post stimulation is significantly increased (p=0.03) which indicates priming of CF neutrophils to release greater levels of primary granule protein upon stimulation compared to HC neutrophils (p=0.16).
Figure 3.2: MPO release determined by Western blot analysis is increased by CF neutrophils post TNFα/fMLP stimulation.

Healthy control and CF purified circulating neutrophils were either unstimulated (time 0) or stimulated with TNFα (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C. Extracellular supernatants were collected at 0 and 20 min. (A) Coomassie blue stained gel of unstimulated and TNFα/fMLP stimulated neutrophil whole cell lysates post 20 min depicting equal protein loading demonstrating equal cell numbers used per reaction. (B) Representative Western blot of extracellular supernatants probed for MPO using a rabbit polyclonal anti-MPO primary antibody. Note, supernatants were loaded on a contiguous gel. Molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was performed using the GeneTools software® (HC t=0 min set as 1 and other time point data normalised to HC t=0 min). Statistical significance was calculated by Student’s t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ±SEM, (n=4 individuals per group).
To further corroborate our finding of aberrant primary granule degranulation, we assessed BPI activity in CF and HC neutrophils both at rest and following exposure to TNFα and fMLP for 20 min at 37°C. Exocytosed neutrophil supernatants were probed for BPI using a mouse monoclonal anti-BPI antibody (Santa Cruz® Biotechnology, 1:1000 dilution).

Figure 3.3 (A) displays a representative Coomassie blue stained SDS PAGE of unstimulated whole cell lysates and those post 20 min stimulation with TNFα and fMLP once again confirming equal protein loading denoting equal cell numbers per reaction. Figure 3.3 (B) displays a representative Western blot showing increased release of BPI from CF primary granules at 20 min post TNFα and fMLP stimulation. Densitometry values of immunoblots from four separate experiments (Figure 3.3 (C), n=4 individuals per group) revealed significantly elevated levels of BPI release from CF neutrophils when compared with HC cells (p=0.02).

There was no statistically significant difference when the relative change was calculated between unstimulated HC (0 min) and 20 min post stimulation (p=0.07) nor CF unstimulated cells (0 min) and 20 min post stimulation (p=0.11).
Figure 3.3: BPI release determined by Western blot analysis is increased by CF neutrophils in response to TNFα/fMLP.

HC and CF circulating neutrophils were either unstimulated (time 0) or stimulated for 20 min with TNFα (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C. Extracellular supernatants were collected at 0 and 20 min. (A) Coomassie blue stained gel of unstimulated and TNFα/fMLP stimulated neutrophil whole cell lysates. (B) Representative Western blot of extracellular supernatants probed for BPI using a mouse monoclonal anti-BPI primary antibody (Santa Cruz®, 1:1000 concentration). Molecular weight (kDa) is displayed on the left. (C) Quantification of immunoband intensity was performed using Image Lab® software (HC t=0 min set as 1 and other time point data normalised to HC t=0 min). Statistical significance was calculated by Student’s t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ±SEM, (n=4 individuals per group). Note: Graph layout differs from preceding graphs to ensure that bars depicted on graph correspond to immunobands on Western blot.
3.3 Excessive NE activity in CF neutrophils analysed by FRET

Thus far we have demonstrated excessive MPO and BPI as markers of increased primary granule release by CF neutrophils using Western blot. Ensuing experiments employed FRET as an alternative and more sensitive method for assessing degranulation patterns of primary granule serine proteases.

FRET is a mechanism describing energy transfer between 2 light sensitive molecules (chromophores). This is a protocol using spectrofluorometric measurement of neutrophil serine protease activities either on neutrophil surfaces or biological fluids using extremely sensitive Abz-peptidyl-EDDnp FRET substrates that fully discriminate between the 3 human neutrophil serine proteases. Specific Abz-peptidyl-EDDnp substrates have been formulated for each neutrophil protease. (Abz=fluorescent group and EDDnp is the quenching group acting as a donor/quenching pair at either end if the peptides). On cleavage and moving away from the quencher the Abz bearing peptides fluorescence can be measured on a microplate spectrofluorometer.

To determine the extent of NE activity in extracellular supernatants, HC and CF neutrophils were isolated and either unstimulated (time=0 min) or stimulated with TNFα (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) for 5, 10 or 20 min at 37°C, n=4 individuals per group. Extracellular supernatants were collected at each time point in the absence of protease inhibitor mix and loaded on a 96-well plate. Fluorescence substrate for human NE (Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp, 3230-v, Peptide Institute, Inc.) was added to each well immediately prior to plate reading at 320-420nm set at 28°C for 1h. The standard curve was obtained from NE standards of increasing concentrations (0, 2.75, 5, 10, 15, 30, 60 and 120 nM) and the slope of the curve was used to extrapolate the unknown NE concentrations. NE activity in extracellular supernatants was quantified by FRET analysis as previously illustrated (Kormaz et al, 2008) (228).
Figure 3.4 displays a graph of NE activity within extracellular supernatants obtained from CF neutrophils compared with HC cells with the difference being significant at 5 and 10 min post stimulation (p=0.03 and p=0.03, respectively).

The relative change in enzymatic activity for HC and CF neutrophil populations has been also calculated, and whilst there is no statistically significant difference, HC neutrophils display a gradual increase in extracellular NE activity in response to stimulation with TNFα and fMLP, whilst CF neutrophils display a sudden increase in NE activity which plateaus off with increasing duration of stimulation confirming priming of CF neutrophils.

In conclusion, NE activity is increased in CF neutrophil extracellular supernatants compared with healthy control cells in response to TNFα/fMLP activation. This is a pertinent finding as it underpins one of the key cellular abnormalities at the core of the CF airway. Previous studies have reported low levels of antiproteases in CF airway samples and in combination with the present study, this corroborates the imbalance of protease/antiprotease balance in vivo in favour of neutrophil serine proteases further fuelling the sustained cycle of inflammation and respiratory tissue damage characteristic of CF. Furthermore, the antiprotease AAT is cleaved and inactivated in the CF airways by Pseudomonas elastase (258), as are SLPI (259) and elafin (180).
Figure 3.4: NE activity in CF extracellular supernatants determined by FRET analysis is increased in response to TNFα/fMLP.

HC and CF circulating neutrophils were either unstimulated (time 0) or stimulated with TNFα (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C. Extracellular supernatants were collected at 0, 5, 10 and 20 min. Quantification of NE activity was carried out using the spectrophotometer (SpectraMax® multi-mode plate reader, Molecular Devices) at a wavelength of 320-420nm at 28°C. Statistical significance was calculated by Student’s t-test, where a p value ≤ 0.05 was regarded statistically significant and shown as mean ±SEM, (n=4 individuals per group).
3.4 Excessive Cathepsin G activity in CF extracellular supernatants analysed by FRET

In addition to NE, Cath G is a second serine protease of the chymotrypsin family also stored in primary granules of neutrophils. We sought to establish Cath G activity in neutrophil extracellular supernatants isolated from HC and CF circulating cells.

To determine the extent of Cath G activity in extracellular supernatants, HC and CF neutrophils were isolated and either unstimulated (time=0 min) or stimulated with TNFα (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) for 10 min at 37°C, (n=4 individuals per group) as the significant difference was observed at 5 and 10 min post stimulation in the NE activity FRET assay in the preceding experiment. Extracellular supernatants were collected at each time point in the absence of protease inhibitor mix and loaded on a 96-well plate. Fluorescence substrate for human Cath G (Abz-Glu-Pro-Phe-Trp-Glu-Asp-Gln-EDDnp, 3231-v, Peptide Institute, Inc.) was added to each well immediately prior to plate reading at 320-420nm set at 28°C for 1h. The standard curve was obtained from Cath G standards of increasing concentrations (0, 1.875, 3.75, 7.5, 15, 30, 40, and 60) and the slope of the curve was used to extrapolate the unknown Cath G concentrations. Cath G activity in extracellular supernatants was quantified by FRET analysis as previously described (228).

Figure 3.5 displays a graph of Cath G activity within extracellular supernatants obtained from CF neutrophils compared with HC cells with the difference being significant at 10 min post stimulation (p=0.02). Once again this significant difference is seen at an earlier time point compared to primary granule release detection by Western blot as FRET is a more sensitive modality.

In conclusion, we have confirmed increased MPO and BPI release as well as increased NE and Cath G activity by CF neutrophils compared with HC cells. The cellular mechanism triggering dysregulated degranulation of primary granules by CF neutrophils is incompletely understood, thus the next experiments were designed in order to investigate the underlying mechanism.
Figure 3.5: Cath G activity in CF extracellular supernatants determined by FRET analysis is increased in response to TNFα/fMLP.

HC and CF circulating neutrophils were either unstimulated (time 0) or stimulated with TNFα (1 ng/10⁷ cells)/fMLP (100 ng/10⁷ cells) at 37°C. Extracellular supernatants were collected at 0 and 10 min. Quantification of Cath G activity was carried out using the spectrophotometer (SpectraMax® multi-mode plate reader, Molecular Devices) at a wavelength of 320-420nm at 28°C. Statistical significance was calculated by Student’s t-test, where a p value ≤ 0.05 was regarded statistically significant and shown as mean ±SEM, (n=3 individuals per group).
3.5 Increased Rac2 activation in CF

3.5.1 Increased Rac2 activity of CF neutrophils analysed by Western blot

In preceding experiments we have confirmed excessive release of MPO and BPI, in addition to excessive NE and Cath G extracellular activity by CF neutrophils. Thus far we have not addressed the underlying molecular basis for this enhanced neutrophil primary granule degranulation. As our initial degranulation experiment using PMA stimulation failed to show any significant difference between CF and HC neutrophils in assessing degranulation activity, we did not continue to use PMA stimulation in subsequent experiments. In this set of experiments TNFα and fMLP were employed as neutrophil activators.

To investigate Rac2 activity of neutrophils following activation as a cause for increased degranulation of primary granules, HC and CF circulating neutrophils were isolated and then either unstimulated (time=0 min) or stimulated with TNFα (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) for 40 sec, (n=3 individuals per group). Neutrophil lysates were obtained after 40 sec stimulation at 37°C. The Abcam Rac activation kit (catalogue # ab 139586) was used to purify active Rac2 from cell extracts via a batch affinity purification step using GST-PAK-CRIB. This exploits the selective interaction of GST-PAK-CRIB with the active Rac-GTP conformation.

The total and active GTP-bound Rac2 content in neutrophil lysates was detected by immunoblotting using a rabbit polyclonal anti-Rac2 antibody (Cell Signalling Technology Rac1/2/3 primary antibody, 1:1000 dilution). Existing data has demonstrated that human neutrophils preferentially express Rac2 rather than Rac1 (260). Conversely, Rac1 and Rac3 are expressed in a wide variety of tissues (261).
We first explored Rac2 activation in unstimulated cells from HC and CF. Figure 3.6 (A) displays a representative Coomassie blue stained SDS PAGE demonstrating equal cell loading. Figure 3.6 (B) displays a representative Western blot showing equal total Rac2 content in HC and CF neutrophil lysates. Figure 3.6 (C) demonstrates increased active Rac2 in CF at baseline (unstimulated cells). Densitometry values of immunoblots of 4 separate experiments (Figure 3.6 (D) n=4) revealed increased Rac2 activation in CF at baseline compared with HC neutrophil lysates (p=0.03). The significant difference observed in resting neutrophils likely represents cellular mechanisms *in vivo*. 
Figure 3.6: Active Rac2 as a percentage of total Rac2 determined by Western Blot analysis is increased in CF neutrophils at baseline.

HC and CF circulating neutrophils were unstimulated (time 0) and neutrophil lysates were obtained. (A) Coomassie blue stained gel of resting HC and CF neutrophil whole cell lysates confirming equal cell numbers used per reaction. (B) and (C) Representative Western blots of neutrophil lysates probed with anti-Rac2 antibody (Cell Signalling Technology, 1:1000 dilution), displaying total (B) and active GTP-bound Rac2 (C). The molecular weight (kDa) is present on the left. (D) Quantification of immunoband intensity was carried out using the Image Lab® software.

Densitometry values for active Rac2 were divided by total Rac2 for resting cells at time point 0 min, and data were normalised to HC time 0. Statistical significance was calculated by Student’s t-test, (n=4 individuals per group).
Of particular interest was to compare Rac2 activation post stimulation to see whether levels were further enhanced in CF cells compared to HC.

Figure 3.7 (A) displays a representative Coomassie blue stained SDS PAGE demonstrating equal cell loading. Figure 3.7 (B) displays a representative Western blot showing equal total Rac2 content in HC and CF neutrophil lysates 40 sec post TNFα and fMLP stimulation. Figure 3.7 (C) demonstrates increased active Rac2 in CF at 40 sec post stimulation compared to HC neutrophil lysates. Densitometry values of immunoblots of 5 separate experiments (Figure 3.7 (D) n=5) revealed significantly increased Rac2 activation in CF at 40 sec post TNFα and fMLP activation compared with HC neutrophil lysates (p=0.004).

Benard et al, (229) demonstrated peaks in Rac2 activity between 30 sec and 1 min following stimulation with fMLP and our results are in keeping with this.

In conclusion, there is increased Rac2 activation in CF neutrophils compared with HC cells both at rest and following TNFα/fMLP stimulation. This novel finding is of specific interest in comprehending the pathogenesis of CF bronchiectasis which is caused by excessive oxidative damage.
Figure 3.7: Active Rac2 as a percentage of total Rac2 determined by Western Blot analysis is increased in CF neutrophils in response to TNFα/fMLP. Healthy control (HC) and CF circulating neutrophils were stimulated with TNFα (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) at 37°C for 40 sec and neutrophil lysates were obtained. (A) Coomassie blue stained gel of resting HC and CF neutrophil whole cell lysates demonstrating equal cell numbers used per reaction. (B) and (C) Representative Western blots of neutrophil lysates probed for anti-Rac2 antibody (Cell Signalling Technology, 1:1000 dilution), displaying total (B) and active GTP-bound Rac2 (C). Molecular weights (kDa) are indicated on the left. (D) Quantification of immunoband intensity was carried out using the Image Lab® software. Active Rac2 was expressed as a percentage of total Rac2. Statistical significance was calculated by Student’s t-test, (n=5 individuals per group).
3.6 Increased degranulation of primary granules in HC neutrophils following treatment with CFTR inhibitor

CFTRinh-172 is a selective inhibitor of CFTR that is an acceptable CF scientific model and has been proven to mimic the CF intrinsic impairment in human tracheal epithelial cells grown in primary culture, as well as native neutrophils in suspension (147). CFTRinh-172 was dissolved in DMSO diluted from a 1:1000 stock and therefore we used DMSO alone at this concentration as the control.

In order to assess if increased primary granule degranulation is intrinsic due to a lack of CFTR function, we isolated HC neutrophils and treated them with 10 µM CFTRinh-172 for 15 min at room temperature. We used HC neutrophils treated with 0.1% (v/v) DMSO for 15 min at room temperature as the vehicle control for this experiment. Neutrophils were centrifuged following the 15 min treatment period and extracellular supernatants were collected and analysed for degranulated proteins.

MPO was detected by Western blot analysis using a rabbit polyclonal primary antibody (Novus Biologicals 1:1000 dilution). Figure 3.8 (A) displays a representative Coomassie blue stained SDS PAGE of unstimulated whole cell lysates post treatment with either the DMSO or CFTRinh-172 depicting equal protein loading confirming equal cell numbers per reaction. Figure 3.8 (B) displays a representative blot showing increased MPO release from CFTRinh-172 treated neutrophils compared to HC cells treated with the DMSO vehicle control. Densitometry values from 3 separate experiments (Figure 3.8 (C), n=3 individuals per group) revealed significantly elevated extracellular MPO levels from HC neutrophils treated with CFTRinh-172 compared with cells treated with DMSO (p=0.01).
Figure 3.8: MPO release determined by Western blot analysis is increased in HC neutrophils in response to CFTRinh-172 treatment.

HC circulating neutrophils were either exposed to DMSO 0.1% (v/v) or 10µM CFTRinh-172 for 15 min at room temperature. Following this, extracellular supernatants were collected. (A) Coomassie blue stained gel of DMSO or CFTRinh-172 treated HC whole cell lysates. (B) Representative Western blot of extracellular supernatants probed for MPO using a rabbit polyclonal anti-MPO primary antibody. The molecular weight (kDa) markers are present on the left. (C) Quantification of immunoband intensity was performed using Image Lab software (DMSO values were set as 1 and other data normalised to DMSO control). Statistical significance was calculated by Student's t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ±SEM, (n=3 individuals per group).
In addition to MPO, we assessed the level of BPI release as another marker of primary granule degranulation in response to CFTRinh-172 treatment. Once again, we isolated HC neutrophils and treated them with either 10 µM CFTRinh -172 or 0.1% v/v DMSO for 15 min at room temperature. Extracellular supernatants were collected post incubation and analysed for degranulated proteins. BPI was detected as before by immunoblotting using a mouse monoclonal primary antibody. Figure 3.9 (A) displays a representative Coomassie blue stained SDS PAGE of unstimulated whole cell lysates post treatment with either DMSO control or CFTRinh-172 depicting equal protein loading once again confirming equal cell numbers per reaction. Figure 3.9 (B) displays a representative blot showing increased BPI release from CFTRinh-172 treated neutrophils compared to HC cells treated with DMSO vehicle control. Densitometry values from 4 separate experiments (Figure 3.9 (C), n=4 individuals per group) revealed significantly elevated extracellular BPI levels from HC neutrophils treated with CFTRinh-172 compared with cells treated with DMSO (p=0.03).
Figure 3.9: BPI release determined by Western blot analysis is increased by HC neutrophils in response to CFTRinh-172 treatment.

HC circulating neutrophils were either exposed to DMSO 0.1% (v/v) or 10µM CFTRinh-172 for 15 min at room temperature. Following this exposure extracellular supernatants were collected. (A) Coomassie blue stained gel of DMSO or CFTRinh-172 treated HC whole cell lysates. (B) Representative Western blot of extracellular supernatants probed for BPI using a mouse monoclonal primary antibody (Santa Cruz ® Biotechnology, 1:1000 concentration). Note supernatants were loaded on a contiguous gel. The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was performed using Image Lab software (DMSO values were set as 1 and other data normalised to DMSO control). Statistical significance was calculated by Student’s t-test, a p value ≤ 0.05 was deemed statistically significant (n=4 individuals per group).
3.7 Increased Rac2 activation by HC neutrophils treated with CFTRinh-172

We have confirmed excessive primary granule release of HC cells following chloride channel inhibition using CFTRinh-172. We next assessed the effect of CFTR inhibition on Rac2 activation as follows: HC circulating neutrophils were isolated and then either treated with 0.1% (v/v) DMSO or 10µM CFTRinh-172 for 15 min at room temperature. Cells were subsequently washed with PBS and neutrophil lysates were obtained for each treatment. Once again the Abcam Rac activation kit (catalogue # ab 139586) was used to purify active Rac2 from cell extracts via a batch affinity purification step using GST-PAK-CRIB.

The total GDP-bound Rac2 and active GTP-bound Rac2 content in neutrophil lysates was detected by immunoblotting using a rabbit polyclonal anti-Rac2 antibody. Figure 3.10 (A) displays a representative Coomassie blue stained SDS PAGE demonstrating equal cell loading. Figure 3.10 (B) displays a representative Western blot showing equal total Rac2 content in HC neutrophil lysates. Figure 3.10 (C) demonstrates increased active Rac2 in HC cells treated with CFTRinh-172 compared with HC cells treated with DMSO. Densitometry values of immunoblots of 4 separate experiments (Figure 3.10 (D) n=4) revealed increased Rac2 activation in HC cells treated with CFTRinh-172 compared to untreated cells (DMSO) (p=0.03).

Collectively these results confirm increased Rac2 activation and dysregulated degranulation by HC neutrophils treated with CFTRinh-172 indicating that enhanced degranulation by CF neutrophils is an intrinsic defect.
Figure 3.10: Active Rac2 as a percentage of total Rac2 determined by Western Blot analysis is increased in HC neutrophils in response to treatment with CFTRinh-172.

HC circulating neutrophils were either treated with 0.1% (v/v) DMSO or 10µM CFTRinh-172 for 15 min at room temperature, and neutrophil lysates were obtained following this exposure. (A) Coomassie blue stained gel of DMSO and CFTRinh-172 treated cells confirming equal cell number per treatment. (B) and (C) Representative Western blots of neutrophil lysates probed for anti-Rac2 antibody (Cell Signalling Technology, 1:1000 dilution), displaying total (B) and active GTP-bound Rac2 (C). Molecular weights (kDa) are denoted on the left. (D) Quantification of immunoband intensity was carried out using the Image Lab® software (Densitometry values for active Rac2 were divided by total Rac2 for cells per treatment. (DMSO values were set as 1 and other data normalised to DMSO control). Statistical significance was calculated by Student’s t-test, (n=4 individuals per group).
In order to confirm the effect of CFTR inhibition on granule release in the presence of physiological stimuli, degranulation assays were subsequently carried following treatment of HC cells with DMSO (D) or CFTRinh-172 (CF). The neutrophils were then washed free of inhibitor and re-suspended in PBSG containing TNFα and fMLP. Extracellular supernatants were harvested at 5, 10, and 20 min post stimulation.

Results are depicted in Figure 3.11 and panel (A) displays representative Coomassie blue stained SDS PAGE of whole cell lysates post treatment with either DMSO (D) or CFTRinh-172 (CF) depicting equal protein loading and confirming equal cell numbers per reaction. Figure 3.11 (B) displays a representative Western blot showing no significant difference in MPO release between DMSO and CFTRinh-172 treated neutrophils for all time points - 5, 10 and 20 min post stimulation. Densitometry values from 4 separate experiments (Figure 3.11 (C), n=4 individuals per group) revealed no significant difference in extracellular MPO levels from HC neutrophils treated with DMSO compared to those treated with CFTRinh-172 following 5, 10 and 20 min stimulation (p=0.3, p=0.4, p=0.3 respectively). Collectively, these results suggest that upon removal of the CFTR inhibitor HC neutrophils reverted back to normal degranulation function in the absence of CFTR pharmacological inhibition, corroborating further our results that this is an intrinsic defect.
Figure 3.11 No difference in MPO release determined by Western blot analysis between CFTRinh-172 and DMSO treated HC cells.

HC circulating neutrophils were exposed to DMSO 0.1% (v/v) or 10µM CFTRinh-172 for 15 min at room temperature then the neutrophil pellets were re-suspended in TNFα and fMLP and supernatants collected at 5, 10 and 20 min post stimulation. (A) Coomassie blue stained gel of DMSO or CFTRinh-172 treated HC whole cell lysates at designated time points. (B) Representative Western blot of extracellular supernatants probed for MPO using a rabbit polyclonal primary antibody (Novus Biologicals®, 1:1000 concentration). Note supernatants were loaded on a contiguous gel. The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was performed using Image Lab software. Statistical significance was calculated by Student’s t-test, a p value ≤ 0.05 was deemed statistically significant (n=4 individuals per group).
In order to support results depicted in Figure 3.11, ensuing experiments analysed BPI release from HC neutrophils treated with DMSO and CFTRinh-172, and then subsequently washed free of the inhibitor. Figure 3.12 (A) displays representative Coomassie blue stained SDS PAGE of whole cell lysates post treatment with either DMSO (D) or CFTRinh-172 (CF) and subsequently washed free of the inhibitor and solvent depicting equal protein loading and confirming equal cell numbers per reaction. Figure 3.12 (B) displays a representative Western blot showing no significant difference in BPI release between cells free of DMSO or CFTRinh-172 for all time points- 5, 10 and 20 min post stimulation. Densitometry values from 4 separate experiments (Figure 3.12 (C), n=4 individuals per group) revealed no significant difference in extracellular BPI levels from HC neutrophils pre-treated with DMSO compared to those pre-treated and washed free of CFTRinh-172 following 5, 10 and 20 min stimulation (p=0.4, p=0.4, p=0.4 respectively), once again suggesting that HC neutrophils reverted to normal in the absence of CFTR pharmacological inhibition.
HC circulating neutrophils were exposed to DMSO 0.1% (v/v) or 10μM CFTRinh-172 for 15 min at room temperature, then the neutrophils pellets were re-suspended in TNFα and fMLP and supernatants collected at 5, 10 and 20 min post stimulation. (A) Coomassie blue stained gel of DMSO or CFTRinh-172 treated HC whole cell lysates at designated time points. (B) Representative Western blot of extracellular supernatants probed for BPI using a mouse monoclonal primary antibody (Santa Cruz Biotechnology®, 1:1000 concentration). Note supernatants were loaded on a contiguous gel. The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was performed using Image Lab software. Statistical significance was calculated by Student’s t-test, a p value ≤ 0.05 was deemed statistically significant (n=4 individuals per group).

Figure 3.12 No difference in BPI release determined by Western blot analysis between CFTRinh-172 and DMSO treated HC cells.
3.8 Discussion

The objectives of chapter 3 were 3 fold: to explore neutrophil primary granule degranulation patterns in CF, to evaluate the activity of the neutrophil serine proteases NE and Cath G in exocytosed supernatants in CF and also to establish the signalling mechanisms involved in aberrant degranulation of primary granules.

Neutrophils are critical inflammatory cells and can cause tissue damage in many diseases and disorders. Neutrophils are initially produced in the bone marrow and migrate through the circulation to the site of insult or injury or inflammation in response to chemotactic signals (262).

Neutrophil degranulation plays a crucial role in innate defence by releasing potent antimicrobial contents of primary, secondary and tertiary granules (83). Degranulation occurs in a highly controlled receptor-coupled manner. Excessive release of neutrophil granule contents can destroy a wide range of extracellular matrix components such as elastin (111), collagen (112), fibronectin (113) and laminin (114). With regards to chronic infection in CF, neutrophils are unable to effectively control bacterial colonisation, despite massive infiltration of these inflammatory cells into the lung parenchyma (57).

Recruited neutrophils release NE amongst other serine proteases which overwhelm the anti-proteases of the lung and cause tissue destruction referred to as “prolonged endobronchial protease activity” (263) leading ultimately to bronchiectasis and obstructive airways disease. Interestingly hypoxia has been proven to augment neutrophil degranulation and enhance the potential for damage to the respiratory airway epithelium (264), a serious consequence in CF patients with chronic respiratory failure.

In this chapter, we have demonstrated that CF circulating neutrophils release excessive levels of MPO, BPI, NE and Cath G determined by Western blot and specific FRET analysis, when compared with HC cells following stimulation with the physiological stimulus combination TNFα/fMLP (p< 0.05).
This is further supported by previous work by Witko-Sarsat et al (1996) (96) who determined increased extracellular MPO release in CF homozygotes by ELISA and supported further by Taggart et al (2000) (155) who confirmed excessive NE release from CF neutrophils in response to pre-incubation with CF BALF and subsequently TNFα and IL-8 when compared with neutrophils from control subjects and bronchiectatic patients.

Interestingly, Brockbank et al (2005) demonstrated that isolated peripheral blood neutrophils from patients with CF spontaneously released more NE than control neutrophils and that spontaneous generation of superoxide was significantly higher from CF neutrophils after recovery from infective exacerbation compared to HC cells indicating that CF exacerbations did not modulate neutrophil function (246).

Excessive MPO levels are found in BALF of patients with ARDS (265, 266); Park et al (2013) (266) established that serum MPO concentrations were significantly related to accelerated decline in FEV₁. MPO has also been found to have a positive correlation with smoking status and may be an early marker of systemic inflammation (267). Moreover, excessive MPO activity is associated with high levels of oxidative stress (93) and has been advocated as potentially a useful biomarker in cardiovascular disease risk stratification.

Ensuing experiments used specific FRET analysis to confirm that there was increased NE activity by CF neutrophils compared with HC neutrophils in response to TNFα and fMLP. The vicious cycle of NE induced inflammation causes upregulation of IL-8 from epithelial cells (117) and LTB₄ from macrophages (268) creating a neutrophil chemo attractant milieu, thereby drawing more neutrophils onto the epithelial surface where they are easily activated to release more NE by cytokines IL-8 and TNFα (155).

Therapeutic strategies aimed at preventing the NE-driven pulmonary destruction in CF have been surmised based on the theory of enhancing the level of anti-proteases present to counter-act NE or by lowering the NE burden in the lung (181, 269).
Following on from this we utilised specific FRET analysis again to confirm that there was increased Cath G activity by CF neutrophils compared with HC cells in response to TNFα/fMLP. Cath G inhibits the actions of macrophages in clearing apoptotic cells from CF airways, leading to an accumulation of neutrophil necrosis thereby further exacerbating the uncontrolled release of proteases into the lung (228).

Cath G in CF BALF has been proven to possess the highest potency of all three neutrophil serine proteases to degrade surfactant protein A (SP-A), a peptide that allows microbial clearance by macrophages, resulting in reduced macrophage phagocytic response and prolonging bacterial survival (270). Despite its abundance in CF, Cath G has been found to interfere with clearance of *P. aeruginosa* from mouse lungs based on a reduction in retrieval of bacteria from Cath G deficient murine lungs due to possible disturbance of airway defences demonstrating that proteases other than NE have roles in the pathogenesis of suppurative lung diseases (249).

In light of our findings of enhanced MPO,BPI, NE and Cath G release and activity by CF neutrophils, the final experiment in this chapter sought to explore if increased Rac2 activation could be an underlying signalling mechanism for aberrant primary granule degranulation in CF. Rac2 is identified as a crucial regulator of neutrophil primary granule exocytosis in mice (208) and in addition to this it plays an important role in regulating NADPH oxidase activation in response to specific signalling pathways (256). Whilst very little is currently known about Rac2 and inflammatory airways disease, one case report described a male infant expressing a dominant negative form of Rac2 suffering from severe bacterial infections, delayed wound healing and culminating in severe life-threatening infections (205, 271) and necessitating neutrophil transfusion from healthy donors to resolve abscesses and surgical wounds. Associations have been described between haplotypes of the Rac2 gene and autoimmunity, specifically increased susceptibility to multiple sclerosis (MS) and early onset Crohn’s disease (272). Thus, therapeutic options aimed at inhibiting or reducing Rac2 expression and/or activity may be useful in this context.
In the final experiment we verified that there was significantly increased Rac2 activity in CF neutrophils both at baseline and 40 sec following exposure to the physiological stimulus combination TNFα and fMLP compared to HC cells (p=0.03 and p=0.004 respectively).

We have also demonstrated the effect of CFTRinh-172 on circulating HC neutrophils, with excess extracellular MPO and BPI release observed by HC cells pre-treated with the CFTRinh-172 compound. Moreover, this resulted in increased Rac2 activation also once again indicating that defective Cl\(^{-}\) channel function is associated with this anomaly. In addition, HC neutrophils reverted to normal in the absence of pharmacologic CFTR inhibition, with no difference in primary granule release between DMSO and CFTRinh-172 treated neutrophils observed.

Collectively, these results illustrate that CFTR Cl\(^{-}\) channel function is important for efficient degranulation and that excessive primary granule release is an intrinsic defect. Pohl et al (2014) (146) observed that pre-treatment of HC cells with CFTRinh-172 caused impaired secondary and tertiary granule degranulation which is in keeping with our finding that aberrant neutrophil degranulation in CF is intrinsic in origin.

In summary, the experiments conducted in this chapter confirm that there is dysregulated primary granule degranulation in CF. Additionally, excessive Rac2 activation has been recognised as a possible molecular basis for the aberrant neutrophil degranulation of primary granules in CF. This anomalous activity could lead to exuberant release of proteolytic enzymes *in vivo* and propagation of pro-inflammatory cytokines resulting in persistent architectural lung damage, and the development of bronchiectasis and airways disease in CF.

One further consequence of enhanced neutrophil degranulation is the development of anti-neutrophilic cytoplasmic antibodies (ANCA) and this phenomenon has been described in both CF (211) and AATD patients (213) and this forms the basis of our experiments in chapter 4.
Chapter 4

The Consequence of Excessive Primary Granule Release by Neutrophils of Individuals with Cystic Fibrosis
4.1 Introduction

An autoantibody is an antibody which is produced by the immune system that is directed against one or more of the individuals’ own proteins. These autoantibodies mistakenly target and react with a person’s own tissues or organs. Autoantibodies largely develop when a person’s immune system fails to distinguish between self and non-self (273). This is a hallmark of autoimmune disorders, however; antibodies directed against self-antigens have also been described in non-autoimmune disorders such as cancer and conditions causing extensive tissue damage (273).

Antinuclear cytoplasmic antibodies (ANCA) are antibodies implicated in the pathogenesis of necrotising vasculitis affecting small vessels. They also serve as serological markers for a wide range of vasculitic diseases such granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MP) (274-276).

The main antigenic targets for ANCA are MPO (276), PR3 (277) and less frequently lactoferrin (278) and NE (279). Autoantibodies against MPO and PR3 are known to activate primed neutrophils in vitro through membrane expressed antigens and FcγRII (215, 216). ANCA signalling can cause neutrophil degranulation as well as free radical and enzymatic release which may lead to tissue injury in vivo (217). Non-MPO p-ANCA are commonly identified in patients with CF, particularly among those with bacterial airway infections (210, 211).

In the preceding chapter we demonstrated increased extracellular release of MPO, BPI, NE and Cath G as markers of primary granule degranulation, and we next sought to determine the potential effects this may have on autoantibody production.

ANCAs may be subdivided by their staining pattern visualised by immuno-fluorescence. The p-ANCA pattern is caused by autoantibodies directed against MPO, Cath G, NE, lysozyme or lactoferrin. Although these proteins are located in neutrophil cytosolic granules, autoantibodies against these antigens produce perinuclear staining because these antigens diffuse away from the granules and become partially bound to negatively charged component of the nucleus during neutrophil preparation causing a peri-nuclear staining pattern (280).
cANCA exhibit a diffusely granular cytoplasmic immunofluorescence staining pattern due to binding of ANCAs throughout the neutrophil cytoplasm with the most common target protein-PR3 seen in patients with GPA (281).

In addition to investigating the development of anti-MPO and anti-PR3 antibody generation in this chapter, the presence of double negative samples led us to examine anti-BPI antibody levels in CF compared with HC and subsequently quantify plasma BPI levels in CF and HC individuals. BPI expression has been found on the neutrophil cell membrane (282) and is amenable to direct binding to anti-BPI antibodies which can cause neutrophil activation potentially leading to tissue damage as seen with anti-PR3 and anti-MPO autoantibodies.

Subsequent to our assessment of ANCA in HC and CF, we explored the effect of Ivacaftor treatment on autoantibody generation in CF individuals receiving this medication. Ivacaftor is a small molecular weight oral drug specifically designed to treat patients who are either homozygous or heterozygous for the G551D mutation. Approximately 14.8% of Irish CF individuals have at least one copy of the G551D mutation leading to the nickname the “Celtic Gene”, whilst 91.7% have at least one copy of the ΔF508 mutation (2).

Ivacaftor functions as a Cl⁻ channel potentiator thereby restoring the function of the mutant CFTR protein (76). Ivacaftor has been proven to result in sustained improvement in pulmonary function as well as reducing frequency of exacerbations and improving weight and sweat Cl⁻ concentration. Dysregulated degranulation of secondary and tertiary neutrophil granule degranulation by CF neutrophils was corrected 1 year post Ivacaftor therapy in vivo (146).
4.2 BPI ANCA

BPI is a single chain, cationic, boomerang-shaped protein with two functionally important domains (283). The N-terminal half of the protein is comprised of basic residues, which possesses the antibacterial and anti-endotoxic activities of the molecule, whereas the hydrophobic (anion) C-terminal half is required for opsonisation (283). In addition, apolar lipid-binding pockets are present in each half of the molecule which are responsible for interactions with LPS acyl chains (253). It plays an integral role in killing gram negative bacteria (GNB) and in neutralisation of bacterial endotoxin. In carrying out this function, BPI can regulate the delivery of complexes which contain endotoxin and bacterial outer membrane proteins to antigen presenting cells (284). Endotoxins are unique glycolipids of the GNB outer membrane that can initiate potent pro-inflammatory responses including release of pro-inflammatory cytokines such as TNFα and IL-1 as well as activating the complement cascade, coagulation factors and fibrinolytic pathways. These effects form essential host defences in response to GNB but may also lead to a number of pathological phenomena such as mounting of fever and chills to fulminant sepsis syndrome when GNB infection is not adequately controlled (285).

The development of autoantibodies against BPI has been implicated as the immunological explanation for the findings in the studies described below, with a dampening down of the immune effect observed following treatment with corticosteroid therapy or ibuprofen:

1- Neutrophils from CF BALF were found to express increased levels of IgA Fc receptor (FcαR), and TNFα further enhanced FcαR expression as well as inducing the production of O₂⁻ residues in response to aggregated IgA and enhanced P. aeruginosa killing (286, 287). Thus the interactions between phagocytic host defence and mucosal IgA in the setting of the CF lung are exacerbated by TNFα inflammatory environment.

2- In the early 1980s an inverse correlation was described between immunoglobulin levels and the clinical course in patients with CF (288). Subsequent studies investigated the effects of chronic pulmonary inflammation as a result of sustained bacterial infection, on the clinical course of CF patients.
The potent antimicrobial activity of neutrophils against GNB is abolished by anti-BPI serum (98) thereby impairing bacterial killing and further propagating the cycle of repeated pulmonary infection. In light of this, we chose to investigate BPI ANCA in our CF population.
4.3 Consequence of BPI ANCA formation

Efforts have been made to discern how GNB exposure and BPI ANCA are generated. Epitope mapping of patients’ BPI ANCA has revealed in a subset of these individuals strong similarity of some BPI epitopes to specific outer membrane proteins of *E. coli* and *P. aeruginosa* (289, 290). As such, molecular mimicry may be a potential factor in initiating BPI autoantibody production. In addition to this, other possible mechanisms influencing normal host tolerance to BPI have been postulated as described below.

Extracellular accumulation of endotoxin and BPI during invasive GNB infection occurs mainly in tissue (291). Investigations using murine models pointed to immature dendritic cells (IDC) rather than macrophages as the targets of BPI coated endotoxin rich particles. IDCs are the most potent antigen-presenting cells in tissue (292). Enhanced delivery of BPI outer membrane blebs to IDCs suggests a link between innate immune recognition of endotoxin by BPI and the generation of adaptive immunity against a selection of integral bacterial surface antigens. Normal control of infection results in accumulation of endotoxin and release of BPI self-limiting events. However, when GNB infection and endotoxin exposure are chronic and sustained as in CF, and accompanied by neutrophil recruitment and activation, prolonged delivery of BPI coated GNB membrane elements to IDCs creates a milieu in favour of processing and presenting BPI by activated dendritic cells (293) leading to autoantibodies to BPI.

In addition to CF (210), ANCA against the neutrophil primary granule BPI as described in a variety of diseases of different aetiologies such as IBD (294), reactive arthritides (295) and transporter for antigen presentation (TAP) deficiency syndrome (296). In each of these conditions there has been a significant and prolonged exposure to GNB and endotoxin, as well as intense local recruitment and activation of neutrophils and extracellular release of BPI.
The presence of BPI ANCA has been associated with reduced pulmonary function in CF (212) and transporter for antigen presentation (TAP) deficient patients, as well as a higher inflammatory burden in IBD patients (290, 297). *In vitro* experiments indicated that anti-BPI autoantibodies can activate PMN and potentially exacerbate the inflammatory cycle (298, 299). Additionally, BPI ANCA are associated with chronic inflammatory status in RA patients and were found to form a positive link to bronchial disease in this group of patients (300).

Once BPI ANCA are formed, BPI mediated bacterial killing is impaired as well as opsono-phagocytosis of GNB. This leads to further neutrophil activation by cross-linking BPI on their cell surface (298, 299) as well as labelling BPI/endotoxin complexes for processing via an Fc receptor mediated pathway (301). These aberrancies in BPI function further promote a pro-inflammatory environment and further exacerbate autoimmunity against BPI (302).

It is important to determine the possible effect anti-BPI antibodies exert on BPI function. It has been reported previously that a rabbit polyclonal anti-BPI IgG could partly inhibit the bactericidal activity of BPI (303). Similarly, a small study assessed neutrophil mediated killing of *P. aeruginosa* by BPI ANCA and found that sera from BPI ANCA positive patients demonstrated significantly impaired bacterial killing when compared with normal HC serum and BPI ANCA negative CF serum (304) further confirming its role in infection suppression and control. Indeed this defect is evident in early life in premature neonates showing impaired ability to mobilise BPI contributing to the susceptibility to infection with GNB (305).

We chose to focus on analysing BPI ANCA expression in HC and CF plasma and BALF as well as quantifying the circulating and airway levels of BPI due to its strong association with bacterial killing particularly in the context of chronic *P. aeruginosa* colonisation.
4.4 Clinical correlation of BPI ANCA in CF

Zhao et al 1995 (306) demonstrated BPI as an important antigen for ANCA autoantibodies in vasculitis and found that anti-BPI positivity correlated with male gender, a mean patient age of 60.4 years, and clinical diagnoses ranging from organ limited vasculitis to widespread systemic vasculitis in a non-CF cohort (306). It is as yet unclear if these autoantibodies are merely serological markers for this group of diseases or whether they play a more specific role in the pathogenesis. Subsequently, this research group investigated BPI ANCA incidence in the CF population, discovering that BPI IgG and BPI IgA ANCA were found in 91 and 83% of CF patients respectively. Anti-BPI titres were directly proportional to the severity of airway destruction and severity of pulmonary compromise (210).

More recently BPI ANCA have been shown to correlate better with reduced pulmonary function and long term patient prognosis than anti-\textit{P. aeruginosa} serology (307) in the setting of CF. A Danish research group described the use of BPI ANCA as a biomarker of a deleterious host-pathogen interaction in CF, with IgA and IgG BPI ANCA levels showing an overall reduction in CF patients post endoscopic image guided sinus surgery and antibiotic irrigation (308) which resulted in a net reduction of bacterial load in the nasal passages.

With advancing age, CF patients are at increased risk of developing extra-pulmonary complications such as gastrointestinal (309) and hepatobiliary complications (310) as well as diabetes (311), vasculitis and CF related arthropathy (312). Indeed, ANCA have been detected in both gastrointestinal and hepatobiliary disorders such as IBD (313) and primary sclerosing cholangitis (PSC) (314) and these observations have prompted the theory that perhaps the extra-pulmonary complications arising in CF in later life may be due to the development of anti-BPI autoantibodies. As yet, it has not been elucidated whether this correlation represents an epiphenomenon or rather a reaction to gram-negative infection with resultant endotoxin release.
BPI ANCA correlated with lower FEV$_1$, chronic *P. aeruginosa* pulmonary infection and increased evidence of bronchiectasis and lung damage on HRCT in a cohort of Norwegian CF patients (315). As well as this, BPI ANCA levels were higher in CF individuals compared with HC, and higher again in the *P. aeruginosa* colonised CF subgroup. Interestingly, treatment with the macrolide antibiotic- azithromycin for a twelve week period did not lower BPI ANCA level in patients with CF indicating that ANCA generation is independent of clinical disease activity (316). In the present study the effect of treatment with Ivacaftor for patients with at least one copy of the *G551D* mutation on BPI ANCA was explored. A potential drawback of our project is that we do not have blood samples from this cohort of patients prior to the initiation of Ivacaftor therapy.
**Aim of this chapter**

In this chapter our aim was to comprehend the consequence of enhanced primary granule release by CF neutrophils.

We hypothesised that individuals with CF exhibit increased levels of anti-MPO, anti-PR3 and anti-BPI autoantibody levels as a result of increased primary granule release. We further envisioned that Ivacaftor therapy could impact upon autoantibody production.

To test this hypothesis, the following objectives were set:

1. To establish if there are increased autoantibodies directed against the contents of neutrophil primary granules in CF
2. To determine BPI levels in CF plasma and BALF
3. To explore the effect of Ivacaftor on autoantibody production
4.5 Investigation of ANCA in CF

4.5.1 Assessment of ANCA directed against neutrophil primary granule proteins in CF

Thus far we have demonstrated increased primary granule degranulation in CF compared with HC cells as well as confirming excessive Rac2 activation at rest and following activation of CF neutrophils with TNFα/fMLP compared to HC cells. This next set of experiments was designed to assess the presence of ANCA against neutrophil primary granule proteins in CF.

Firstly we assessed the presence of autoantibodies directed against MPO and PR3. For this, plasma samples were collected from non-smoking HC (n=35), CF (n=29) and CF individuals receiving Ivacaftor therapy (n=8). The Department of Clinical Immunology in Beaumont Hospital, Ireland carried out ELISA to evaluate autoantibodies in this cohort. Assessment of anti-MPO and PR3 autoantibodies was performed using the “Routine use of the Phadia® 250 for anti-MPO and anti-PR3 antibody testing” protocol established by the Clinical Directorate of Laboratory Medicine, Beaumont Hospital, Ireland (document no. LP-IMM-ICAP004, revision 3.6) as described in section 2.9.1. Positivity was set by the ELISA manufacturer to 3 standards deviations (SDs) above the mean of the HC individuals and was identified as > 5 IU/mL and >3 IU/mL for anti-MPO and anti-PR3 autoantibodies, respectively.

Figure 4.1 displays the plasma levels of anti-MPO autoantibodies detected in HC and CF individuals, revealing that there was no significant increase in anti-MPO autoantibodies in CF individuals compared with HC (p=0.09). There was also no difference detected between CF and CF+IVA groups (p=0.05). When comparing HC to CF+IVA we see a significant difference (p=0.03) which is unexpected as Ivacaftor therapy has been shown to preserve lung function and reduce exacerbation rate as well as maintaining BMI, in addition to correcting defective degranulation of secondary and tertiary granules and cytosolic homeostasis (146).
Figure 4.1: No increase in anti-MPO autoantibodies in CF individuals determined by ELISA.

Plasma samples from HC individuals (n=35), CF (n=29) and CF patients on Ivacaftor (CF+IVA) (n=8) were analysed for anti-MPO autoantibodies using the Phadia® 250 system. The dashed line represents the cut-off for anti-MPO antibody positivity set by the manufacturer at 5 IU/mL (3 SDs above the mean for healthy control individuals). Overall there was no significant difference in anti-MPO autoantibody titres detected between HC and CF (p=0.09) nor CF and CF +IVA (p=0.05). Statistical significance was calculated by Student’s t test, where a p-value of ≤ 0.05 was deemed statistically significant.
In addition to anti-MPO autoantibodies, the ensuing experiment investigated the presence of anti-PR3 autoantibodies, another neutrophil primary granule protein. Anti-PR3 autoantibody levels are demonstrated in Figure 4.2. Analysis confirmed that there was no significant difference in anti-PR3 autoantibody titres between CF and HC individuals \((p=0.46)\) nor between CF and CF+IVA \((p=0.45)\). Unexpectedly we see a difference when comparing HC and CF+IVA \((p=0.02)\) which may be attributed to the heterogeneity of the CF+IVA group.

We did not quantify plasma MPO and PR3 levels in our patient population in ensuing experiments as no autoantibodies to these proteins were detected.
Figure 4.2: No increase in anti-PR3 autoantibodies in CF individuals determined by ELISA.

Plasma samples from HC individuals (n=35), CF (n=29) and CF+IVA (n=8) were analysed for anti-PR3 autoantibodies using the Phadia® 250 system. The dashed line represents the cut-off for anti-PR3 antibody positivity set by the manufacturer at 3 IU/mL (3 SDs above the mean for healthy control individuals). There was no significant difference overall in anti-PR3 autoantibody titres detected between these HC and CF (p=0.46), nor CF and CF+IVA (p=0.45). Statistical significance was calculated by Student’s t test, where a p-value of ≤ 0.05 was deemed statistically significant.
Next we employed a specific ELISA for the detection of anti-BPI autoantibodies in the plasma of HC (n=35), CF (n=29) and CF individuals receiving Ivacaftor therapy (n=8). This technique exploits the immobilisation of antigen to a solid surface, and incubating the antigen coated surface with the specific anti-BPI antibody so that it can bind to the antigen, and linking this antibody to an enzyme conjugate (contains anti-human IgG antibodies, HRP linked). In the final step, TMB substrate is added that the enzyme can convert to a detectable signal. We employed this technique as only a small amount of antigen is required and it allows for the quick and simple analysis of multiple plasma samples on the same ELISA plate. The intensity of the colour change correlates with the concentration of the antigen-antibody complex and is measured by spectrophotometry at 650nm.

As demonstrated in Figure 4.3 the results obtained revealed significantly elevated anti-BPI autoantibody titres in CF individuals compared to HC (p=0.002). CF individuals receiving Ivacaftor treatment also exhibited significantly increased anti-BPI autoantibodies when compared to HC (p=0.0001). Interestingly, we found no difference between anti-BPI antibody levels in CF and CF individuals receiving Ivacaftor treatment (p=0.39), this perhaps is due to the wide variation in antibody levels between the two groups as well as the heterogeneity of the Ivacaftor treatment group (Table 2.10).
Figure 4.3: Increased circulating IgG BPI antibody level in CF individuals determined by ELISA.

Plasma samples from HC individuals (n=35), CF (n=29) and CF individuals receiving Ivacaftor therapy (n=8) were analysed for anti-BPI antibody level using the anti-BPI ELISA kit-ORG 523 (ORGENTEC®). Positivity was set by the manufacturer at a level of ≥ 10 U/ml. CF individuals had significantly higher circulating anti-BPI antibody levels compared with HC individuals (p=0.002). There was no difference between the levels of circulating anti-BPI antibody levels between CF and CF individuals receiving Ivacaftor treatment (p=0.39).
As BPI autoantibodies were present in significantly higher levels in CF plasma, we next wanted to evaluate circulating levels of BPI. We used a BPI ELISA to quantitatively determine circulating BPI levels in vivo. In plasma of healthy individuals BPI is present at levels less than 500pg/ml, which can increase up to ten-fold during an acute phase response (317). Figure 4.4 reveals significantly elevated circulating BPI levels in CF individuals (p=0.01) compared with HC individuals, however once again, there was no difference between CF and CF individuals on Ivacaftor treatment (p=0.23).

It is particularly surprising that there was no difference detected between CF individuals and CF individuals in receipt of Ivacaftor therapy as Ivacaftor has been shown to correct dysregulated degranulation of secondary and tertiary granules and cytosolic ion concentrations as well as improving bacterial killing (146). An explanation for this is perhaps due to the heterogeneity of the Ivacaftor treatment group, with large differences in patients’ baseline lung function; sputum cultures (Table 2.10) and associated co-morbidities as well as CF complications. There was certainly a subset of 4 patients who were either non-compliant or have failed to respond to Ivacaftor therapy in the conventional manner.
Figure 4.4: Increased circulating BPI level in CF individuals determined by ELISA.

Plasma samples from HC individuals (n=35) and CF (n=29) and CF individuals receiving Ivacaftor therapy (n=8) were analysed to determine the plasma BPI level using the human BPI ELISA kit (Hycult Biotech®). Plasma from CF individuals exhibit significantly increased BPI levels compared with HC individuals (p=0.01). There was no difference identified between circulating BPI levels of HC and CF individuals receiving Ivacaftor (p=0.42). There was also no significant difference between the CF patients and those CF individuals receiving Ivacaftor therapy (p=0.23).
Progressive pulmonary disease and chronic pulmonary infection result in secondary bronchiectasis; which drives neutrophil recruitment to the airways in CF (318). Therefore it was important to ascertain what is happening in BALF as a surrogate marker for CF airway cells. To complete our investigation, we set out to determine BPI concentrations in CF BALF (n=6) compared to HC individuals (n=4) and inflammatory controls (n=5) (including individuals with COPD (n=2) and those with non CF bronchiectasis (NCFB) (n=3)). We pooled BALF samples from patients with COPD and NCFB and classified these pooled samples as “inflammatory control” as we sought to investigate if BPI levels were elevated in other chronic inflammatory lung diseases.

We employed the same BPI ELISA as used for BPI measurement in plasma. Following a pilot run, the optimal dilutions were established to ensure our results were within the standard range. This included a 1 in 500 dilution of the CF BALF and aliquoting both HC and inflammatory control BALF neat onto ELISA plates.

Figure 4.5 demonstrates that CF BALF had significantly greater concentrations of BPI compared with HC BALF (p=0.0083). Interestingly, CF BALF exhibited greater levels of BPI compared to inflammatory control BALF (p=0.005) indicating that chronic inflammation alone is not causing BPI elevation but that this may be specific to CFTR dysfunction.
Figure 4.5: Increased BPI level in CF BALF determined by ELISA.

BALF samples from HC individuals (n=4) and CF (n=6) and inflammatory control individuals (n=5) were analysed to determine the BPI level using the human BPI ELISA kit (Hycult Biotech®) as previously. BALF from CF individuals exhibited significantly increased BPI levels compared with HC individuals (p=0.0083) and inflammatory controls which included pooled samples from COPD and NCFB patients (p=0.005). One way ANOVA was carried out to establish a difference between the three groups, and Student’s t-test was used to calculate the difference between 2 groups.
4.6 Discussion

The focus of this chapter was threefold; to explore if there were increased autoantibodies directed against neutrophil primary granule proteins in CF as well as determining the BPI levels in CF and HC plasma and BALF, in addition to exploring the effect of Ivacaftor treatment on the development of autoantibodies.

The Phadia® 250 system for anti-MPO and anti-PR3 antibody testing were utilised to determine the presence of IgG autoantibodies directed against neutrophil primary granule proteins in plasma samples of HC, CF and CF individuals receiving Ivacaftor therapy. These ELISA assays determined that there was no increase in anti-MPO and anti-PR3 autoantibody levels between HC and CF individuals, suggesting that CFTR dysfunction alone was insufficient to induce ANCA-positive vasculitis. This is supported by original results from Zhao et al (1996) who found that in an adult group of 66 patients with CF, none were positive for autoantibodies to the major ANCA antigens PR3 or MPO. Nevertheless, this result was surprising given our previous finding of increased extracellular MPO from CF neutrophils in response to TNFα/fMLP activation compared with HC cells. One explanation could be that MPO and PR3 bind to cell surfaces and therefore cannot be detected as an antigen for autoantibody production.

Next we employed the ORGENTEC Alegria ® assay to quantitatively measure IgG class autoantibodies against BPI in HC and CF plasma. We observed a significant increase in the anti-BPI levels in CF plasma compared to HC (p=0.002).

A fault in the present study is the modest number of patients on Ivacaftor therapy as well as the lack of plasma from patients who are either homozygous or heterozygous for the G551D mutation before commencing Ivacaftor therapy as a comparator. There are approximately 150 patients attending the CF service in Beaumont hospital with only 14 of these receiving Ivacaftor treatment currently.
It would also be informative to assess the effect of the new Ivacaftor/Lumacaftor (Orkambi®) combination drug on circulating BPI levels and anti-BPI autoantibodies for the majority of our patients who are homozygous for the ΔF508 mutation.

We have confirmed increased circulating BPI levels in CF as well as significantly increased anti-BPI autoantibodies in this patient population. It is as yet unclear why autoantibodies to BPI are produced. One possible theory is related to chronic *P. aeruginosa* infection and colonisation. It is thought that steady increasing bacterial load over time results in the accumulation of necrotic and apoptotic neutrophils in the CF airways (causing lung destruction) which in turn cause changes in the interactions between bacteria and BPI perhaps resulting in the formation of a new epitope which could initiate autoantibody response to BPI (319). We also demonstrated increased BPI levels in CF BALF compared to HC and to patients with COPD or NCFB as the inflammatory controls. This further reinforces the assertion that autoantibody production is related to the CF intrinsic defect as opposed to the chronic inflammation and infection associated with this disease.

As a consequence, the killing of *P. aeruginosa* mediated by normal neutrophils was inhibited in the presence of BPI ANCA in sera of children with CF (304). The degree of inhibition reflected the titre of BPI ANCA detected in serum confirming that BPI ANCA development is a poor prognostic indicator and potentially contributes substantially to the persistence of *P. aeruginosa* in the CF lung.

Moreover, as BPI has potent antimicrobial function, its exogenous administration has been suggested as a novel anti-infective agent (320) and has been trialled in the setting of human meningococcemia management due to overwhelming *Neisseria meningitidis* (*N. meningitidis*) infection and the use of recombinant BPI has been evaluated for early treatment of gram negative pneumonia (321) with favourable results. This raises the question as to why the high levels of BPI in CF BALF recorded in this study do not kill bacteria and patients suffer from recurrent *Pseudomonas* infections. It is possible that BPI present in BALF is degraded or rendered inactive due to excessive levels of protease enzymes within CF airways.
Chapter 5

General Discussion
5.1 Discussion

In this study we have demonstrated that there is enhanced degranulation of primary granules by CF circulating neutrophils, as well as excessive serine protease activity by CF neutrophils compared with HC cells upon activation with the physiological stimulus combination TNFα and fMLP. This was associated with excessive Rac2 activation, the central regulatory protein responsible for primary granule trafficking. Additionally, HC cells treated with the pharmacological CFTR inhibitor – CFTRinh-172 compound, exhibited significantly increased primary granule degranulation compared to HC cells treated with the vehicle control. Furthermore, we illustrated that HC cells treated with CFTRinh-172 also displayed enhanced Rac2 activation as a cause for increased primary granule degranulation. As a consequence of increased primary granule release, plasma and BALF BPI levels were found to be greater in CF and autoantibodies against the primary granule protein BPI were significantly elevated in individuals with CF compared to HC. Collectively, these results provide further evidence for an intrinsic defect in circulating neutrophils of individuals with CF.

The findings of this study contribute to the on-going debate on whether dysregulated neutrophil activity in CF is caused by chronic inflammation or intrinsic changes. Countless studies have emphasised the effects of inflammation and exposure to the harsh lung environment on the neutrophil’s ability to fight bacterial pathogens including receptor cleavage by high protease concentrations in CF airways (183, 322, 323), as well as elevated levels of pro-inflammatory cytokines (324, 325). Moreover, various reports have illustrated intrinsic aberrations including changes in gene expression (38, 42, 43), altered apoptosis in heterozygous carriers of a single mutated gene (154), and chlorination defect of phagocytosed bacteria due to defective Cl− transport. The role of the neutrophils in the pathogenesis of CF is very complex as both inflammation and intrinsic dysregulation could potentially contribute to the CF phenotype that promotes chronic bacterial infection of the airways.
To minimise changes in neutrophil activity due to inflammation, circulating neutrophils were isolated from clinically stable CF patients as these cells have not been previously activated or exposed to bacteria or the harsh conditions of the CF lung, and inflammatory markers have been shown to be lower in stable patients compared to individuals with acute exacerbations (243, 246). Moreover, we chose to investigate circulating neutrophils as opposed to airway cells for our experiments as many studies have confirmed evidence of airway inflammation in CF BALF present as early as 4 weeks of age (47). Perhaps a drawback of the present study however is the sole use of adult circulating CF neutrophils as opposed to neutrophils from children with CF where levels of inflammation are significantly lower, or alternatively the lack of use of CFTR animal model such as the CFTR pig without infection.

Whilst neutrophil degranulation plays a crucial role in innate immunity (83), dysregulated neutrophil degranulation plays an essential role in the pathogenesis of CF associated airways disease as well as in other respiratory conditions such as AATD (213) or asthma (326). As discussed in previous chapters, the observed enhanced primary granule degranulation is not only problematic because of increased antibacterial proteins being released capable of exacerbating oxidative damage to epithelial cells due to HOCl production (94), but also due to associated uncontrolled serine protease activity. Our results are in keeping with previous studies illustrating increased release of MPO (156) and NE (155) from primary granules. This is a concerning finding as excess MPO levels have been associated with worse airflow obstruction and increased sputum production in CF homozygous individuals (92) as well as enhanced oxidative stress in the setting of cardiovascular disease (93). Furthermore, increased levels of neutrophilic MPO in the BALF of ARDS patients have been previously described (265), as well as elevated levels of MPO detected in both serum and BALF of patients with lung cancer compared to HC (327), positively correlating with increased ROS in peripheral blood neutrophils of lung cancer patients. Of note, use of AZ1 (a 2-thioxanthine compound), an MPO inhibitor, slowed progression of emphysema and small airway modelling whilst partially protecting against pulmonary hypertension in a cigarette smoke exposure model in guinea pigs proposing a potential therapeutic role for MPO inhibitors in the management of obstructive airways disease (328).
To verify our result of increased MPO release by CF circulating neutrophils, we investigated BPI as an additional marker of primary granule degranulation (Figure 5.1). We demonstrated significantly increased BPI release by CF cells in response to TNFα and fMLP activation by immunoblotting compared to HC neutrophils. Furthermore, resting HC neutrophils treated by the pharmacological CFTR inhibitor compound- CFTRinh-172 also displayed increased BPI levels compared to HC cells suspended in the vehicle control. BPI is a potent antimicrobial protein and BPI and its utility in the treatment of life threatening infections and conditions associated with bacteraemia and endotoxaemia has been advocated (101).

Interestingly, genetic variations in BPI expression were found to influence the risk of development of rapid airflow decline after haematopoietic cell transplantation (HCT) (329). However, BPI ANCA are generated as a consequence of increased BPI levels in CF and these autoantibodies are recognised as interfering with the antimicrobial effects of BPI (330), hampering bacterial killing in CF. Collectively, this reinforces the view that excessive primary granule degranulation is defective and contributes to disease pathogenesis in CF. This is of particular significance as we confirmed increased BPI levels in CF BALF compared to HC and to inflammatory control (COPD/ NCFB) BALF.

We know that significant neutrophil influx and increased degranulation of primary granules lead to a protease/antiprotease imbalance that favours the development of bronchiectasis. The role of neutrophil breakdown and protease activity in CF lungs have been well described (52) with particular deleterious effects on destruction of lung architecture, promotion of oxidative stress, alterations in viscosity of endobronchial material further enhancing airway obstruction and infection. In addition to MPO and BPI as markers of neutrophil primary granule release, this study also concentrated on NE. We employed FRET analysis as an alternative modality to quantify extracellular NE activity. We documented increased extracellular NE activity by CF neutrophils in response to TNFα and fMLP stimulation. This result is in keeping with existing literature indicating that peripheral blood neutrophils from patients with CF spontaneously release more NE than HC cells (246), which is not corrected by antibiotic use indicating that NE activity is continuous even following resolution of clinical infection.
It has previously been shown that children as young as 1 year of age with CF have significantly elevated levels of active NE in epithelial lining fluid (ELF) whereas ELF of normal individuals displayed an absence of active NE. Additionally, there was a distinct modification of AAT and SLPI, naturally occurring antiproteases, including degradation and complex formation (131). Indeed excessive levels of NE have been shown to positively correlate with poor prognoses in CF (331), causing damage to structural proteins including elastin, collagen and proteoglycans (158, 159) in airways disease. This has led to the proposal of treating CF patients with aerosolised AAT augmentation therapy in order to restore the protease/antiprotease imbalance. The clinical benefits of AAT augmentation therapy in the setting of AATD have been advocated (332), and emerging evidence of its utility in other conditions such as RA (333) and type 1 diabetes mellitus are promising (334). Unfortunately, whilst trials in CF have endorsed both safety and tolerability of AAT use, they have failed to demonstrate improvements in lung function (184, 185). However, as the study drug was administered for 4 weeks only, it is possible that prolonged use of AAT in this CF population may prove efficacious in preserving lung function (184). Fortunately, the authors were able to demonstrate that use of inhaled AAT in CF exhibited improved bacterial killing capacity of airway neutrophils and resultant decreased *P. aeruginosa* bacterial load in CF sputum (184). Results from a study carried out by Wainwright et al (2015) encouraged the therapeutic intravenous use of AAT in combating inflammation in CF (335).

Cath G, a second neutrophil serine protease was examined in our project. In keeping with excessive primary granule release by CF circulating neutrophils, we have also demonstrated increased extracellular Cath G activity by CF neutrophils in response to TNFα and fMLP activation (Figure 5.1) compared with HC cells as measured by FRET analysis. Cath G along with NE is a potent secretagogue for airway submucosal glands, increasing sputum production and exacerbating airway hyper-secretory conditions of CF (250).
Cath G activity has also been proven to interfere with airway defences and the clearance of *P. aeruginosa* from mouse lungs (249) and to degrade SP-A thereby diminishing innate pulmonary antimicrobial defence mechanisms (270), worsening an already tenuous situation in CF. To combat excessive Cath G activity and mucus hyper-secretion in CF, use of recombinant DNase has demonstrated a beneficial effect by depolymerisation of DNA, lysis of bronchial secretions and clearance of NE and Cath G (336).
Figure 5.1: Neutrophils in CF.

Schematic illustration demonstrating the proposed effect of CFTR dysfunction in neutrophils. Neutrophils contain three types of granules: primary (1°), secondary (2°) and (3°) granules. There is exuberant degranulation of 1° granules by CF neutrophils, in response to inflammatory mediators such as TNFα and fMLP (step 1). Excessive activity of the GTP-binding protein Rac2 occurs (step2), driving increased degranulation of 1° granules and excessive release of 1° granule proteins - MPO and BPI and the serine proteases NE and Cath G (step 3). Furthermore, enhanced release of BPI extracellularly leads to the production of BPI autoantibodies (step 4).
Thus far we have corroborated increased MPO and BPI release by CF circulating neutrophils by immunoblotting as well as confirmed enhanced extracellular NE and Cath G activity by FRET analysis. To test the hypothesis that increased primary granule release is intrinsic due to the CFTR defect, we employed CFTRinh-172 to inhibit the CFTR function of HC neutrophils (257). Inhibition of CFTR function resulted in increased primary granule degranulation and increased Rac2 activation of resting cells similar to the observation made in CF cells. This result suggests that CFTR function is directly involved in regulation of primary granule release. Indeed, CFTR function and its inhibition by CFTRinh-172 treatment have been described in bronchial epithelial cells as HC cells illustrated upregulation of T cell immunoglobulin and mucin domain containing 3 (TIM-3) and its ligand galectin-9 mimicking levels detected in CF (337). In addition, CFTRinh-172 treatment of primary airway epithelial cells for up to 5 days resulted in a heightened inflammatory response akin to CF as evidenced by increased IL-8 production and NF-κB nuclear translocation post TNFα stimulation (147). Use of both CFTR inhibitors, CFTRinh-172 and N-(2-naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene) glycine hydrazide (GlyH-101) (145) to treat circulating control neutrophils also resulted in reduced secondary and tertiary granule degranulation and impaired Rab27activation mirroring the defects observed in CF neutrophils (146).

Given our findings of enhanced MPO and BPI release as well as increased NE and Cath G extracellular activity by CF circulating neutrophils, we hypothesised that increased activity of the GTP-binding protein Rac2 was the underlying molecular mechanism for excessive degranulation of primary granules in CF. Primary granule release is regulated by Rac2, as Rac2 deficient mice exhibited impaired release of primary granules whereas secondary and tertiary granule release remained intact (208). In this study we focused on the involvement of Rac2 in regulation of primary granule degranulation and determined increased activity of Rac2 potentially causing the excessive primary granule release by CF neutrophils both at rest and in response to TNFα and fMLP stimulation. We confirmed a link between CFTR function and Rac2 activity as control cells treated with CFTRinh-172 demonstrated enhanced Rac2 activation.
Rac2 has been implicated in F-actin formation required by neutrophils for chemotaxis and O$_2^-$ release (338) as well as its function in regulating primary granule release from neutrophils. Moreover, a murine model of IgG immune-complex mediated acute lung injury ALI, (339), identified that ALI was attenuated in Rac2 knockout mice compared with wild type mice, suggesting that lung injury in response to immune complex deposition was dependent on Rac2 in both alveolar macrophages and neutrophils.

Collectively, our findings add further to our understanding of neutrophil involvement in the pathogenesis of CF by describing aberrations in neutrophil function which certainly tip the protease/antiprotease balance in favour of unrestrained protease activity leading to lung parenchymal destruction, progressive airflow obstruction and irreversible bronchiectasis.

In addition, these defects described lead to increased autoantibodies mounted against the primary granule protein BPI, which in turn may well have implications for inefficient bacterial killing and further complicating and exacerbating chronic bacterial infection characteristic of CF (290). Moreover, BPI ANCA titres in individuals with CF have been shown to be directly proportional to the severity of airway destruction, lower FEV$_1$ and chronic $P. \text{aeruginosa}$ colonisation (210, 315, 340, 341). Autoantibodies against neutrophil proteins have been described in other airways diseases. In this regard, a higher incidence of anti-lactoferrin IgG autoantibodies in the plasma of AATD individuals compared with HC, whilst no significant differences were detected in the level of anti-hCAP 18 or anti-MMP 9 IgG autoantibodies (213). Interestingly, the authors found that anti-lactoferrin IgG autoantibody levels were reduced significantly following long-term AAT augmentation therapy, further supporting its potential use in autoimmune disorders (213). Whilst BPI antibody levels were significantly greater in CF plasma compared to HC, we did not detect a significant difference between CF patients and those CF individuals receiving Ivacaftor treatment. We propose that this is due to the heterogeneity of the Ivacaftor treatment group in terms of baseline lung function, sputum microbiology and co-morbidities.
5.2 Future Direction

Despite providing further evidence for intrinsic abnormalities in neutrophil function, the results of this translational research project have raised a host of additional questions and vast possibilities to further elucidate the role of neutrophils in the pathogenesis of CF.

This study focused on degranulation to the outside of the cell, however, bacterial killing occurring within the phagosome of the neutrophil is a very important process (342). Therefore future experiments will investigate the pattern of degranulation of various types of granules into the phagosome. Furthermore, the role of recombinant anti-proteases should be evaluated in future studies, with particular emphasis on modulating the inflammatory cycle and preservation of lung function. We hypothesize that treatment with synthetic anti-proteases would attenuate the inflammatory response of CF and in turn maintain pulmonary function.

In addition, the consequence of anti-BPI autoantibodies on the bacterial killing capacity of CF plasma and BALF on *P. aeruginosa* requires further exploration.

Much of the major clinical consequences, morbidity and mortality of CF are related to progressive airways destruction. It would seem logical that modulation of neutrophil function may remedy this, and studies investigating various therapeutic options are required. To this end, non-steroidal anti-inflammatory drugs have been examined with disappointing results and an unacceptable side effect profile (343-345). Similarly, chronic use of systemic corticosteroids is largely limited by the development of significant side effects including development of diabetes mellitus and osteoporosis (220). Macrolide antibiotics have emerged as useful agents in this patient cohort due to their antimicrobial action and immunomodulatory effects with evidence that macrolides can inhibit superoxide generation by activated neutrophils *in vitro* (346) and reducing cytokine concentrations (347).

Future studies will assess phagosomal killing of bacteria and investigate whether degranulation and activation of proteolytic enzymes occurs to the same extent in CF neutrophils as in HC cells. This could in turn, lead to the identification of therapeutic targets that are independent of the underlying mutation as opposed to the current development of genotype specific compounds that may not become available for all mutations.
References


195


196


299. Schultz H, Csernok E, Schuster A, Schmitz TS, Ernst M, Gross WL. Anti-neutrophil cytoplasmic antibodies directed against the bactericidal/permeability-increasing protein (BPI) in pediatric cystic fibrosis patients do not recognize N-terminal regions important for the anti-microbial and lipopolysaccharide-binding activity of BPI. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2000;11(2):64-70.


Appendix 1

Patient Information Leaflet

Study title: Neutrophil dysfunction in patients with Cystic Fibrosis

Principal investigator’s name: Professor N.G. McElvaney

Principal investigator’s title: Professor of Medicine

Telephone number of principal investigator: 01 8093763

You are being invited to take part in a clinical research study to be carried out at Beaumont Hospital.

Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don’t feel rushed and don’t feel under pressure to make a quick decision.

You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as ‘Informed Consent’.

You don’t have to take part in this study. If you decide not to take part it won’t affect your future medical care.

You can change your mind about taking part in the study any time you like. Even if the study has started, you can still opt out. You don’t have to give us a reason. If you do opt out, rest assured it won’t affect the quality of treatment you get in the future.
Why is this study being done?

This study is being carried out to examine the behaviour of immune cells in people who have cystic fibrosis. Neutrophils are the main white blood cells that are responsible for fighting infection in the human body. Patients with cystic fibrosis suffer from recurrent infections of the lung. The neutrophils of people who have cystic fibrosis behave differently from those of healthy individuals; they are not as effective as normal neutrophils in killing bacteria during the time of infection. We would like to find out the reasons why this is so.

Neutrophils also contain and release granules. We plan to investigate the mechanism by which the granules are released in patients with cystic fibrosis to assess whether this process is working properly and what treatments may influence this.

Who is organising and funding this study?

Professor McElvaney and his research team are organizing this study. This study will be carried out to the highest standard. Our research team will provide you with any information you require and are happy to answer any queries you may have.

Why am I being asked to take part?

You have been selected as a potential study candidate because you follow up in our clinic for the management of your cystic fibrosis.

How will the study be carried out?

This study will commence in January 2015 and run over 3 years. You will be asked to donate a sample of blood for scientific experimentation on blood cells, a maximum of twice over a 24 month period. We will attempt to co-ordinate taking blood from you with your routine bloods required for you in the CF unit as much as possible.

We hope to recruit approximately 10 patients with CF from Beaumont Hospital for this study and you have been identified as a potential patient for inclusion.
What will happen to me if I agree to take part?

After meeting with a member of Professor McElvaney’s research team you will be given the time you wish to come to a decision about enrolling in this study. You are invited to contact us with your decision. If you are happy to go ahead and participate we will ask you to sign a consent form. At all times we are happy to provide additional information and answer any further queries that may arise.

You will have blood samples taken from you before you leave the CF clinic, and the amount of blood taken will be equivalent to six teaspoons.

What other treatments are available to me?

There are no alternative treatments available at the moment. The results if this study may provide important information for developing new treatments in the future.

What are the benefits?

The findings of this study may help to improve existing treatments for cystic fibrosis as well as for developing new treatments in the future. Knowledge may also be gathered during this process which may alter your medical management or open up new areas of research which may prove beneficial for cystic fibrosis in the future.

What are the risks?

There are minor risks in relation to having blood taken for example mild pain during blood withdrawal and minor bruising or infection at the site where the blood is drawn.

What if something goes wrong when I’m taking part in this study?

If you experience any of the above complications the appropriate care will be provided to you.

Will it cost me anything to take part?
There are no extra costs involved with your participation in this study. You will continue to receive your regular medical care by your GP or respiratory team at the cystic fibrosis unit in Beaumont Hospital.

_is the study confidential?

Your information and samples will be stored for up to 10 years. Any information will be coded so that the data does not identify you by name. You will be given a unique code for the purpose of identification during the study. Beaumont Hospital medical staff can only access your medical records. All research personnel and medical staff are obliged to maintain confidentiality at all times. Your GP will not be notified of your participation in the study unless you request otherwise.

_where can i get further information?

If you have any further questions about the study or if you want to opt out of the study, you can rest assured it won't affect the quality of treatment you get in the future.

If you need any further information now or at any time in the future, please contact:

Name 
Dr Fatma Gargoum
Address
Education and Research Centre, 
Smurfit Building, 
Beaumont Hospital, 
Dublin 9.

Phone No
01 8093796
01 8094852


**Appendix 2**

**Patient Consent Form**

*Study title: Neutrophil Dysfunction in patients with Cystic Fibrosis*

<table>
<thead>
<tr>
<th>I have read and understood the Information Leaflet about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>I understand that I don’t have to take part in this study and that I can opt out at any time. I understand that I don’t have to give a reason for opting out and I understand that opting out won’t affect my future medical care.</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>I am aware of the potential risks of this research study.</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>I give permission for researchers to look at my medical records to get information. I have been assured that information about me will be kept private and confidential.</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>I have been given a copy of the Information Leaflet and this completed consent form for my records.</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

**Storage and future use of information:**

I give my permission for information collected about me to be stored or electronically processed for the purpose of scientific research and to be used in related studies or other studies in the future but only if the research is approved by a Research Ethics Committee.

| | |
|---|---|---|

**Patient Name (Block Capitals)** | **Patient Signature** | **Date**
To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

| | | | |

Name (Block Capitals) | Qualifications | Signature | Date

3 copies to be made: 1 for patient, 1 for PI and 1 for hospital records.