

Investigating the Role of the Eosinophil in Chronic Obstructive Pulmonary Disease



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Abstract

Now the third leading cause of death worldwide, chronic obstructive pulmonary disease (COPD) is characterised by unpredictable acute worsening of symptoms. A third of these crisis events are associated with an eosinophilic phenotype that has an unknown mechanism of injury. In this thesis, I have shown that RNA-sequencing of eosinophils isolated during exacerbations revealed upregulation of genes related to redox systems. Furthermore, eosinophils isolated during crisis events had increased capacity to produce reactive oxygen species (ROS), had a higher resting state of ROS generation, and released more mitochondrial specific ROS compared to neutrophils. Eosinophils also had comparatively low levels of the antioxidant enzyme glutathione, which appeared to be less oxidised, indicating that eosinophils have a weak ability to scavenge excess ROS. Finally, in a co-culture system, eosinophils were shown to disrupt epithelial barriers and induce hyperpermeability, which was further increased upon eosinophil stimulation. These findings would suggest that when COPD patients experience an eosinophilic exacerbation and consequently, an influx of activated eosinophils into the airways, there is damage caused to the local tissue milieu due to the release of ROS. This mechanism proposes a new therapeutic avenue at the point of an eosinophilic exacerbation. Additionally, analysis of serum and sputum samples highlighted IL-5 as a key eosinophil mediator released during eosinophilic exacerbations. Flow cytometry analysis identified the IL-3 receptor as a potential marker of eosinophil activation status.

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Abbreviations

Abbreviation	Long format
8-OHdG	8-hydroxy-2'- deoxyguanosine
ABRA	Acute exacerbations treated with Benralizumab
ADCC	Antibody dependent cellular cytotoxicity
AERIS	Acute exacerbations and respiratory infections
β -ME	β -mercaptoethanol
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CFU	Colony forming units
CITE-seq	Cellular indexing of transcriptomics and epitopes by sequencing
CLC	Charcot-Leyden crystal
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CXCL	Chemokine (C-X-C motif) ligand
COPD	Chronic obstructive pulmonary disease
DHE	Dehydroepiandrosterone
DCFH ₂ DA	Dichlorodihydrofluorescein
DMEM	Dulbecco's Modified Eagle's Medium
DUOX	Dual oxidase
DTT	Dithiothreitol
ECACC	European Collection of Authenticated Cell Cultures
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
EET	Eosinophil extracellular trap
ELISA	Enzyme linked immunosorbent assay
EPX	Eosinophil peroxidase
FACS	Fluorescent associated cell sorting
FEV ₁	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FPKM	Fragments per kilobase of transcript sequence
FVC	Forced vital capacity
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GO	Gene ontology
GOLD	Global initiative for COPD guidance
hEOS	Homeostatic eosinophil
H ₂ O ₂	Hydrogen peroxide
Lcsbp	interferon consensus sequence binding protein
IFN	Interferon

Ig	Immunoglobulin
ILC	Innate lymphoid cell
IL	Interleukin
iEOS	Inflammatory eosinophil
LPO	Lactoperoxidase
LPS	Lipopolysaccharide
LT	Leukotriene
mAb	Monoclonal antibody
MBP	Major basic protein
MCP	Monocyte chemoattractant protein
MDA	Malonic dialdehyde
MMP	Matrix metalloprotease
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NK	Natural killer
NET	Neutrophil extracellular trap
NTHi	Non-typable Haemophilus influenzae
Padj	P-adjusted value
PBS	Phosphate buffered saline
PK	Protein kinase
PPM	Potentially pathogenic microorganisms
PRDX	peroxiredoxin
PGD	Prostaglandin
PMA	Phorbol 12-myristate 13-acetate
RIN	RNA integrity number
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RT-qPCR	Reverse transcription quantitative chain reaction
RV	Rhinovirus
sc-RNA-seq	Single cell RNA sequencing
STAT	Signal transducer and activator of transcription
Tc	Cytotoxic T
Tc1	Type 1 cytotoxic T
Tc2	Type 2 cytotoxic T
Th	T helper
Th1	Type 1 T helper
Th17	Type 17 T helper
Th2	Type 2 helper
TNF	Tumour necrosis factor
TMB	Tetramethylbenzidine
TSLP	Thymic stromal lymphopoietin
TXN	Thioredoxin
VCAM	Vascular cell adhesion molecule

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Chapter 1

General Introduction

1 General Introduction

1.1 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is characterised by chronic obstruction of pulmonary airflow that is not fully reversible and interferes with normal breathing, often resulting from bronchitis and parenchymal destruction (1). Exposure to smoke, or a history of smoking, are major risk factors and the most common aetiology in the developed world. Diagnosis is based on symptoms of exertional breathlessness, chronic cough, regular sputum production, frequent winter bronchitis and wheeze. However, COPD is heterogenous and will affect individual patients in different ways (2).

Acute periods of deterioration in symptom severity are known as exacerbations and further weaken lung function. These periods can be extremely distressing for the patient and are the major cause of patient hospitalisation (3,4). There is no cure for COPD with treatment aiming to slow progression, improve symptoms, and prevent acute exacerbations (5).

COPD poses a global healthcare burden and is associated with significant morbidity and mortality with 251 million cases reported in 2016 and 3.17 million deaths (6). Alongside the burden on human health, COPD is responsible for large economic costs. Both directly to healthcare and the wider economy (7). It is vital that more is understood surrounding the pathogenesis of COPD. This will aid in the development of more effective treatments to improve the quality of life of sufferers and reduce the burden on healthcare systems.

1.2 Diagnosis of COPD

Aspects of this multifaceted disease have been recorded for centuries. The French physician Renè Laënnec is considered the father of clinical auscultation and documents various descriptions of pulmonary diseases in his book 'Treatise of the chest', published in 1821. He writes of the sounds he hears from his invention, the stethoscope, which allowed him to classify pulmonary conditions including emphysema:

'... since I have made use of the stethoscope, I have verified [emphysema's] existence as well on the living as the dead subject, and I am led to consider it as by no means infrequent'.

He goes on to describe the anatomical characteristics of 'air cells' in this condition, their rupture, enlargement and of air trapping (8).

More modern lung function assessments are measured by spirometry. Clinicians will use spirometry to assist in diagnosing COPD by measuring the volume of air that can be expired in one second (FEV₁) and its ratio to the forced vital capacity (FVC) to reflect the degree of airway obstruction. Following bronchodilator treatment this ratio is calculated with 0.70 being the lower acceptable limit for non-obstructed airways (9). FEV₁ % predicted is used to assess airflow limitation, with the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria applied to categorise the severity of airway obstruction by considering the number of exacerbations and the COPD Assessment Test Symptom score (1,10) (see table 1):

Table 1. Severity of airflow limitation based on GOLD criteria

FEV₁ (%predicted)	
GOLD 1	> 80
GOLD 2	50-79
GOLD 3	30-49
GOLD 4	< 30

1.3 Pathophysiology

A spectrum of three main pathologies can be observed in COPD patients: chronic inflammation of the bronchi and bronchioles (bronchitis), small airway obstruction and emphysema. A common feature of COPD includes inflammation within the airways and lumen. This is understood to be initiated by toxic gases and smoke particles damaging the airways and stimulating an immune response (11).

The larger airways are located centrally where the internal diameter is more than 4 mm and extends along the gland ducts and into the mucus producing glands. Inflammation here is associated with disruption of the epithelial barrier and an increase in mucus production from the mucociliary system, which prevents proper clearance of particles out of the airways due to ciliary dysfunction. Thickening of the bronchial walls also occurs due to the deposition of connective tissue and decline in function. Patients therefore present with a chronic cough and excessive sputum production. Chronic bronchitis is diagnosed if these symptoms continue for three months over two consecutive years (12).

The first line of contact for noxious particles entering the airways is an epithelial cell barrier which can become disrupted due to contact with these particles. The mucociliary clearance system also increases the amount of exudates into the small airway lumen in response to noxious particle infiltration (12,13). Other cellular components of the immune system thought to contribute to COPD pathogenesis include neutrophils, cytotoxic CD8⁺ T cells and alveolar macrophages. Neutrophilia is a dominant inflammatory pattern seen in patients with COPD however, another cell type found to be elevated in up to 30% of patients is the eosinophil, although its contribution to disease progression is still debated (14)(15). Inflammation also increases mucus production from goblet cells and

consequently the formation of mucus plugs within the already damaged airways. This presents as dyspnoea, cough, and sputum production in the patient. Despite smoking cessation being a major first line intervention to prevent disease progression, airway inflammation has been shown to persist in patients (16–19).

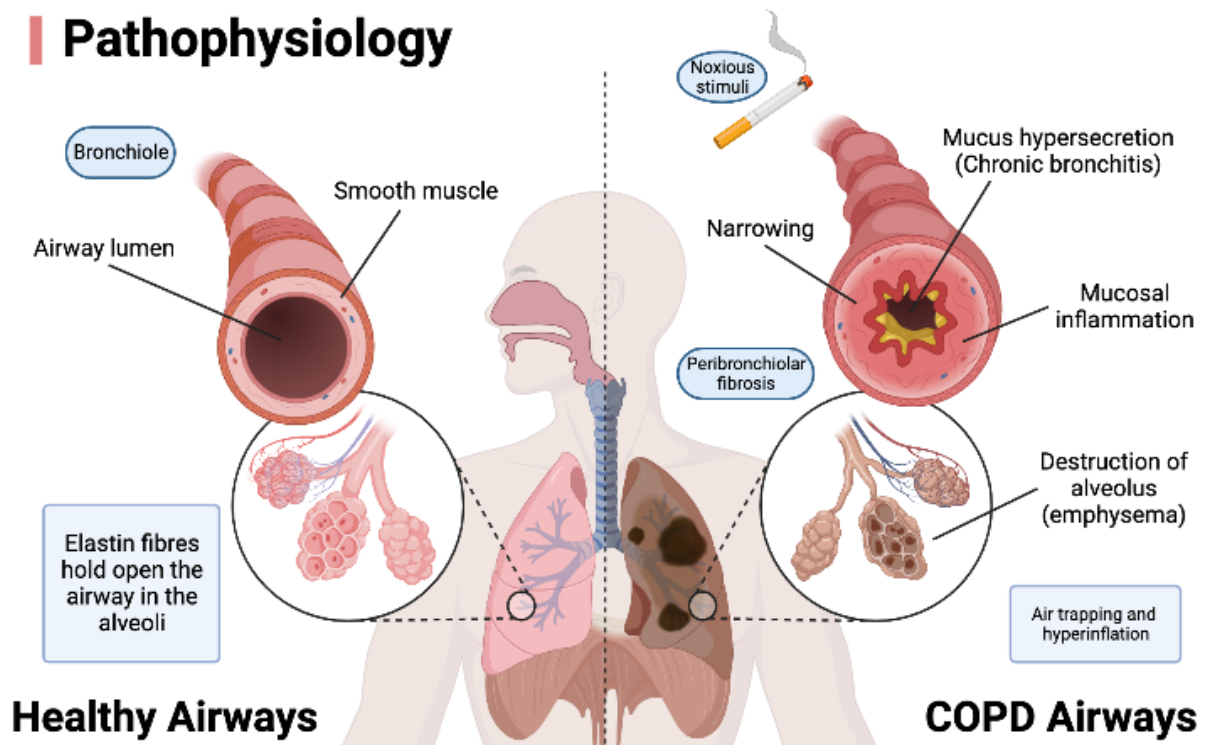


Figure 1. Pathophysiology of COPD.

Chronic exposure to noxious stimuli often associated with smoking induces inflammation within the airways. This causes thickening of the bronchiolar periphery wall along with fixed narrowing due to fibrosis. Alveolar attachments are disrupted through emphysema and the airway lumen occluded by mucus hypersecretion. Created in Biorender.com.

1.4 Exacerbations of COPD

As described previously, acute, and sustained periods of worsening respiratory symptoms and lung function are termed exacerbations. This includes any increase or onset of cough, sputum production, wheezing, or dyspnoea that requires additional treatment. GOLD classifies exacerbations as mild, moderate, and severe. Mild and moderate exacerbations may be treated at home whereas severe events

require hospitalisation. If a patient is hospitalised due to an exacerbation, the prognosis is usually poor with an increased risk of death. Patients usually experience symptoms for between two days to two weeks, but some may continue beyond this. Around 20% of patients will not recover to a state of health equivalent to how they felt prior to their exacerbation event (20).

Surprisingly, the exacerbation definition dates to Laënnec and has remained relatively unchanged for nearly 200 years (8). The reader would be forgiven for finding this definition vague, at best, as it does little to inform the clinician of the underlying nature of the exacerbation or the appropriate treatment needed to remedy the cause. Clinicians are heavily reliant on patient self-diagnosis of their symptom severity and reporting their exacerbation. This is compounded with individuals having their own perceived tolerance thresholds for when they require additional treatment to relieve their symptoms (21).

Patient exacerbation rates depend on the individual and GOLD grading systems have shown no consensus on the likelihood of an exacerbation presenting. There are no tests to predict when exacerbations will occur, and the strongest indicator of frequent exacerbations is a history of previously treated episodes as shown by Hurst *et al.*, in the ECLIPSE study, an observational longitudinal study investigating biomarkers in COPD (22).

1.5 Aetiology of exacerbations

1.5.1 The role of bacteria

One of the known infectious triggers of exacerbations is bacterial infection. Bacteria may be the primary infection or alternatively they can co-exist with viruses; both

instances will stimulate a characteristic immune response by activating the hosts innate immune system. This includes release of inflammatory cytokines, recruiting neutrophils to the airways, mucus hypersecretion, disrupted mucociliary clearance, and damage to the epithelial cells (23). Bacteria have been isolated from patients with stable COPD, identifying lower airway bacterial colonisation (LABC). Bacterial colonisation may be implicated in the progression of airway obstruction as it has been noted by multiple investigators that airway inflammation increases in response to higher bacterial load measured by 16S qPCR (24–28). It is estimated that 50% of COPD exacerbations are caused by bacterial infections. The major pathogens isolated in these exacerbations include *Haemophilus influenza*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* (29). Pathogenic and host factors are thought to contribute to occurrence of a bacterial driven exacerbation, as not all bacterial infections are followed by exacerbations. Contributors may include an impaired innate response from the host and genomic differences between bacterial strains. A strain specific immune response to an invading pathogen may also result in the host being susceptible to invasion from another pathogenic strain (30).

Fifty-years ago, Fletcher and colleagues investigated the relationship between respiratory infections, sputum, and loss of lung function as measured by FEV₁. They found that chronic mucus secretion and plugging, in combination with recurrent bacterial infections, led to further airway obstruction. They argued this explained why some smokers developed progressive airways obstruction where others did not (31). This contrasted with the ‘Dutch hypothesis’ that postulated asthma and COPD to be manifestations of the same disease and that genetic interactions with the environment influenced disease progression (32). In fact, more

recent biological clustering of sputum cytokine profiles from COPD and asthma patients identified distinct and overlapping groups of subjects with either diagnosis, possibly providing evidence in support of both hypotheses (33).

Sputum itself is a reliable indicator of bacterial infection; whilst traditional bacterial culture is considered the gold standard for clinical diagnosis of lower bacterial respiratory infections and utilises semi-quantitative colony forming units (CFU) to estimate the number of viable bacterial cells in a sputum sample, qPCR is commonly used to quantify bacterial load, and 16S rRNA gene sequencing to identify taxonomic composition (25). Potentially pathogenic microorganisms (PPMs) including *Haemophilus influenzae* (*H. influenzae*) may chronically colonise the airways of COPD patients. *H. influenzae* is associated with increased airway and systemic neutrophilic airway inflammation, and less eosinophilic airway inflammation (34). However, this association is currently only causal. Further studies are required to elucidate the molecular mechanisms involved to fully understand the relationship at play between inflammatory cells and bacterial phyla and species.

Multiple reports have explored whether sputum colour is influenced by PPM infection, but these studies have often resulted in mixed outcomes (35–37). Miravittles *et al.*, reported that green and yellow sputum colour was a predictor of PPMs but without necessarily predicting the need for antibiotic treatment (35). This was a pooled study from six multicentre trials consisting of 4,089 sputum samples. Although many samples were gathered, one major issue is standardisation of colour definition. There is often ambiguity regarding the colour of sputum and colours can be defined differently depending on the person who describes it, making the utility of PPM detection in sputum based only on colour unreliable at best.

Wilkinson *et al.* showed that bacterial load measured by 16S was significantly associated with disease progression, measured by lung function (FEV₁) decline (26). This small study included 30 patients with COPD and collected sputum samples during recruitment and at the end of the study. The investigators also showed that changes in bacterial colonisation, otherwise known as dysbiosis, also increase lung function decline compared with patients who have persistent strains colonising their airways. This study did not aim to investigate the relationship between COPD exacerbations and bacterial dysbiosis however, a Chinese cohort of 152 individuals showed that microbial dysbiosis may be used as a biomarker in the diagnosis of acute exacerbations (38).

Although bacterial identification by PCR has major advantages including time and the ability to identify non-culturable bacteria, agar plate culture remains the gold-standard for bacterial identification. Limitations of PCR may include the requirement of technical replicates from a sample that may vary in amount, in the case of sputum where the expectorate volume is highly unpredictable, this may prove problematic. The presence of low copy numbers, close to the lower detection limit, might also require adjustment to higher assay sensitivity depending on the chosen primers. Furthermore, although the PCR amplification process is much quicker than the time required to culture bacterial colonies, analysis of the sequencing data may require other specialist expertise and access to equipment with additional costs.

1.5.2 The role of viruses

Viruses are the most frequent cause of an exacerbation, which aligns with a seasonal distribution pattern and influx of winter hospital admissions seen in

patients with COPD. Rhinovirus (RV) and respiratory syncytial virus (RSV) are the most commonly found but influenza A may also be responsible for exacerbations (39).

Modern molecular techniques have improved virus detection and have changed our understanding of COPD exacerbations with increased specificity and sensitivity that was not previously possible. Viruses can be detected by direct antigen tests such as immunofluorescence by virus isolation, or the nucleic acid amplification tests which are mostly PCR based or nucleic acid sequence-based amplification tests (40,41).

Once in the airways, viruses stimulate epithelial cells to release pro-inflammatory mediators similar to the pathways seen in bacterial infection. This includes the secretion of mediators such as TNF- α , CXCL1 and CXCL8 which are elevated during viral infection (42). IL-6 is also reported to be significantly increased in viral exacerbations compared with exacerbations of other causes and negatively correlated with FEV1 (26,43). Bafadhel *et al.* reported serum CXCL10 measured by ELISA to be predictive marker of viral exacerbations which may be induced by interferon (IFN)- γ release (44). Additionally, Papi and colleagues found that increased sputum eosinophils during an exacerbation were also predictors of viral exacerbations and viral-bacterial co-infections (45).

Seemungal *et al.* found almost 40% of exacerbations in their study of 83 patients tested positive for respiratory virus infections by PCR. These infections were also associated with prolonged symptom recovery and increased episode frequency with a median recovery time of 13 days for viral associated exacerbations and 6 days for non-viral exacerbations (39). However, colds are known to precede

exacerbations often by several days, potentially reducing the numbers of positive samples detected in this study thus this may be an underrepresentation (46).

1.5.3 Non-infectious related exacerbations

Although our understanding of the aetiology of COPD exacerbations has improved significantly in recent years, up to 30% are of unknown cause. Environmental factors may play a role in triggering or amplifying exacerbations (47). While the precise mechanisms are unknown, it appears that high levels of urban air pollution may be an associated risk factor, albeit much lower in comparison to cigarette smoking. It is reported that exposure to fine matter particles less than 2.5 mm in diameter and NO₂ levels may increase COPD prevalence particularly during lung development (48,49). Acquiring appropriate samples suitable for laboratory tests that can identify potentially pathogenic bacteria, viruses, or fungi may also limit certain studies.

1.6 Pharmacological interventions to treat exacerbations

The severity of the exacerbation episode will determine whether the patient is treated as an outpatient or inpatient and mainstay treatments include systemic corticosteroids. However, as the triggers for exacerbations vary as much as the clinical presentation, response is unpredictable. Nevertheless, systemic corticosteroids are associated with numerous harmful side effects, including steroid myopathy, and the very medication prescribed to treat the exacerbation may lead to adverse events that require hospitalisation (50).

Ordinarily, antibiotics should be administered to resolve bacterial infections

however, it may prove difficult to define whether the exacerbation is bacterially driven when a patient presents requiring urgent care. Clinical signs of bacterial infection include the colour of sputum and increase in sputum purulence. Stockley *et al.* reported that purulent (green) sputum was 94.4% sensitive and 77% specific for identifying high bacterial load and in identifying patients who would benefit from antibacterial therapy. This is opposed to patients who presented with mucoid (white) sputum who improved without antibiotic intervention and whose sputum characteristics remained constant following a return to stable state (37). However, Brusse-Keizer and colleagues did not establish such a correlation and questioned the usefulness of sputum colour in identifying the subgroup of patients who would respond to antibiotics (36). Still, clinicians are conscious of the widespread use of antibiotics contributing to microbial resistance and may be hesitant to prescribe antibiotics without a more reliable indicator for infection.

Guidelines advocate a unilateral approach of using glucocorticoids to treat exacerbations regardless of the clinical presentation. This is to shorten recovery time, improve lung function and prevent treatment failure (51). It is advised that 40 mg oral prednisone is prescribed for five days; any longer use increases the risk of pneumonia and mortality (52).

The use of systemic corticosteroids is supported by a Cochrane review of eight studies that investigated the risk of outcome failures. This review established the beneficial use of systemic corticosteroids to prevent treatment failures and was incorporated into guidelines (53). However, the overall outcome conclusion is skewed by two small, single-centre studies showing favourable outcomes, with the confidence intervals of all other studies crossing 1, thus providing insufficient evidence that the non-steroid groups were significantly different to the steroid-

treatment groups.

1.6.1 Experimental interventions to treat exacerbations

Benralizumab is a humanised afucosylated antibody that targets cells expressing IL-5R α , crosslinking with natural killer (NK) cells and inducing antibody-dependent cellular cytotoxicity (ADCC) and eosinophil depletion (see figure 2) (54). Benralizumab therefore binds to eosinophils with high selectivity and results in rapid reduction of circulating eosinophil levels (55). Marketed as Fasenra, it is currently approved for the treatment of severe eosinophilia asthma to prevent exacerbations. In two phase III trials, Benralizumab was investigated for its efficacy in reducing exacerbations in COPD patients with moderate to severe exacerbations with blood eosinophil counts of 220 per cubic millimetre or greater (56). Unfortunately, Benralizumab did not reach its primary endpoint. A small trial of Benralizumab to treat acute exacerbations of asthma as an adjunct to systemic corticosteroids has also proven to be negative (57), but the study investigators did not look at only eosinophilic exacerbations of asthma. Several case reports have demonstrated efficacy in using Benralizumab for acute exacerbations of asthma (55,58). The acute exacerbations treated with Benralizumab (ABRA) trial (see methods 2.1.1 for study outline), is a phase IIb proof-of-concept study to test the efficacy of 100 mg of Benralizumab as a single injection subcutaneously in patients with an acute eosinophilic exacerbation of asthma or COPD in comparison to corticosteroids and placebo. Experiments undertaken throughout my DPhil projects will utilise blood and sputum samples from this trial (ClinicalTrials.gov Identifier: NCT04098718).

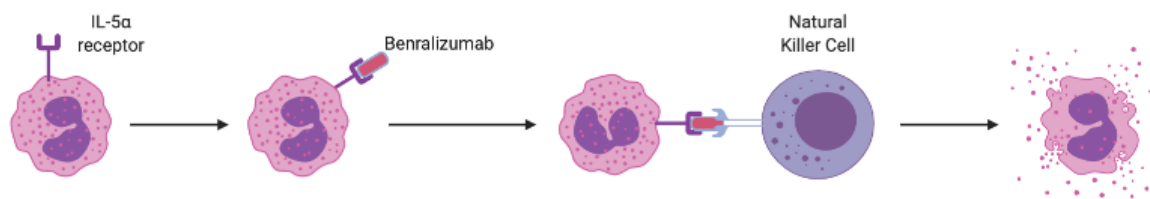


Figure 2. Benralizumab mechanism of action.

Benralizumab binds to the IL-5Ra expressed on eosinophils and crosslinks with natural killer (NK) cells to induce antibody dependent cellular cytotoxicity (ADCC) resulting in apoptosis of the target cell. Created in Biorender.com.

In summary, bacterial exacerbations are treated with antibiotics, where infections are the presumed aetiology. Systemic glucocorticoids are commonly used but their efficacy is under question and associated with various side effects. There is urgent need for effective interventions that are directed at the cause of an exacerbation to resolve the episode quickly. Benralizumab is currently approved as a maintenance therapy for severe eosinophilic asthma and may provide a much needed and superior alternative in the treatment of eosinophilic exacerbations in COPD, a hypothesis being tested in the ABRA trial.

1.7 Type 1 / Type 2 immunity

The distinction of type 1 and type 2 immunity can be traced back to Parish when he described dose dependent immune responses in mice elicited by *Salmonella flagellin*. He recorded the antibody titre as a measure of the humoral response and footpad swelling for cell-mediated responses (59–61). These observations led to the development of the type 1 / type 2 immune response hypothesis, that the antibody titre (humoral immunity) is inversely proportional to cell-mediated immunity.

Following this discovery, two T lymphocyte subsets were later described as T-cytotoxic cells and T helper cells. T-cytotoxic cells express the CD8 surface

marker and are directly involved in the destruction of autologous cells infected with intracellular pathogens. Conversely, T helper cells express CD4 and induce B cells to produce antibodies, thus implicating them in humoral responses. Further subpopulations of CD4⁺ T helper cells were categorised based on their cytokine profiles; Th1 cells are capable of producing IFN-gamma whereas Th2 cells can secrete IL-4 (62,63). The two subsets of T helper cells share a naïve precursor known as the Th0 cell. These cells must first become activated in response to different stimuli before they polarize and mature into either subset (64). Of the different stimuli that induce T helper cell differentiation, IL-12 is critical for the Th1 phenotype whereas IL-4 results in Th2 commitment (65). These cytokines are secreted by innate immune cells in response to gram-negative LPS, gram-positive lipoteichoic acid, prokaryotic CpG DNA motifs, heat-shock proteins, DHEA, and the interferons alpha and gamma to produce IL-12. Alternatively, IL-4 is produced in response to frustrated phagocytosis, corticosteroids, and catecholamines (see figure 3). Release of either of these cytokines will suppress the release of the other however, if both are present in the milieu, IL-4 will dominate (66).

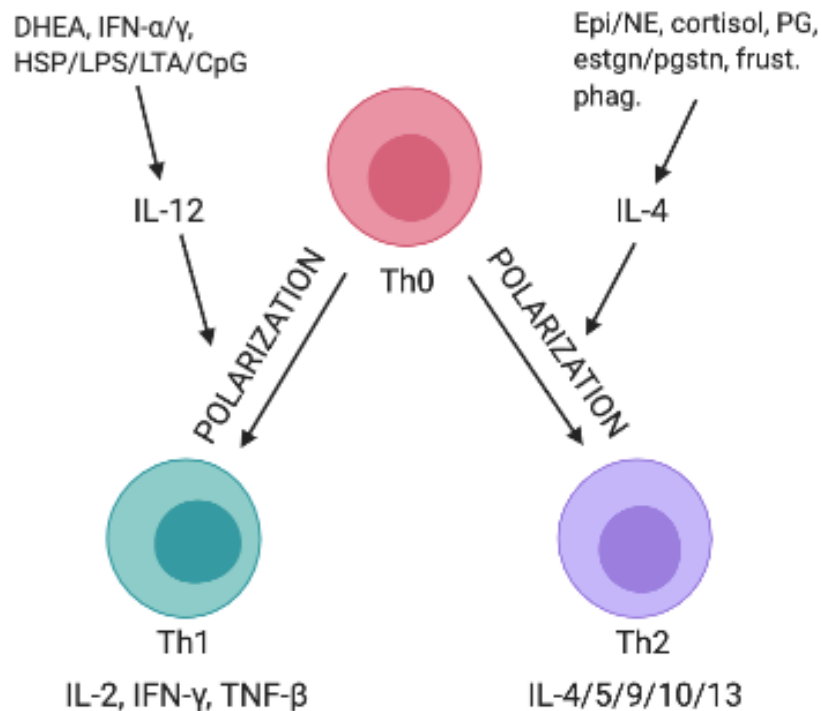


Figure 3. Summary of Th1/Th2 differentiation.

Adapted from (66). Th0 stimulation by various hormones, microbial products, and cytokines in the local milieu activate Th0 cells. IL-12 is responsible for Th1 differentiation whereas IL-4 induces the Th2 phenotype. DHEA, dehydroepiandrosterone; HSP, heat-shock protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; CpG, purine-purine-C-G-pyrimidine-pyrimidine DNA hexamer; Epi/NE, epinephrine/norepinephrine; PG, prostaglandin; estgn/pgstn, oestrogen/progesterone; frust. Phage, frustrated phagocytosis. Created in Biorender.com.

1.7.1 Type 2 regulation

As described in section 1.7, type 2 inflammation is orchestrated by CD4⁺ T helper 2 cells. Once differentiation is complete, Th2 cells will respond to cytokines released from epithelial cells known as alarmins; IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). Other lymphocytes integral to the type 2 immunological response are the more recently discovered group 2 innate lymphoid cells (ILC2s). These cells do not express surface proteins consistent with the other lymphocyte subsets and alternatively express CD278 (ICOS), ST2 (IL-33 receptor), IL-17B, and proliferate in response to the alarmins IL-25 and IL-33. Along with Th2 cells, ILC2s prominently secrete type 2 cytokines regulated by the transcription factor GATA3

(67)(68).

Airway inflammation in COPD is thought to be the result of an amplified response to inhaled irritants, proteases and pathogens that persists long after the stimulant has passed. These inhaled irritants activate dendritic cells which release CCL17 and CCL22 involved in the activation of Th2 and ILC2s. Secretion of type 2 cytokines consequently results in increased eosinophil recruitment to the airways (see figure 4). Smith *et al.* reported that ILC2 numbers were increased in the blood and sputum of patients with severe asthma and were the predominant source of type 2 cytokines due to their uncontrolled activation (69). A subset of CD8+CRTH2+ type 2 cytokine secreting lymphocytes (Tc2s) are enriched in the patients with severe eosinophilic asthma. The lipid cytokines PGD2 and LTE4 were found to recruit and activate these cells, implicating Tc2s in promoting eosinophil recruitment (70).

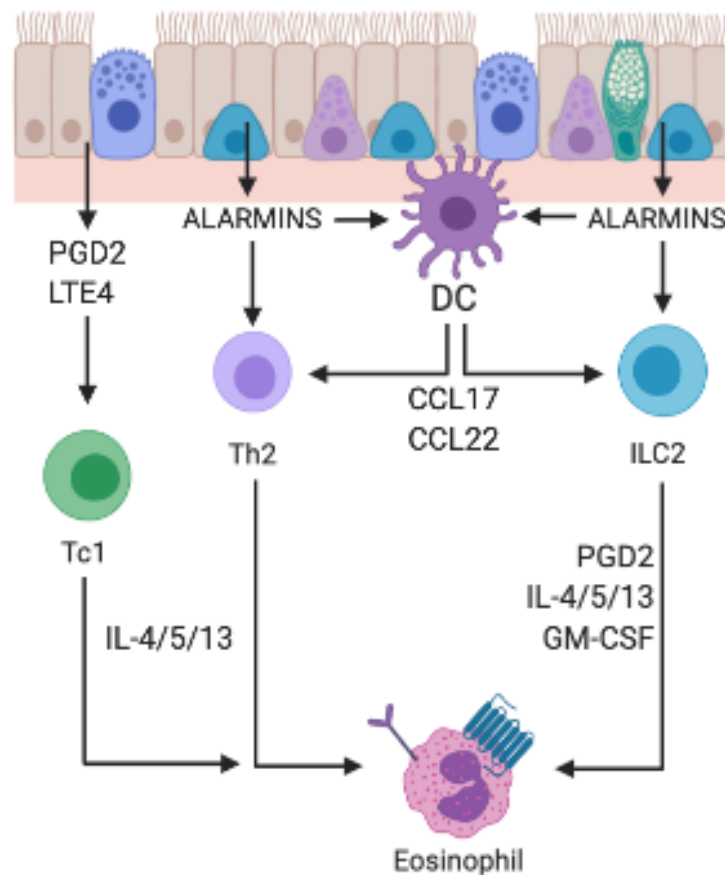


Figure 4. Type 2 mediated eosinophil regulation.

Inhaled irritants activate dendritic cells (DC) to secrete CCL17 and CCL22, initiating type 2 T helper cells (Th2s) and from type 2 innate lymphoid cells (ILC2s) recruitment along with alarmin (IL-25, IL-33, and TSLP) release from epithelial cells. Epithelial cells also release PGD2 and LTE4 which recruit the type 2 cytotoxic lymphocyte subset Tc1. Type 2 mediator secretion from recruited cells include IL-4, IL-5, and IL-13 along with PGD2 (ILC2s) and epithelial cells and GM-CSF from ILC2s which all contribute to eosinophil recruitment in the airways. Created in Biorender.com.

1.8 Oxidative stress

Reactive oxygen species (ROS) encompass a range of highly reactive chemicals formed from O_2 including singlet oxygen, superoxide, hydroxyl radicals, peroxides, and hydroperoxides (71). Generated species in healthy cells occur as a product of aerobic respiration and are actively scavenged to prevent excessive accumulation (72). At a controlled rate, they can act as signalling molecules involved in selective posttranslational modifications. For example, hydrogen peroxide interaction may cause p66Shc phosphorylation by protein kinase C (PKC) leading to mitochondrial

translocation and proapoptotic activation or act as iron chelators to increase IRP/IRE interaction (71).

Immune functions can also be impacted by oxidative stress. In COPD, alveolar macrophages have been shown to be defective in phagocytosing *H. influenzae* and *S. pneumoniae* following exposure to H₂O₂. This was found to be due to reduced mitochondrial ROS production, an essential feature of respiratory bursts required in bacterial killing (73).

Excessive accumulation of ROS can result in nucleic acid modification from DNA double-strand breaks and subsequent alterations to proteins and lipids if the damage is not repaired (74,75). Cigarette smoke is known to cause DNA damage partly through oxidative stress. There is evidence that specific DNA alterations may persist following smoking cessation, potentially accounting for the persistence in inflammation seen in patients who develop COPD and stop smoking. This may also explain why only a subset of smokers develop COPD, as certain people may have genetic traits that could make them more vulnerable to DNA damage which results in developing COPD (76).

Their ubiquitous generation has led ROS to be associated with various pathologies including endothelial dysfunction, tissue injury, and excessive inflammatory cell infiltration (75,77). Regulation of endothelial cell adhesion molecule expression is controlled by direct activation of redox-sensitive transcription factors and tight junction disassembly. Increases in ROS upregulate adhesion molecules, inducing increased influx of leukocytes across endothelial membranes, increasing local tissue inflammation (75).

ROS generally have a short lifetime and there are no sensors that can reversibly monitor their levels though, various compounds exist that can measure

the location of their formation and modifications they induce. DNA damage markers include 8-hydroxydeoxyguanosine (8-OhdG), the oxidised version of guanosine that is removed from amino acid chains during the DNA repair process, and detectable in various bodily excretions through ELISAs. Lipid peroxidation is another indicator of ROS induced damage. Malonic dialdehyde (MDA) is a common marker detectable through the thiobarbituric test. Intracellular markers include DCFH₂DA, a sensitive non-fluorescent probe capable of crossing cell membranes. Once it comes in contact with ROS, DCFH₂DA is converted into fluorescent DCF, providing a sensitive measure of ROS activity (78).

To counter the deleterious effects of ROS, cells employ numerous antioxidant enzymes to scavenge excess ROS. Proteomics analysis of sputum samples collected from smokers, individuals exposed to asbestos, and lung cancer patients has shown some promise as a diagnostic tool with increased abundance of peroxiredoxin 2 (PRDX2) and thioredoxin (TXN) when compared to healthy controls (79). In combination, detection of ROS production and antioxidant activities can provide valuable information into the effects of ROS generation and its relation to disease processes.

1.9 Airway inflammation in COPD

Both innate and adaptive immunity is implicated in response to smoking however, an exaggerated response is seen in patients with COPD. An arsenal of inflammatory cells are recruited to the lungs in response to the activation of epithelial cells, endothelial cells, and fibroblasts releasing inflammatory mediators.

Macrophages are prominent conductors orchestrating the inflammatory response in COPD. Sputum and bronchoalveolar lavage (BAL) samples from patients with COPD have been reported to have increased levels of CCL2 (MCP-1)

and chemokine ligand (CXCL) -1, crucial mediators for the recruitment of monocytes to the airways. This is coupled with monocytes showing increased chemotactic response to these factors when smokers are compared with non-smokers (80,81). Once these monocytes have migrated to the airways from the blood, they then differentiate into macrophages and release a host of inflammatory mediators themselves. This includes IL-1 β , IL-6, IL-23, tumour necrosis factor- α (TNF- α), CXCL1, CXCL8, CCL2, and LTB₄ which further recruit other inflammatory cell types (82). One of the primary functions of macrophages is phagocytosis of apoptotic cells and bacteria but this appears to be attenuated in COPD patients, possibly blunting their ability to resolve inflammation leading to a prolonged response and chronic bacterial colonisation (83) (see figure 5). Dendritic cells, responsible for antigen presentation to T lymphocytes in the lymph nodes, are crucial for differentiating lymphocytes and of particular importance helper T cells (Th2) (84).

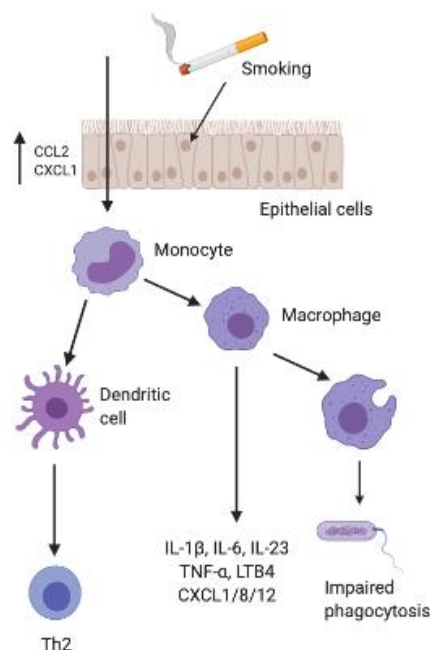


Figure 5. Schematic representation showing monocyte recruitment and differentiation.

Smoking stimulates increased CCL2 and CXCL1 release which recruits monocytes. Once in the airways, monocytes differentiate into macrophages which secrete IL-1 β , IL-6, IL-23, tumour necrosis factor- α (TNF- α), CXCL1, CXCL8, CCL2, and LTB₄. Macrophages may have impaired ability to phagocytose invading pathogens and resolve infections. Monocytes may alternatively differentiate into dendritic cells responsible for antigen presentation in the lymph nodes and recruitment of Th2 cells. Created in Biorender.com.

T lymphocytes are largely implicated in the pathology of COPD as their numbers correlate with both alveolar destruction and airflow obstruction. A subset known as cytotoxic T cells (Tc1) have been found to have elevated levels in patients with COPD, possibly exacerbating the inflammatory response within these individuals. Patients also have another Th1 cell subset found in an activated state due to the expression of STAT-4 (85). Cytokine release from macrophages recruit Th17 cells through IL-6/-23 and have increased cytokine expression in the bronchial mucosa of certain COPD patients (86,87). These cells are thought to be implicated in neutrophil recruitment in the lungs. Cytotoxic CD8⁺ cells release mediators including granzyme B, perforins, and TNF- α and are involved in bacterial defence. As bacterial clearance is found to be impaired in patients with COPD, CD8⁺ T cell numbers may be increased in response to this (85).

Th2 cells release the known type 2 mediators including IL-4, IL-5, and IL-13 which promote eosinophil recruitment and survival. An innate lymphoid cell (ILC)-2 subset also releases type 2 cytokines and responds to alarmins or 'damage signals.' (69) These cells release lipid mediators such as prostaglandin D₂ (PGD₂), also capable of recruiting eosinophils to the airways through binding to the chemotactic receptor type 2 (CRTh2) expressed on these cells (67) (see figure 6).

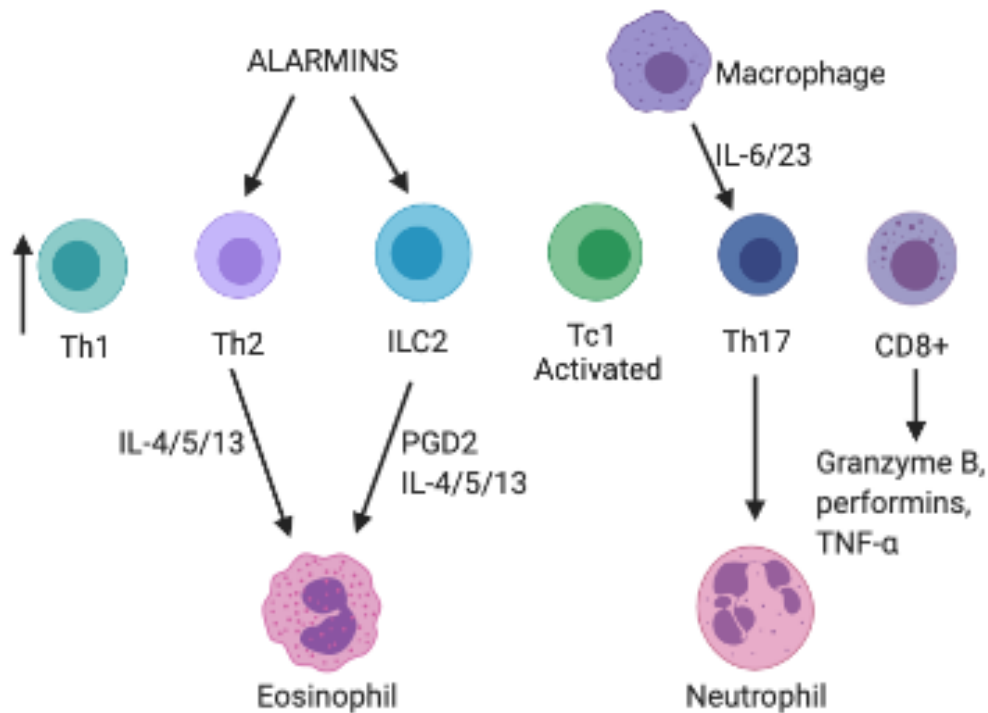


Figure 6. Lymphocyte activation and recruitment in COPD.

Lymphocyte numbers are increased in patients with COPD. Tc1 cells are also activated and CD8+ cells release mediators including granzyme B, perforins, and TNF- α which recruit inflammatory cells. Alarmin release from epithelial cells activate ILC2s to secrete PGD2, IL-4, IL-5, IL-13 along with Th2 cells to recruit eosinophils. Macrophages release IL-6 and IL-23 which stimulate Th17 cells to recruit neutrophils. Created in Biorender.com.

1.9.1 Neutrophilic inflammation

The most abundant leukocyte of the immune system is the neutrophil. Equipped with highly cytotoxic granule proteins that include myeloperoxidase (MPO), human neutrophil lipocalin, and numerous other proteases, these granulocytes have a multifaceted role in host defence (see figure 7). Once recruited to the site of inflammation, neutrophils clear pathogens through several mechanisms including degranulation of their intracellular proteins (23)(88). They can eject their granules along with chromatin DNA to trap and kill pathogens in so called neutrophil extracellular traps (NETs). Neutrophils are also capable of phagocytosing pathogens intracellularly in a process that is mediated by reactive oxygen species (ROS) production (23).

Neutrophilic airway inflammation, defined as >61% of non-squamous cells in sputum (89), is a dominant pattern of inflammation observed in COPD patients (14)(90). Sputum and BAL samples both show increased numbers of neutrophils that correlate with the severity of disease state (91)(92). Migration of neutrophils into the airways is mediated by E-selectin, which is reportedly upregulated on endothelial cells in patients with COPD (88). Other chemotactic factors that are increased in COPD to promote neutrophil recruitment include LTB₄, CXCL1, CXCL5 and CXCL8 (IL-8) (93).

Bacterial infections driving exacerbations have been associated with a neutrophilic phenotype which is documented in sputum expectoration produced by patients testing positive for bacterial infection (14). These samples are classified as being purulent, a sputum type associated with mucus hypersecretion from goblet cells and stimulated by neutrophils. Neutrophils are found to be highly activated in response to bacterial infection and release their cytotoxic granule proteins on the airways to destroy the invading pathogens. This has been hypothesised to stimulate local damage and alveolar destruction over prolonged instances (88).

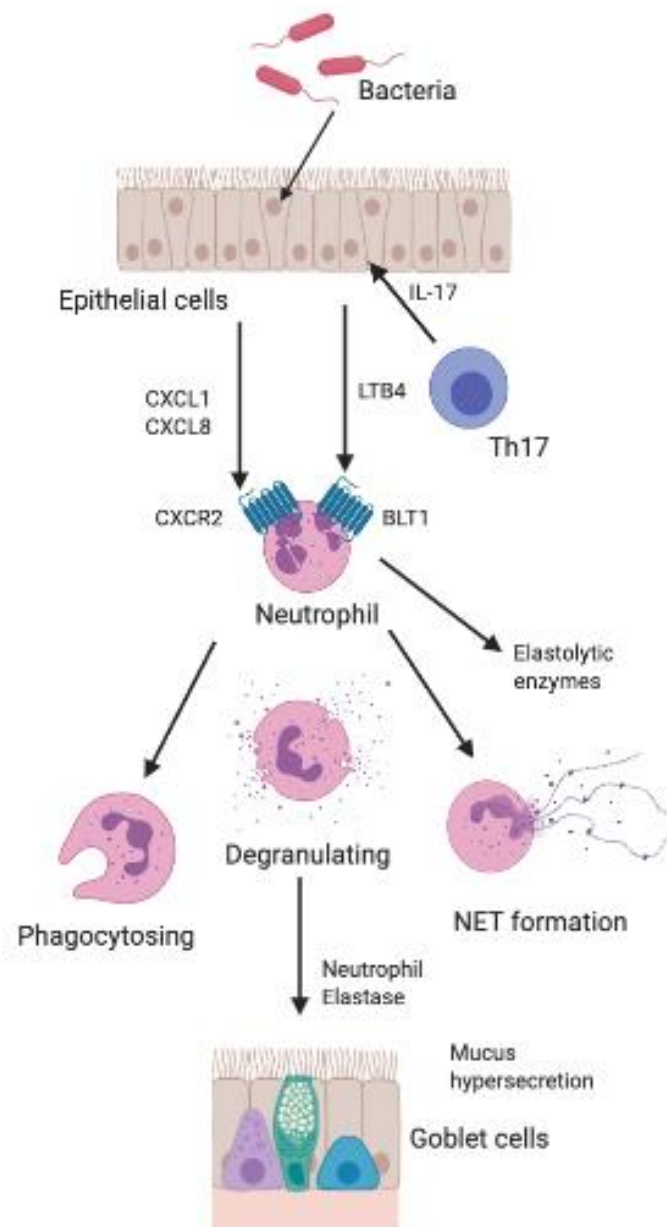


Figure 7. Neutrophil recruitment to the airways.

Bacterial infection stimulates neutrophil proliferation and release of CXCL1 and CXCL8 (IL-8) which recruits neutrophils through binding to the CXCR2 receptor on neutrophils. Th17 cells further recruit neutrophils through IL-17 release which stimulates epithelial cells. LTB4 release activates BLT1 receptors on neutrophils. Neutrophils resolve infections through several mechanisms including phagocytosis, neutrophil extracellular trap (NET) formation and degranulation of intracellular proteins. Neutrophil elastase release stimulates mucus hypersecretion from goblet cells. Created in Biorender.com.

1.9.2 The lung microbiome

Although it has long been accepted that the lungs are constantly exposed to bacteria, the concept that healthy lungs are sterile went unchallenged until the

advent of culture-independent techniques. It was not until Hilty *et al.* published their findings that healthy lower airways contain bacterial communities distinct from the upper respiratory tract in 2010 that researchers began to challenge this long held notion (94). The term microbiome is elegantly defined by Dickson *et al.* and the distinction between 'microbiota' and 'microbiome' outlined:

'... we use the term "microbiota" to mean the collection of microbes from a site. By microbiome, we mean the community of microscopic cells, viruses, and phages that occupy a site and are under environmental pressures from that site. The microbiome includes microbial and host cells, as well as the biotic and abiotic factors that modulate the interactions of these cells.' (95)

In healthy lungs, the microbiome is delicately balanced by immigration and elimination of microbial cells that make up the total microbial burden. However, bacterial infection develops when the relative reproduction rates overcome the hosts' defences. Chronic lung diseases, such as COPD, modify this balance through increased activation and infiltration of host inflammatory cells and chronic cough to eliminate microbes (96). Aspects of COPD histopathology may also increase the microbial burden within the lungs. Emphysema reduces the surface area of the lungs (97) without reducing the microbial burden, providing the ecosystem with a reduced terrain, thus increasing the burden. Selective pressures may therefore be introduced depending on the effectiveness of the host's immune cells at clearing microbes and strains (95).

In COPD, the microbiota varies depending on the severity of the disease (98). Patients with mild and moderate COPD have airway microbiota that represents healthy airways. However, patients with severe COPD have consistently altered airway microbiota (99). Importantly, it has been reported that microbial diversity decreases with the severity of disease with a shift from the Bacteroidetes phylum towards Proteobacteria (99,100). Patients may be predisposed to this shift as a

similar microbiota found between COPD patients and healthy volunteers reportedly displayed a proteobacteria skew following viral exposure that was not observed in healthy individuals (101).

1.9.3 Patterns of inflammation at the time of an exacerbation of COPD

Seminal work by Bafadhel *et al.* determined three biological clusters that drive exacerbations in COPD and one cluster that showed limited changes in its inflammatory profile. An extensive panel of measured sputum biomarkers were applied to factor and cluster analysis algorithms to identify the differential inflammatory profiles between the groups. These inflammatory profiles were broadly identified as bacteria-predominant, eosinophil-predominant, and viral-predominant (44).

The bacterial-predominant exacerbation cluster identified by Bafadhel *et al.*, showed an increase in IL-8 production and the resulting neutrophilic inflammation is a characteristic response to bacterial infection (44). The eosinophil-predominant exacerbation cluster showed increased levels of IL-5. Interestingly, these two patterns of inflammation were independent of each other. This inflammatory pattern is independent of the eosinophilic profile displayed in COPD both at exacerbation and during periods of stability. High blood eosinophil counts are associated with increased risk of future exacerbations and further eosinophilic exacerbations but without an infectious component (102,103). Additionally, analysis of the AERIS cohort found that eosinophilic exacerbations did not follow the known seasonal trends of viral and bacterial infections seen in the winter months. In fact, the odds ratio of patients with blood eosinophils equal to or more than 2% at the time of an

exacerbation were 2.65 times higher in the summer season (104). These observations in combination with findings that eosinophils possess potent antibacterial properties (105) may suggest that the eosinophil confers protection against pathogenic infection. Thus, these cells are a worthy topic of investigation.

1.10 Eosinophils

Eosinophils are inflammatory leukocytes with roles in both innate and adaptive immune responses (106). Their characteristic bi-lobed nucleus and cationic cytoplasmic granules allowed them to be identified by Paul Ehrlich in 1879 with use of the acidic aniline eosin stain, to which they owe their name (107). Circulating levels of eosinophils are typically low, comprising between 1 and 5% of the total white blood cell count however, this increases during helminth infections and atopic allergic disease (108).

Even to this day, the role of the eosinophil in health and disease is widely debated. Indeed, the nonspecific abilities of the eosinophil, low circulating numbers, and the advent of eosinophil depleting drugs with no obvious adverse effects (109,110), may make one question whether their role within the body is as anything more than as an auxiliary cell. Nevertheless, as our understanding of eosinophil biology has evolved, so too have the hypotheses relating to where the eosinophil fits within the immune system. Eosinophils are now implicated in an array of diseases, host defence against helminths, modulation of immune events and even in tissue remodelling.

Paul Ehrlich's contributions to not only immunology but also haematology, oncology, and chemotherapy cannot be overstated. In fact, his contributions were considered so profound that in 1908 he was awarded the Nobel prize in

Physiology and Medicine with Ilya Mechnikov. Although a clinician by training, Ehrlich maintained a strong research interest. The ability of dyes to stain tissue particularly fascinated him and became the topic of his dissertation in 1878 when he used aniline dyes to stain microscopic specimens (107). Once perfected, he used haematoxylin to stain nuclei blue and eosin to stain granules red in a similar manner that we use to stain eosinophils today. He used this technique to identify eosinophils in a variety of different animals and identified that there was a storage depot located in the bone marrow, correctly speculating that this was where they were formed. Staining granules allowed Ehrlich to differentiate neutrophils and basophils from eosinophils by observing their morphology in greater detail. He also reports on diseases associated with eosinophilia at that time including pemphigus, helminthiasis, acute and chronic skin-diseases, and asthma. He even speaks of eosinophils in the sputum of asthma patients in his book 'Histology of the blood: normal and pathological':

'... sputum of asthmatic patients contains, in addition to Charcot-Leyden's crystals, eosinophil cells only. One must therefore assume that within the bronchial tree there exists material which attracts the eosinophils. This supposition is also supported by the close connection that obtains, according to many observations, between the severity of the disease and the eosinophilia. Thus V. Noorden records that the eosinophil cells are more numerous about the time of an attack.'

He rightly argues against others who believed eosinophils originated locally in tissues, using inductive reasoning as an argument to support his claim that they emigrated from the blood to the organs (111). Although investigators at the time were correct to assume eosinophil presence correlated with certain diseases, a causative relationship between eosinophils and their direct involvement in disease progression was yet to be established. This trend would continue long after Ehrlich's era.

It was not until the end of the twentieth century when molecular biology

techniques became sophisticated enough to identify and characterise specific genes, along with monoclonal antibodies (mAbs) that would allow specific binding to receptors, that eosinophils could be studied in enough detail to investigate their functions more precisely. Gerald Gleich was at the forefront of these discoveries and rode the wave into this new era. He contributed extensively to our understanding of the properties and functions of eosinophil secondary granules, purifying each distinctive protein to homogeneity (112) and allowing a more in-depth investigation into their abilities (113).

Further studies found that granule proteins were commonly cationic, which explained why these proteins had such affinity for eosin. It was now possible to isolate individual proteins and investigate *in vitro* their toxicities against helminths, for which eosinophils had already been implicated in host defence. Major basic protein (MBP) and eosinophil cationic protein (ECP) were found to be particularly efficient at killing the larvae of *Trichinella spiralis* whereas eosinophil derived neurotoxin (EDN) was much less so (114). Similar results were seen in other helminths (115–117), firmly establishing eosinophils as end-stage effector cells involved in innate host defence against multicellular parasites. The destructive properties of eosinophil granules were quickly implicated in detrimental effects within the host tissues upon degranulation, particularly within asthma where accumulation of eosinophils was associated with lung dysfunction (118,119).

More recently, a group of researchers spearheaded by James J. Lee promoted the LIAR hypothesis – eosinophils being critical mediators of tissue homeostasis through Local Immune and Remodelling/Repair (120). This was in response to certain observations that did not quite match with the established paradigm of the eosinophil being a solely destructive end-stage effector cell.

Curiously, mouse models failed to repeat many of the *in vitro* observations including their involvement in many aspects of host defence.

Specht *et al.* reported that knockout mice for MBP and eosinophil peroxidase (EPX) did not show deficiencies in host defence rather, they alluded to other roles of eosinophils as regulators of immune response (121). Astoundingly, eosinophil deficient mice strains (PHIL and DdblGATA) have also provided evidence that eosinophils modulate the host immune system to be beneficial for sustained *Trichinella spiralis* survival (122). Eosinophil depletion had no difference on *Schistosoma mansoni* burden, egg deposition or other eosinophil immunological directed responses thought to be critical to host defence against parasites, including granuloma or fibrosis (123). Knock-out mice in asthma allergen challenge models added to the confounding data surrounding eosinophil functions. Humbles *et al.*, reported that eosinophils were not directly implicated in asthma airway pathogenesis but instead contributed to collagen and smooth muscle changes seen in tissue remodelling (124). These studies failed to provide evidence for eosinophil driven host cellular destruction because of nonspecific immune responses but alluded to a more complex role for eosinophils in mediating and directing immune responses.

Although the role of the eosinophil in disease pathogenesis is yet to be confirmed unequivocally, evidence is pointing away from eosinophils being solely responsible for end-stage effector functions and destruction. Indeed, the vast array of reported functions include communication with T cells (125,126), supporting glucose homeostasis in adipose tissue (127,128), tissue remodelling (124), and mediation of Th2 responses (129), providing convincing evidence in support of the LIAR hypothesis.

1.10.1 Eosinophil maturation

Derived from CD34+ myeloid progenitors, eosinophil differentiation and maturation is promoted by IL-3, granulocyte / macrophage-colony stimulating factor (GM-CSF), and IL-5 cytokines, which additionally induce functional properties in mature cells (130).

The bone marrow is the source of progenitor myeloid cells that must first undergo stages of differentiation and maturation before being released into the peripheral blood. The eosinophil shares its committed haemopoietic progenitor with basophils. These cells then differentiate in response to soluble mediators, such as the aforementioned cytokines, and transcription factors (131). There are various transcription factors involved in eosinophil commitment (see figure 8) with CCAAT/enhancer-binding protein (C/EBP) expression inducing eosinophil differentiation whereas dominant negative expression will inhibit commitment. C/EBP is required for commitment into the eosinophil progenitor and maturation into an eosinophil (132). An ETS transcription factor, PU.1, is critical myeloid lineage commitment and works in synergy with GATA-1 to produce eosinophils (133,134). The interferon consensus sequence binding protein (Icsbp) is another transcription factor essential for eosinophil commitment, the loss of which was found to produce eosinophil deficient mice (135). GATA-1 binds to the palindromic GATA sequence located in a multitude of eosinophil genes and importantly, the GATA-1 promoter sequence (134).

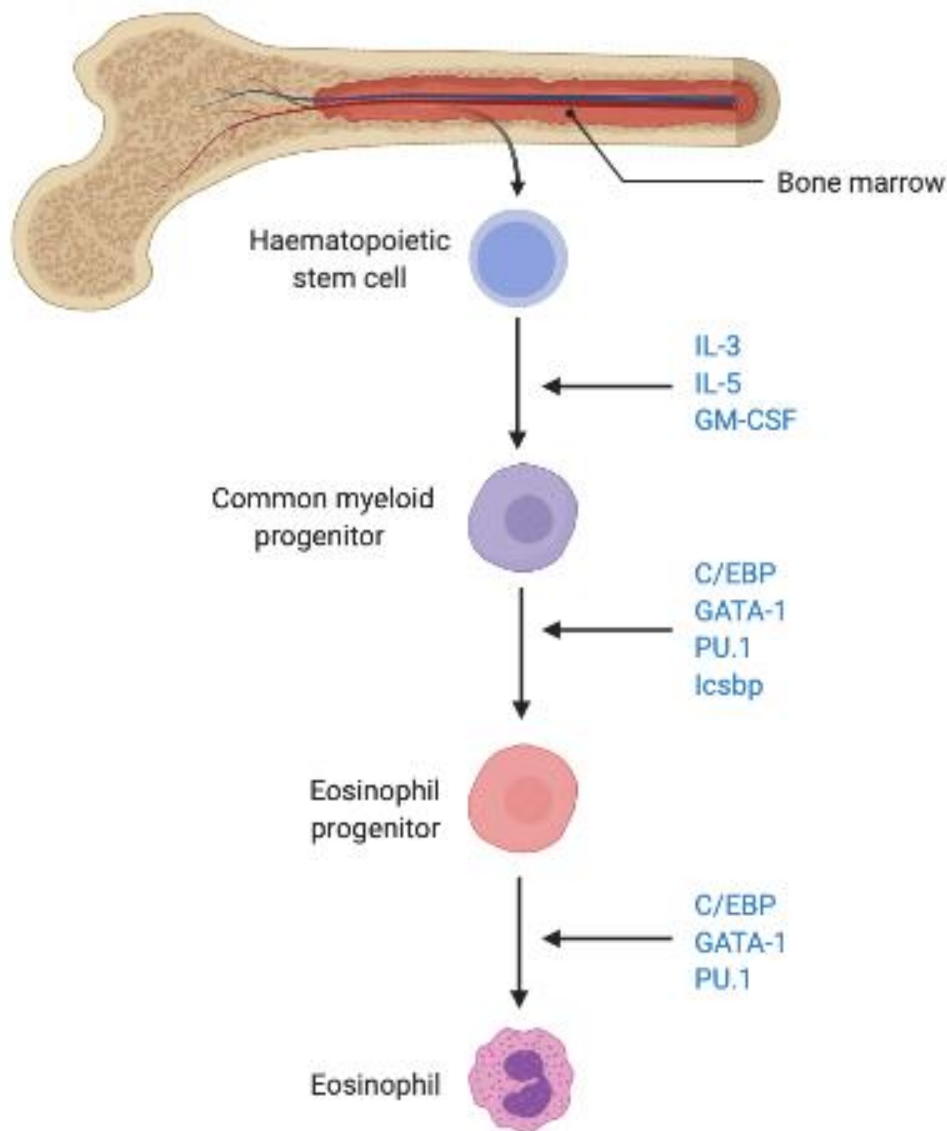


Figure 8. Cytokines and transcription factors involved in eosinophil lineage commitment. The bone marrow produces haematopoietic stem cells that differentiate depending on mediators and transcription factors that shift lineage commitment. C/EBP: CCAAT/enhancer-binding protein; Icsbp: interferon consensus sequence binding protein. Created in Biorender.com.

1.10.2 Eosinophil recruitment

Recruitment of eosinophils to specific tissues is controlled through a complex interaction of antigen-presenting cells, mast cells, T cells, B cells and cytokine release. IL-5 is the most eosinophil specific cytokine and recruits mature eosinophils from the bone marrow to the blood. In healthy individuals, eosinophils will predominantly localise to the gastrointestinal tract (136), but will also reside within

the thymus (137), uterus, and mammary glands.

The type 2 cytokines include IL-4, IL-5, and IL-13 and are commonly associated with allergy and eosinophil responses (138). As mentioned previously, IL-5 has a direct effect on eosinophil recruitment whereas IL-4/13 activate a STAT6-dependent pathway, releasing eotaxin-1 (CCL11), eotaxin-2 (CCL22), and eotaxin-3 (CCL26) that initiate chemotaxis. These three chemokines are released from tissue resident cells and from other cells recruited to the site of inflammation and are selective for eosinophils. Eotaxin-1 is related to early eosinophil recruitment whereas eotaxin-2 and -3 are implicated in later stages eosinophil recruitment. The common receptor for eotaxins is the G-protein coupled CC chemokine receptor 3 (CCR3) (139).

Arachidonic acid metabolites including the leukotrienes; LTB₄, LTC₄, LTD₄, LTE₄, and prostaglandin (PG) D₂ are also involved in eosinophil recruitment. The chemoattractant receptor Th2 (CRTH2) has high affinity for PGD₂ and, as its name suggests, is implicated in type 2 responses. In disease states and allergy, an increased release of cytokines is responsible for driving recruitment of elevated numbers of eosinophils to the site of inflammation.

Adhesion molecules are essential for trafficking cells across membranes. Eosinophils express various adhesion molecules and, depending on the molecule, will interact with specific proteins expressed on the surface of tissues that facilitate cell arrest and migration (see figure 9 for an example of eotaxin initiated cell trafficking and tables 2 and 3 for a summary of adhesion molecules and their characteristics).

Table 2. Summary of endothelial adhesion molecules and their characteristics

Endothelial cell adhesion molecule	CD designation	Inducing stimuli	Leukocyte ligands
ICAM-1	CD56	IL-1, TNF, IFN-g	CD11a/CD18, CD11b/CD18
ICAM-2	CD102	NONE	CD11a/CD18
VCAM-1	CD106	IL-1, TNF, IL-4	CD49d/CD29
E-selectin	CD62E	IL-1, TNF	sLe ^x
P-selectin	CD62P	Histamine, thrombin	sLe ^x

Table 3. Summary of eosinophil adhesion molecules

Adhesion molecule	CD designation	Ligands
LFA-1 (aLb2)	CD11a/CD18	ICAM-1 (domain 1)
Mac-1 (aMb2)	CD11b/CD18	ICAM-1 (domain 3), C3bi
p150,95 (aXb2)	CD11c/CD18	UNKNOWN
VLA-4 (a4b1)	CD49d/CD29	VCAM-1, fibronectin
VLA-6 (a6b1)	CD49f/CD29	Laminin
Act-1 (a4b7)	NONE	VCAM-1, MAdCAM-1, fibronectin
L-selectin	CD62L	Gly-CAM-1, CD34, MAdCAM-1
sLe^x	CD15s	E-selectin, P-selectin

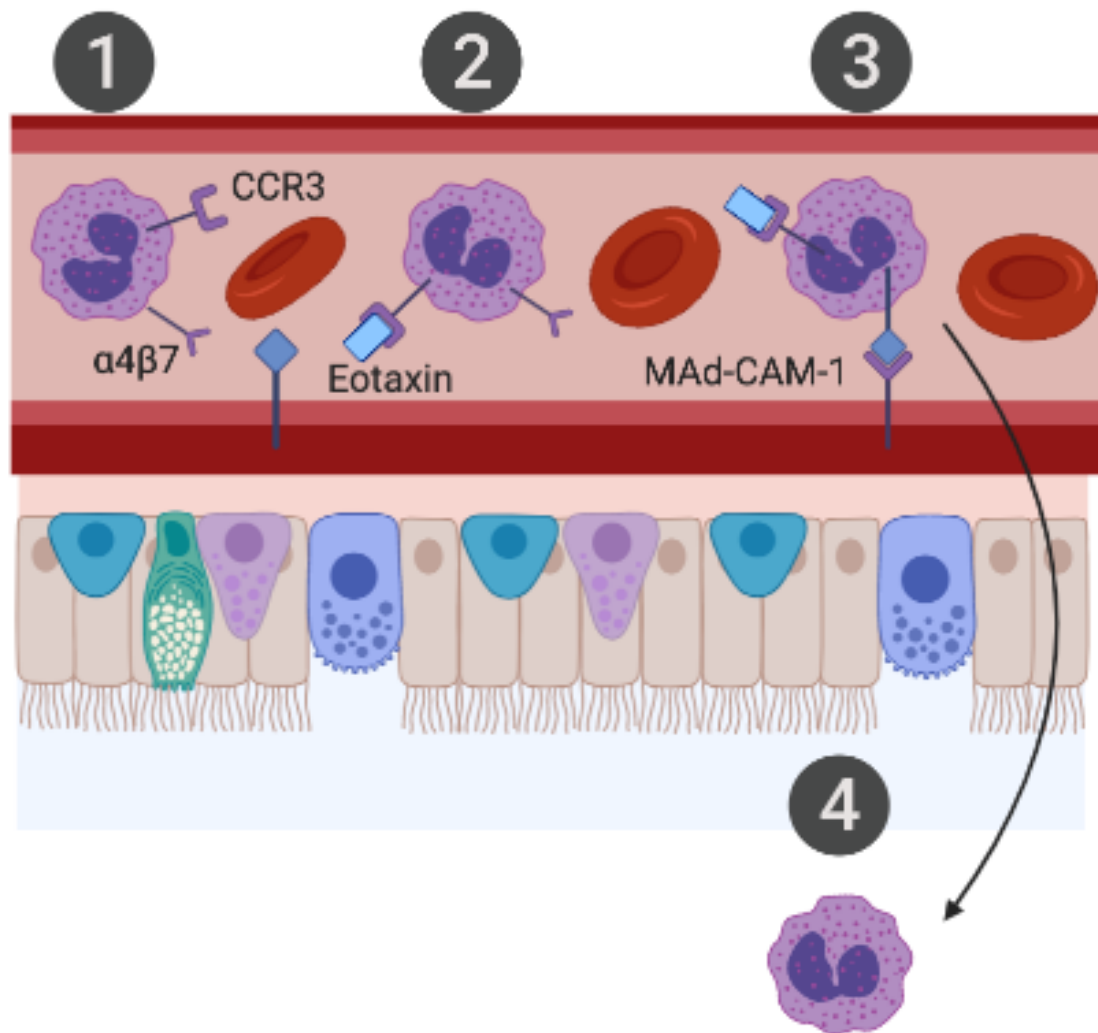


Figure 9. Eosinophil trafficking into tissue.

(1) Circulating eosinophils with $\alpha 4\beta 1$ integrin in the inactive conformation are (2) Primed by eotaxin (3) $\alpha 4\beta 1$ integrin adheres to MAdCAM-1 leading to eosinophil arrest on the endothelium. (4) $\alpha 4\beta 1$ integrin in the high activity conformation allows eosinophils to extravasate the endothelium into the tissue. Mad-CAM-1: mucosal addressin cell adhesion molecule-1; IL-5: interleukin-5. Created in Biorender.com.

1.10.3 Eosinophil granule proteins

As shown by Paul Ehrlich, eosinophil granule proteins are highly basic as they bind to acidic stains. At the core of these granules lies a crystalloid complex of major basic protein (MBP)-1 and MBP-2 surrounded by a matrix of eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), and eosinophil peroxidase (EPX).

Two separate genes encode MBP-1 and MBP-2 with MBP-2 selectively

expressed in eosinophils (140). The relatively small 14-kDa MBP is 117 amino acids long and has an isoelectric point of 11, making it extremely cationic (141). The precursor of MBP is lost in differentiating eosinophils indicating that mature eosinophils are unable to produce more of this protein once differentiation is complete (142). The cationic charge of MBP allows it to bind to anionic surfaces, disturbing the target lipid milieu and increasing the membrane permeability (141). These properties make MBP toxic to helminths and gave rise to eosinophils' role in host defence. However, these same properties are indiscriminate to host membranes, giving them the capacity to damage tissues also.

Eosinophil peroxidase (EPX) is a haem protein composed of a heavy chain subunit between 50 and 57 kDa and a light chain of 11-15 kDa. Making up around 25% of eosinophil granules, it has similar properties to neutrophil myeloperoxidase, indeed, it has largely the same sequence identity (143,144). EPX is involved in the catalysis metabolism of oxygen species generation that attack nucleophiles, inducing cell death (145).

Eosinophil cationic protein (ECP) also has two isoforms (ECP-1/2) and ranges between 16 and 21.3 kDa in molecular weight with an isoelectric point of 10.8. ECP directly binding to membranes will cause disruption and induce ion-selective pores, which may allow other cytotoxic molecules to enter the target cell. Like MBP, the basic properties of ECP are toxic to the targets they meet, including bacteria and helminths. The ribonuclease A (RNase A) superfamily includes ECP, also known as RNase-3, making RNA viruses particularly susceptible to its affects. *In vitro* experiments have implicated ECP in host defence against respiratory syncytial virus.

Another granule belonging to the RNase A superfamily is eosinophil derived

neurotoxin (EDN) or RNase-2. EDN is one of the most abundant RNases in humans and can induce the Gordon phenomenon by destroying cerebellar Purkinje cells, causing the paralytic syndrome. As both ECP and EDN are part of the same superfamily, they share 67% sequence homology giving them similar abilities. However, in comparison to the other eosinophil granules, EDN does not have the same cationic properties.

1.10.4 Eosinophil extracellular traps

A more recent discovery was that eosinophils have the capacity to eject their mitochondrial DNA in combination with the granule proteins ECP and MBP to produce so called eosinophil extracellular traps (EETs). These traps are similar to neutrophil extracellular traps (NETs) however, the selective release of only mitochondrial DNA prevents eosinophils from undergoing cell death as neutrophils do. EETs were first described by Yousefi *et al.*, in 2008 and were shown to bind and kill bacteria both *in vitro* and *in vivo*. The authors reported that this process suggested a novel mechanism vital for maintaining intestinal barrier function following epithelial cell damage, preventing invasion of bacteria (146). Following this discovery, several articles have since reported unique combinations for priming and stimulating eosinophils to release EETs, possibly providing protection from various bacterial species (147–149) and implicating traps in disease pathologies (150–153).

1.10.5 Eosinophil subtypes

Commonly thought of as end-stage effector cells due to their cytotoxic granules and associations with helminth infections, eosinophils are often implicated with increased patient morbidity and mortality. However, recent findings are causing a

shift in this paradigm. With the rise of the LIAR hypothesis, eosinophil function has extended beyond innate end-stage immune cells (120). Indeed, eosinophils do accumulate and degranulate in locations of tissue damage, as seen in asthma however, conclusive clinical evidence is yet to be reported to definitively link the two (154).

Eosinophils are known to reside in multiple locations throughout the body in healthy individuals including the gastrointestinal tract, thymus, and uterus with no detrimental effects (155). Depending on the local tissue milieu, it appears eosinophils are carrying out a range of functions and far exceeding their normal half-life in circulation (3-24 h) to 36 h in the lung and up to 6 days in the intestines, thymus, and uterus (156).

So called 'homeostatic (regulatory) eosinophils' (hEos) are reported to have various functions both in mice and humans (106), from maintaining mucus production and Th17/Treg balance in the intestines (157,158) to mammary gland development (159) and prevention of allergic Th2 responses in the lung (160). This contrasts with an 'inflammatory eosinophil' (iEos) subset which is abundantly recruited to the lung during allergic responses. These lung subtypes were first reported by Mesnil *et al.*, 2016, who found that in mice models of asthma, these subtypes presented discrete surface marker expression and a marker distinctly expressed on iEos - CD101. hEos even display a morphologically distinct ring-shaped nucleus whereas iEos retain the characteristic segmented nucleus. They also reported other modifications in humans, including Siglec-8⁺CD62L⁺IL-3R^{lo} expression in homeostatic eosinophils in the lung tissue of non-asthmatics and Siglec8⁺CD62L⁻IL-3R^{hi} expression in inflammatory eosinophils isolated from the sputa of asthmatic patients. hEos were also found to be IL-5 independent, in that

their numbers did not decrease after IL-5-a therapy and conversely recruitment to the lung was unaffected by recombinant IL-5 in naïve mice (160).

1.10.6 Eosinophilic inflammation

As described previously, an alternative pattern of inflammation observed in COPD is eosinophilic inflammation, recorded both at stable state and during exacerbations (15,44). The normal circulating level of eosinophils is much lower than neutrophils, only making up around 1-5% of the total white blood cell count. Eosinophilic COPD may occur in up to 30% of patients although the role of the eosinophil in the pathogenesis of COPD is still largely debated (161). Rather arbitrary thresholds are used to define peripheral blood eosinophilia; 350 cells per cubic millimetre of blood is generally considered to be the upper limit of normal circulating levels. Mild eosinophilia ranges between 351 – 1500 cells per cubic millimetre, moderate between >1500 – 5000 cells per cubic millimetre, and severe being above 5000 cells per cubic millimetre (162). In the sputum, a normal eosinophil count is found below 1.1% with a count made up of more than 3% eosinophils considered eosinophilic. Around 50% of asthma cases are identified as eosinophilic by following this procedure whereas between 20 and 40% of COPD cases are found to be eosinophilic (44).

Various disease states can induce eosinophilia both in the peripheral blood and in tissues. Parasitic helminth infections are the most common cause of eosinophilia in developing countries but with exposure rates to helminths comparatively low in industrialised countries, atopic diseases have the highest incidence in the west. Examples of allergic diseases with an eosinophilia component may include allergic rhinoconjunctivitis, eczema, and asthma however, eosinophilia

need not be present in both the peripheral blood and tissue concurrently for all diseases (108)(163).

Eosinophils differentiate in the bone marrow from progenitor cells in a process that is tightly regulated by transcription factors and three cytokines: IL-3, IL-5, and GM-CSF. Responsible for selective differentiation and eosinophil release from the bone marrow, it has been demonstrated that IL-5 is the most specific cytokine for the eosinophil lineage. This includes transgenic mice models that overproduce IL-5, which results in eosinophilia (164).

Eosinophilic allergic conditions may be driven by Th2 mediated responses that overproduce the cytokines IL-4 and IL-13, both involved in eosinophil recruitment to tissue through IgE and VCAM-1 production, and survival through IL-5 release (165). The same three cytokines that promote eosinophil proliferation in the bone marrow are also responsible for their survival in tissues and can prevent apoptosis for up to 14 days *in vitro*. In addition to Th2 pathways, eosinophils can also operate in an autocrine mechanism to stimulate their own survival under certain circumstances.

Mature eosinophils are recruited to the airways from the blood by activated epithelial cells secreting eotaxin (CCL11) and RANTES (CCL5). Alarmin release from epithelial cells stimulates Th2 cells and ILC2s to promote the survival and activation of eosinophils by the local release of IL-5 (67) (see figure 10). Eosinophils have the capacity to release an arsenal of cytokines, chemokines, and growth factors along with their own cytotoxic granule proteins capable of airway remodelling and host antibacterial defence.

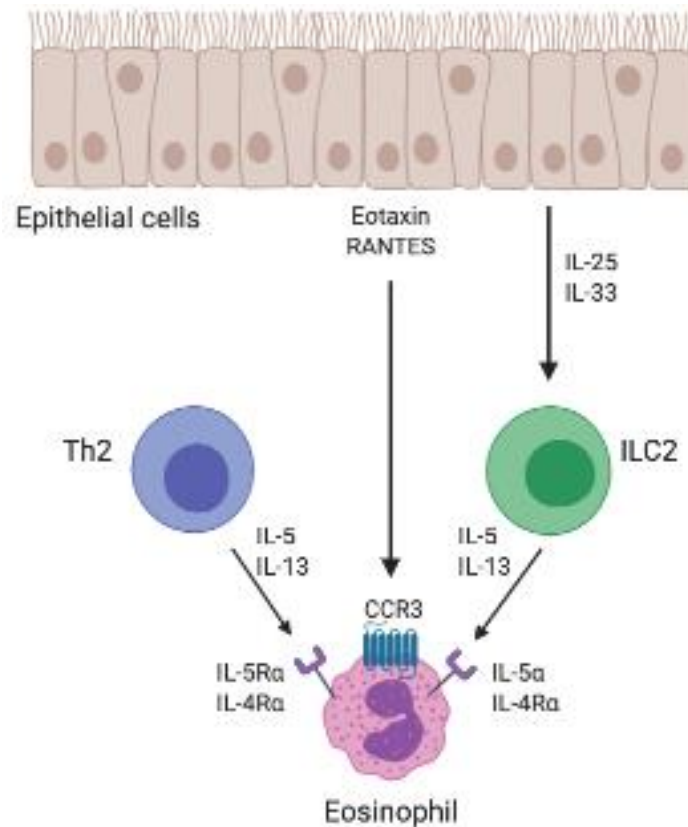


Figure 10. Eosinophil recruitment to the airways.

Eosinophils are recruited to the airways through eotaxin (CCL11) and RANTES (CCL5) release from epithelial cells which bind to the CCR3 receptor on eosinophils. IL-25 and IL-33 release stimulates ILC2s to release IL-5 and IL-13 which are also released from Th2 cells. Binding to the IL-5 receptor alpha and IL-4 receptor alpha, these cytokines promote eosinophil activation and survival. Created in Biorender.com.

Although the mechanism initiating eosinophilic airway inflammation in COPD is unclear, some studies have reported an inverse relationship between lung function decline and the ratio of activated eosinophils (166)(167). More recently, studies have linked COPD patients presenting at exacerbation with an eosinophilic phenotype and a more favourable response to steroid treatment (168,169), implicating the eosinophil in disease pathogenesis. This has led to the Global Initiative for COPD Guidance (GOLD) updating their criteria in 2019 to include the patient blood eosinophil count as a biomarker for directing the use of inhaled corticosteroids (51).

1.11 Study hypothesis

Chronic obstructive pulmonary disease is a progressive, irreversible disease characterised by inflammation of the airways. Eosinophils are a type II effector cell with elevated levels observed in up to 30% of COPD patients during acute exacerbations.

I hypothesise that eosinophils drive acute exacerbations through uncontrolled innate functions that have deleterious effects on the host.

1.11.1 Study aims and objectives

Aim 1: Determine the gene expression profile of eosinophils isolated from the peripheral blood of healthy volunteers, patients with COPD at stable state and during an eosinophilic exacerbation.

Eosinophils are notoriously difficult to extract RNA from due to the RNAses they produce compounded with their relatively low circulating cell number. As such, very few studies have attempted to sequence the eosinophil genome, and none have investigated the transcriptional changes during an eosinophilic COPD exacerbation.

Objectives to investigate Aim 1:

- i. Determine the optimum RNA extraction method to secure high quality RNA from eosinophils.

- ii. Compare the differential gene expression between healthy volunteers, stable state COPD and during an eosinophilic exacerbation using mRNA-seq.
- iii. Validate any potential genes of interest through PCR, surface protein assessment, and cytokine analysis.

Aim 2: Assess eosinophil-derived oxidative stress and its relation to eosinophilic exacerbations.

RNA-seq analysis revealed differential gene expression of genes related to oxidative stress between patients undergoing an eosinophilic exacerbation and healthy volunteers. This chapter will aim to explore these mechanisms and the effect on epithelial barrier.

Objectives to investigate Aim 2:

- i. Measure the production of mitochondrial, intracellular, and extracellular ROS production following stimulation and during an eosinophilic exacerbation. Neutrophils from the same donors will be included as a control.
- ii. Assess oxidative stress through the detection and quantification of the reduced and oxidised glutathione ratio.
- iii. Measuring any changes in epithelial permeability as an indicator of tight junction damage following extracellular ROS production from eosinophils.

Aim 3: Characterising eosinophilic inflammation in the context of COPD.

Although around a third of COPD exacerbations are eosinophilic, the pathophysiological drivers of an eosinophilic exacerbation are unknown. Measuring the cytokine profile and eosinophil surface marker expression during an exacerbation will contribute towards understanding a role for eosinophils in promoting exacerbations.

Objectives to investigate aim 3:

- i. Measurement of eosinophil specific cytokines in serum and sputum to characterise the cytokine profile during an eosinophilic exacerbation.
- ii. Measurements of eosinophil granule protein (EPX) as a marker of degranulation to determine its utility as a biomarker of exacerbations.
- iii. Surface marker assessment of eosinophils from whole blood and sputum and measurement of reported inflammatory eosinophil subtypes (iEOS) that may be a driver of exacerbations.

Chapter 2

Methods

2 Methods

2.1 Clinical sample collection

2.1.1 Clinical trials

2.1.1.1 Acute exacerbations treated with benralizumab (ABRA) trial

To address the role of eosinophils in COPD, clinical samples were utilised from two separate clinical trials. The acute exacerbations treated with Benralizumab (ABRA) study investigated whether administration of Benralizumab alone, or in combination with prednisolone, will improve clinical outcomes compared to prednisolone alone at the point of an exacerbation. Sampling for blood and sputum was performed as per study flow diagram (Fig. 11).

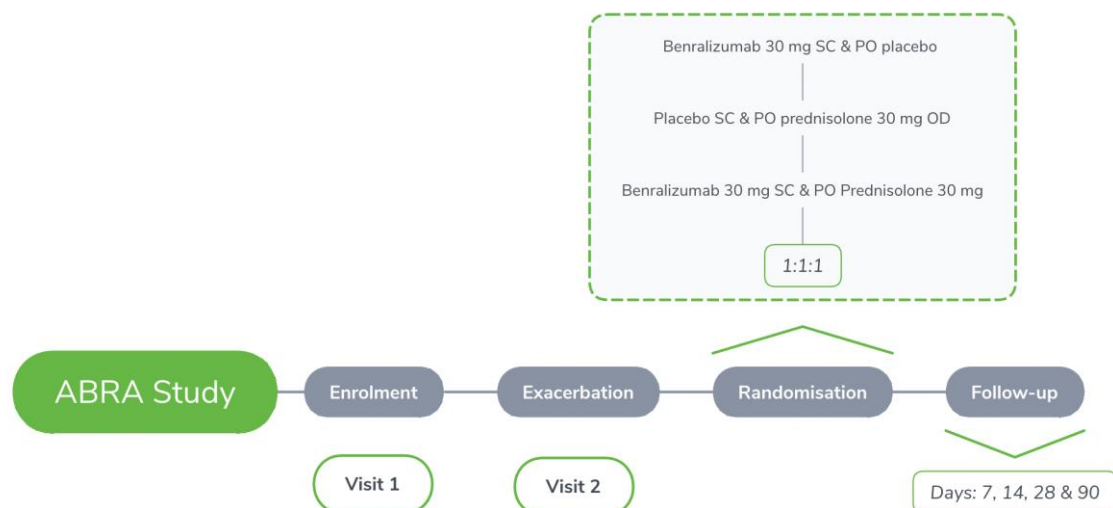


Figure 11. ABRA study.

Flow diagram showing the visits and randomisation schedule for the ABRA study. Created in Biorender.com.

Trial Participants

Adult participants were aged ≥ 18 with a diagnosis of asthma and/or COPD.

Inclusion Criteria

- Participant was willing and able to give written informed consent for participation in the trial.
- Male or Female, aged ≥ 18 years or above.
- A diagnosis made in primary or secondary care, of:
 - COPD with current or historic evidence of spirometry confirming airflow obstruction (FEV_1/FVC ratio <0.7) and a smoking pack year history of ≥ 10 . Or,
 - Asthma with current or historic evidence of spirometry confirming variable airflow limitation (any one of airflow reversibility FEV_1 change >200 mL; and/or $FEV_1\%$ change of 12%; and/or $P_{c20} \leq 8$; and/or peak flow diurnal variation; and/or variable FEV_1/FVC ratio) and a smoking pack year history <10 . Or;
 - COPD and asthma (as defined above)
- A history of at least 1 exacerbation requiring oral/intravenous corticosteroids in the previous 12 months.
- Prior (within 2 years) evidence of eosinophilic inflammation; including an elevated exhaled nitric oxide (F_{ENO}) ≥ 25 ppb; and/or peripheral blood eosinophil count ≥ 250 cells/ μ L; and/or sputum eosinophils $\geq 3\%$ of the total cell count.
- Female participants of childbearing potential unless surgically sterile and/or at least 2 years post-menopause must agree to use effective measures of birth control (including sexual abstinence, vasectomised sexual partner,

female sterilization by tubal ligation, any effective intra-uterine device, Depo-Provera injections, oral or transdermal contraceptive) from study recruitment to 16 weeks of the last dose of IMP.

- Male participants who were sexually active with partner(s) of child-bearing potential must have used an adequate method of contraception (condom) or be surgically sterile from the first dose of IMP until 16 weeks after this dose.
- In the Investigator's opinion, was able and willing to comply with all trial requirements.

Exclusion Criteria

- A known allergy to IMP (Benralizumab or prednisolone).
- Clinically important and significant pulmonary disease other than asthma or COPD (e.g., lung cancer, pulmonary fibrosis, bronchiectasis as primary respiratory problem, active pulmonary tuberculosis, cystic fibrosis, obesity hypoventilation syndrome).
- Another clinically significant pulmonary or systemic disease associated with an elevated peripheral blood eosinophil count (e.g., allergic bronchopulmonary aspergillosis, eosinophilic granulomatosis with polyangitis, hyper-eosinophilic syndrome, and helminth infection).
- Unstable ischaemic heart disease, arrhythmia, cardiomyopathy, heart failure, significant renal or hepatic impairment, uncontrolled hypertension, or ECG abnormality as defined by the investigator, which in the judgement of the investigator may put the patient at risk or negatively affect the outcome of the study.

- An alanine aminotransferase (ALT) or aspartate aminotransferase (AST) level that is persistently ≥ 1.5 times the upper limit of normal.
- Regular use of immunosuppressive medication (including but not limited to maintenance daily prednisolone ($>10\text{mg}$ per day), hydrocortisone, azathioprine, or weekly methotrexate).
- Established use (greater than 3 months) of long-term oxygen therapy (i.e., receiving oxygen therapy for >15 hours per day).
- The presence of hypercapnic ventilatory failure and/or the requirement of nocturnal non-invasive ventilation therapy.
- Participant with life expectancy of less than 6 months.
- Any other unstable significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the trial, or may influence the result of the trial, or the participant's ability to participate in the trial.
- Receipt of any licenced (e.g., omalizumab, mepolizumab or benralizumab) or other monoclonal antibody or polyclonal antibody therapy (e.g., gamma globulin) within 6 months.
- A history of known immunodeficiency disorder (including HIV-1 or HIV-2).
- Positive hepatitis B surface antigen, or positive hepatitis C virus antibody serology or a known medical history of hepatitis B or C.
- A history of drug or alcohol abuse in the previous 12 months, which in the opinion of the investigator, may compromise study data interpretation.
- A current (or within 5 years) history of solid organ or haematological malignancy.
- Female participant who is pregnant, lactating or breast-feeding.

1.1.1. Additional exclusion criteria at Exacerbation Visit 2

- *Fever recorded as $>38^{\circ}\text{C}$ measured using the tympanic temperature and/or a suspected pulmonary bacterial infection (chest radiograph demonstrating consolidation).
- *Type 2 respiratory failure necessitating non-invasive or invasive ventilation
- Any clinically significant abnormal findings in physical examination, vital signs, haematology, clinical chemistry or urinalysis, which in the opinion of the investigator, may put the subject at risk because of their participation, or may influence the results of the study, or the ability to complete the duration of the study.
- *An alternative cause for the increase in symptoms that are unrelated to an exacerbation such as i) suspicion or clinical evidence of pneumonia; ii) high probability and suspicion of pulmonary embolism; iii) suspicion or clinical evidence of a pneumothorax; iv) primary ischaemic event – ST or Non ST elevation myocardial infarct and left ventricular failure [i.e. not an exacerbation of asthma and/or COPD].
- *Treatment with oral corticosteroids and/or hospitalisation for an exacerbation of asthma and/or COPD in the previous 4 weeks prior to randomisation.
- *A confirmed (radiological) diagnosis of pneumonia 8 weeks prior to Exacerbation Visit, based on the last date of antibiotic treatment or hospitalisation date.
- *More than 12 hours of oral corticosteroid treatment for a current exacerbation
- Pregnancy or a positive urinary βHCG

- Donation of blood, plasma, or platelets within 90 days prior to Visit 2.
- Receipt of blood products within 30 days prior to Visit 2.
- Participants who had participated in another research trial involving an investigational product in the previous 4 weeks or 5 half-lives prior to visit 2
- Scheduled elective surgery or other procedures requiring general anaesthesia, within 3 months of the randomisation visit.
- Treatment with allergy immunotherapy, actively or within 90 days prior to Visit 2.

**asterisked additional exclusion criteria note: participants with these criteria could have entered in the 'exacerbation pool' for future exacerbation events that may have been suitable for randomisation to the interventional arm*

2.1.1.2 A 12-week exploratory study to characterise the relationship between changes in inflammatory markers, lung function, symptoms and reliver use in chronic pulmonary disease patients (CORAL)

The CORAL study was a phase IV, open-label, single-arm 12-week observational longitudinal study that monitored the relationship between changes in inflammation, lung function, symptoms and medication use in patients with COPD. Sample collection is outlined in figure 12.

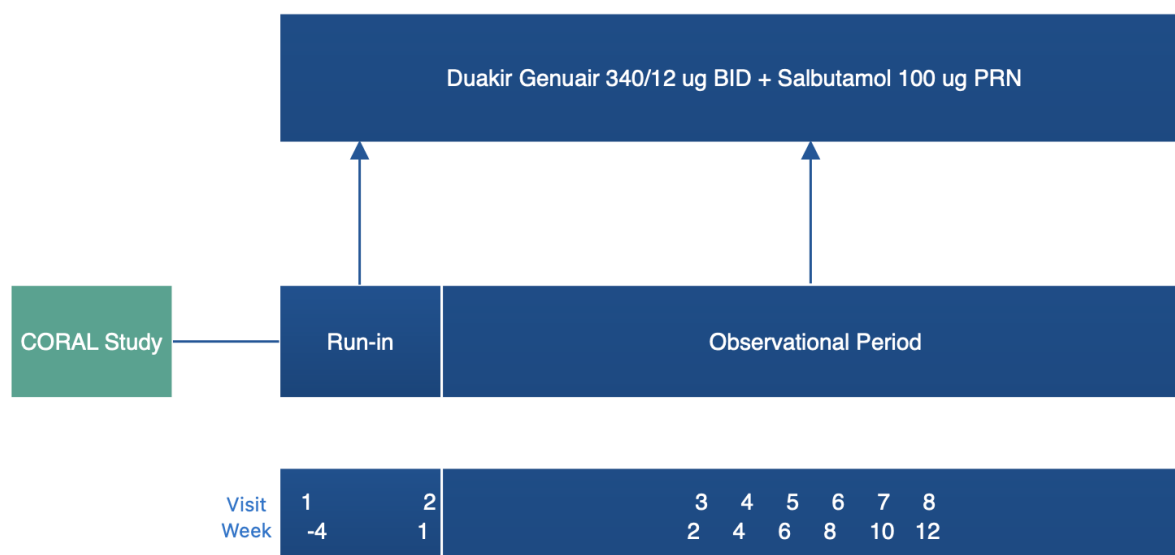


Figure 12. CORAL study.

Flow diagram showing the visits and observational period schedule for the CORAL study. BID, twice per day. PRN, as needed. Created in Biorender.com.

Study Population

The study included adult male or female subjects (at least 40 years of age) diagnosed with symptomatic COPD and blood eosinophil count $>0.1 \times 10^9$ cells/L, who were receiving maintenance treatment with a LABA in combination with ICS and/or a LAMA and required use of an inhaled short-acting bronchodilator (β_2 agonists or anticholinergics) as rescue medication. The study will include both current and former smokers (with at least 10 pack-year history) and intends to recruit a maximum of 30% current smokers.

Inclusion Criteria

Criteria for Entry in the Run-in Period

Subjects must have met the following criteria for enrolment in CORAL:

- 1 Provision of signed and dated, written consent form prior to any study specific procedures, sampling, and analyses (at Visit 1).
- 2 Male or female subjects ≥ 40 years of age at the time of signing the consent form.

3 A physician diagnosis of COPD and receiving maintenance treatment with a LABA in combination with an ICS, a LAMA, or both (ie, LABA+ICS, LABA+LAMA, or LABA+ICS+LAMA), for at least 3 months prior to Visit 1.

4 Physician-documented prescription and subject-confirmed use of an inhaled short-acting bronchodilator (β_2 agonists or anticholinergics) as rescue medication prior to study.

5 Current or ex-smoker with ≥ 10 pack-year history.

6 Post-bronchodilator FEV1/FVC < 0.7 recorded in medical history.

7 Blood eosinophils $> 0.1 \times 10^9$ cells/L.

8 The subject must have been able to read, speak, and understand English; and be able to, in the Investigator's judgment, comply with the Study Protocol.

9 Negative pregnancy test (urine) for female subjects of childbearing potential at Visit 1.

Criteria for Entry in the Observational Period

After completion of the run-in period, for entry in the observational period (at Visit 2), subjects must have met the following criteria:

1 Frequent use of SABA (≥ 200 μg of Ventolin per day, for at least 75% of days in the 3 weeks prior to Visit 2).

2 At least 70% compliance with daily FeNO, spirometry, EXACT-RS, and BCSS during the last 3 weeks prior to Visit 2.

3 Blood eosinophils $> 0.1 \times 10^9$ cells/L at Visit 2.

4 Negative pregnancy test (urine) for female subjects of childbearing potential at Visit 2.

Exclusion Criteria

- 1 As judged by the Investigator, any evidence of drug/substance abuse which would have posed a risk to subject safety, interfered with the conduct of study, have an impact on the study results, or affect the subject's ability to participate in the study.
- 2 Concurrent Respiratory Disease: Presence of a known pre-existing, clinically important lung condition other than COPD.
- 3 Concurrent diagnosis of asthma (subjects with a history of asthma may have been included).
- 4 Any disease currently treated with chronic oral/systemic corticosteroids.
- 5 Subjects with a history/treatment of malignancy, and which in the Investigator's opinion could compromise the safety of the subject.
- 6 Other Concurrent Medical Conditions: Subjects who had known, pre-existing, clinically significant endocrine, autoimmune, metabolic, neurological, renal, gastrointestinal, hepatic, haematological or any other system abnormalities that were uncontrolled with standard treatment.
- 7 Subjects currently treated with eosinophil-depleting medications (ie, mepolizumab, benralizumab), or who had been treated within 5.5 half-lives of eosinophil-depleting medications.
- 8 Subjects who have had a COPD exacerbation requiring oral corticosteroids and/or antibiotics in the last 30 days before the run-in period. Subjects were able to be re-screened 30 days following an exacerbation.
- 9 Participation in another clinical study with any marketed or investigational biologic drug within 4 months or 5 half-lives (whichever is longer) prior to Visit 1.

10 Participation in another clinical study with a non-biologic investigational product or new formulation of a marketed non-biologic drug during the previous 30 days prior to Visit 1 that, in the opinion of the Investigator, could have impacted the results of the current study.

11 Subjects with a known hypersensitivity to the treatment or any of the excipients of Duaklir and Ventolin.

12 Involvement in the planning and/or conduct of the study (applies to both AstraZeneca staff and/or staff at the study site).

13 Judgment by the Investigator that the subject should not participate in the study if the subject was unlikely to complete the observational period (e.g., literacy, substance abuse, life-threatening comorbidity).

14 Previous entry into the observation period in the study.

15 For women only: at the time, they were pregnant (confirmed with positive pregnancy test), breast-feeding or planned pregnancy during the study. Women of childbearing potential who were not using acceptable contraceptive measures, as judged by the Investigator.

16 As judged by the Investigator, planned hospitalisation/vacation during the study that interferes with study assessments.

The clinical characteristics of the patients sampled at different disease states (stable versus eosinophilic crisis), in addition to healthy controls are shown in table 4.

Table 4. Clinical characteristics of COPD patients and volunteers

	Stable (n = 54)	Eosinophilic Crisis (n = 24)	Healthy (n = 10)
Males, n (%)	31 (59)	12 (50)	7 (70)
Mean age (range), years	68 (35-85)	68 (35-84)	31 (26-33)
Ex-smokers, n (%)	38 (70)	22 (91)	-
Pack year history	32 (3.1)	20 (2.1)	-
Long-acting beta agonist usage, %	100	100	-
Inhaled corticosteroid usage, %	40.7	87.5	-
FEV1% predicted	45.6 (3.1)	38.0 (3.84)	-
FVC measured (L)	3.6 (0.2)	2.5 (0.2)	-
FEV ₁ /FVC (%)	64.2 (4.5)	44.1 (3.8)	-
White cell count (x10 ⁹ /L)	8.02 (0.4)	8.4 (0.5)	7.15 (1.2)
Peripheral blood neutrophil count (x10 ⁹ /L)	5.2 (0.3)	5.5 (0.4)	4.45 (1.1)
Peripheral blood eosinophil count (x10 ⁹ /L)	0.5 (0.1)	0.76 (0.45)	0.11 (0.04)

Data are presented as means ($\pm$ SEM)

2.2 Sample processing

2.2.1 Peripheral blood granulocyte isolation

Heparinised whole blood (minimum 40 mL) was diluted 1:1 with Dulbecco's phosphate buffered saline (DPBS; Sigma) and granulocytes collected using density gradient centrifugation. Diluted blood was layered over 1.07 ficoll-hypaque (Sigma) and centrifuged for 25 minutes, 500 x g, acceleration 1 and deceleration 0, following

separation of plasma, PBMC and ficoll were discarded leaving the red blood cell/granulocyte layer. To separate red blood cells from granulocytes, the cell pellet was resuspended in an equal volume of 3% dextran (GE Healthcare, New York, USA) and incubated with the granulocyte / red blood cell pellet for 30 minutes in the dark at room temperature. The leukocyte-rich upper layer was harvested and washed in DPBS and centrifuged at $300 \times g$ for 10 minutes at 4°C . Residual red blood cells were lysed using cold low endotoxin distilled water (Gibco, USA) for 20 seconds with the addition of DPBS to stop the hypotonic lysis. Cells were pelleted as above, resuspended in DPBS and cell number determined using trypan blue (sigma, USA) exclusion for viability.

2.2.2 Enrichment of eosinophils by magnetic associated cell sorting

Eosinophils were enriched using CD16 negative selection, cells were resuspended in 50 μL of MACS buffer (PBS, 0.5% BSA and 2 mM EDTA) per 5×10^7 total cells and 50 μL of CD16 MicroBeads (Miltenyi, Germany). The cell/bead mix was incubated for 30 minutes at 4°C then washed in 2 mL of MACS buffer and centrifuged at $300 \times g$ for 10 minutes. The cell pellet was resuspended in 500 μL of MACS buffer and magnetic cell separation was then carried out by applying the suspension onto the using MACS LS column (Miltenyi). Unlabelled cells were collected, and the LD column washed with 2 x 1 mL with MACS buffer. The total effluent was then centrifuged at $300 \times g$ for 10 minutes.

To determine the optimum method of eosinophil isolation, a MACSxpress whole blood eosinophil isolation cocktail mix was also used. Briefly, the lyophilized pellet was reconstituted with 'buffer A' which was then mixed with 'buffer B'. Whole blood was mixed with this solution in a Falcon tube and incubated for 5 minutes at

room temperature whilst rotating. The tube was then placed in the MACSxpress separator for 15 minutes allowing the magnetically labelled cells to adhere to the wall of the tube and the supernatant containing unlabelled eosinophils to be pipetted into a new tube. Red blood cells were lysed as described above.

2.2.3 Sputum processing

Spontaneous sputum samples were weighed, plugs selected and transferred to a new petri dish to reduce saliva contamination. Each individual plug was then transferred to a Falcon tube and eight times the plug's weight of DPBS added and a single plug removed and stored neat at -80°C for microbiome analysis. This was then centrifuged at 400 x *g* for 5 minutes at 4°C. Four times the plug's weight of supernatant was transferred into a new tube and centrifuged at 17000 x *g* for 5 minutes at 4°C, the supernatant was aliquoted and stored at -80°C for later use. Four times the sample weight of 0.2% sterile DTT (Sigma) was added and incubated on ice for 15 minutes with intermittent vortexing to break up the sample. The sample was then filtered to remove any debris and weighed. A 1:2 trypan viability count was carried out to calculate the total number of live cells and squamous contamination. To determine sputum differential cell counts cytopspins were performed, the sample was centrifuged at 400 x *g* for 5 minutes at 4°C and re-suspended in DPBS to 1×10^6 / mL, 7.5×10^5 was removed, applied to a cytopsin slide and spun at 450 rpm for 6 minutes. The slide was air-dried, fixed in methanol for 10 minutes, and 'Buffer B' from the Rapi-diff kit applied for 5 minutes followed by washing with deionised water and 'Buffer C' applied for a further 5 minutes.

2.3 RNA-seq transcriptomics

2.3.1 RNA isolation

RNA was extracted from isolated peripheral blood eosinophils using the RNeasy Mini Kit (Qiagen, Germany). Cells were pelleted by centrifugation at 300 x *g* for 10 minutes and supernatant removed. The cells were resuspended in buffer RTL with β -mercaptoethanol (β -ME) (1:100) using vortexing to homogenise the cell pellet lysate. 1 volume of 70% ethanol was added and mixed well by pipetting. Up to 700 μ L of the sample was transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 seconds at >8000 x *g* with the flow-through discarded. 700 μ L of buffer RW1 was then added to the RNeasy spin column and the centrifugation step repeated to wash the spin column membrane. The flow-through was discarded and 500 μ L of buffer RPE was added to the spin column and the sample again centrifuged for 15 seconds at >8000 x *g*. Once the flow-through was discarded, 500 μ L of buffer RPE was added to the spin column and centrifuged for 2 minutes at >8000 x *g*. The RNeasy spin column was then placed in a new collection tube and 30 μ L of RNase-free water added directly to the spin column membrane. The column was then spun for 1 minute at >8000 x *g* to elute the RNA. Isolated RNA was then assessed by pipetting 1 μ L on a NanoDrop spectrophotometer (Thermo) to check for RNA amount. RNA purity was assessed on the Agilent 4200 TapeStation for RNA integrity number (RIN) by pipetting 5 μ L of sample buffer and 1 μ L sample or RNA ladder in a tube strip. The strip was then heated at 72°C for 3 minutes and cooled on ice before vortexing. The samples could then be analysed on the ScreenTape device. RNA taken for poly A enrichment library preparation had OD_{260/280} $\geq$ 2.0 and RIN greater than 6.8.

2.3.2 RNA library generation and sequencing

RNA-seq library preparation was carried out by Novogene (Cambridge). Total RNA quality was confirmed using a Bioanalyser 2100 RNA Pico kit (Agilent) and quantitated using a Qubit 2.0 (Life Technologies). Once sample QC had been checked, mRNA library preparation using the NEBNext poly A enrichment module (NEB, USA) with the Ultra II RNA library prep kit for Illumina (NEB, USA) was carried out to construct a 250-300 bp insert cDNA library. To perform library QC checks, the Bioanalyser 2100 DNA 1000 kit for size distribution detection and Qubit 2.0 and real-time PCR quantification was used. The quantified library was pooled and then loaded onto the flow cell on the Illumina NovoSeq 6000 instrument using a high output paired end 150 bp sequencing strategy (Illumina, USA). 30×10^6 to 50×10^6 paired-end reads were generated per sample.

2.3.3 Transcriptomics analysis

Quality control. Raw data was converted from raw sequence reads to hit counts by bioinformaticians at Novogene. The original output data file was transformed to sequence reads by CASAVA base recognition and stored in FASTQ format before being processed through in-house perl scripts. Clean reads were acquired by removing reads containing poly-N and any low-quality reads from the raw data to calculate Q20, Q30 and GC content. The *homo sapiens* reference genome and annotation files were downloaded from ENSEMBL. Hisat2 v2.0.5 was used to index the reference genome and align the paired-end clean reads.

Quantification of gene expression levels were performed using featureCounts v1.5.0-p3 with the options (the line of code): “-T 18 -s 2 -Q 255.” to count the read numbers mapped to each gene in the human reference genome.

The fragments per kilo base per million mapped reads (FPKM) of each gene were calculated using the gene length and reads count mapped to each gene to estimate the expression levels.

Differential expression analysis was carried out using the DESeq2 R package 1.20.0 utilising a model based on the negative binominal distribution of the gene expression data. To control the false discovery rate, p-values were adjusted using the Benjamini and Hochberg approach and any genes with an adjusted p-value ≤ 0.5 were assigned as differentially expressed.

Enrichment analysis of differentially expressed genes by the clusterProfiler R package corrected gene length bias. Gene ontology terms with corrected p-values less than 0.05 were considered significantly enriched. The clusterProfiler R package was used to compare the enrichment of differentially expressed genes to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways to explore the higher-level functions of eosinophils.

2.4 Flow cytometry

Flow cytometry acquisition was performed with a BD LSR Fortessa (BD Biosciences) or a Cytex full spectrum analyser (Cytex Biosciences). Data analysis was performed using FlowJo v10 (Tree Star Inc.). 200 μ L of whole blood and 1×10^6 sputum cells were stained with mAbs directed to siglec-8 for the identification of eosinophil populations, CD16 for neutrophil populations, LIVEDEAD Blue was used to discriminate between live and dead cells in sputum samples. Additionally, the leukocyte common antigen, CD45, was included in sputum samples. Dilutions were performed in FACS buffer: PBS + 0.1% BSA + 0.5 M EDTA and can be found in table 5. Antibodies were added to samples and incubated for 30 minutes at 4°C

followed by pelleting at 500 x g for 5 minutes. 2 mL 1X RBC lysis buffer (BioLegend) was added to whole blood for 15 minutes followed by two wash steps in FACS buffer. Sputum proceeded directly to the wash steps and was filtered through a FACS tube cell strainer then fixed in 200 µL IC fixation buffer (BioLegend) for 15 minutes and diluted 1:2 in FACS buffer. Gating strategies for sputum and whole blood are presented in figure 13 and 14 respectively.

Table 5. Antibodies and stains used for flow cytometry

Antibody Specificity	Dilution	Conjugate	Clone	Supplier
CD45	1:80	BUV395	H130	BD Pharmingen
Viability	1:1000	LIVEDEAD Blue	-	Thermo Fisher Scientific
CD16	1:40	Pacific Blue	3G8	Biolegend
Siglec-8	1:100	PE-Cy7	7C9	Biolegend
CD123/IL3R	1:50	AF647	6H6	Biolegend
CD11b	1:100	PE	CBRM1/5	Biolegend
CD63	1:100	AF700	H5C6	Biolegend

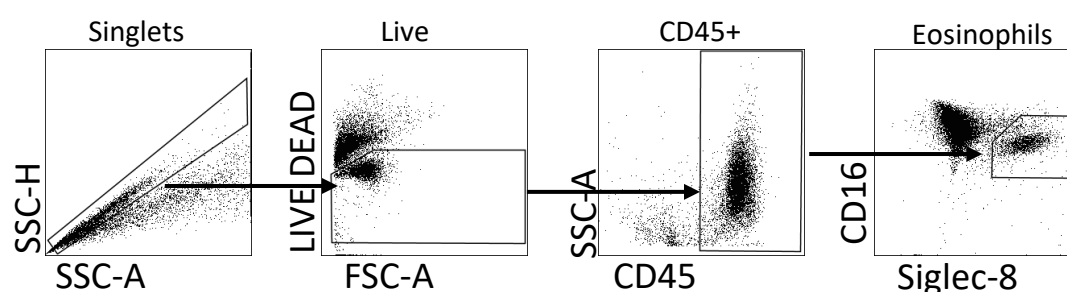


Figure 13. Sputum gating strategy.

Eosinophils were identified as live, CD45⁺, siglec-8⁺CD16⁻.

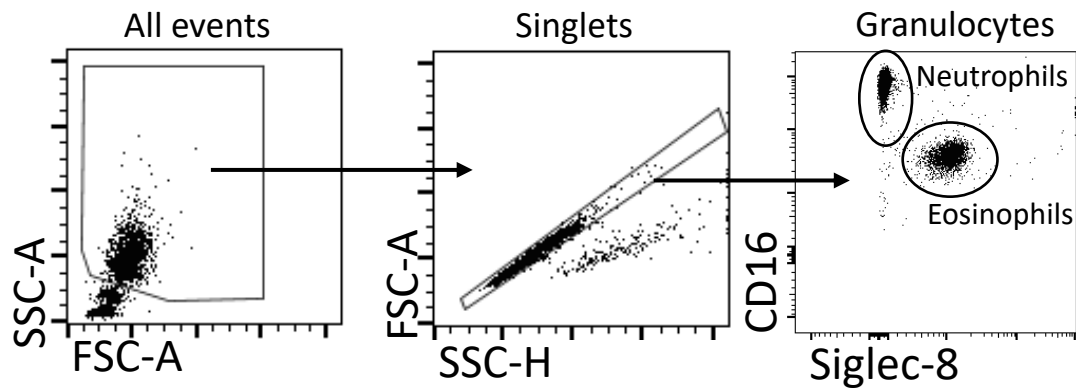


Figure 14. Whole blood gating strategy.
Eosinophils were identified as siglec-8⁺CD16⁻.

2.5 NTHi eosinophil interactions

2.5.1 Preparation of NTHi

2×10^8 /mL NTHi-398 stock was thawed and centrifuged at 13,000 rpm for 5 minutes. The glycerol was aspirated, and the bacterial pellet resuspended in 1 mL PBS prior to streaking 50 μ L on chocolate agar plates. This was then cultured in an incubator at 37°C and 5% CO₂ overnight before several colonies were picked (to account for phase variation) into Brain heart infusion (BHI) broth (Thermo Scientific) supplemented with haemin (1 μ g/mL) and β -NAD (0.2 μ g/mL). This was then cultured in an incubator at 37°C and 5% CO₂ (200 rpm).

Before any proceeding experiments, it was essential to know the characteristic growth phase profile of NTH-398 and the approximate time taken to grow a known number of bacteria. Figure 15 shows how OD490, measured on a FLUOstar OPTIMA (BMG Labtech), related to CFU/mL. For example, OD490 was typically measured after 1 h incubation for the required 2×10^8 CFU/mL once an OD490 of 1 was reached.

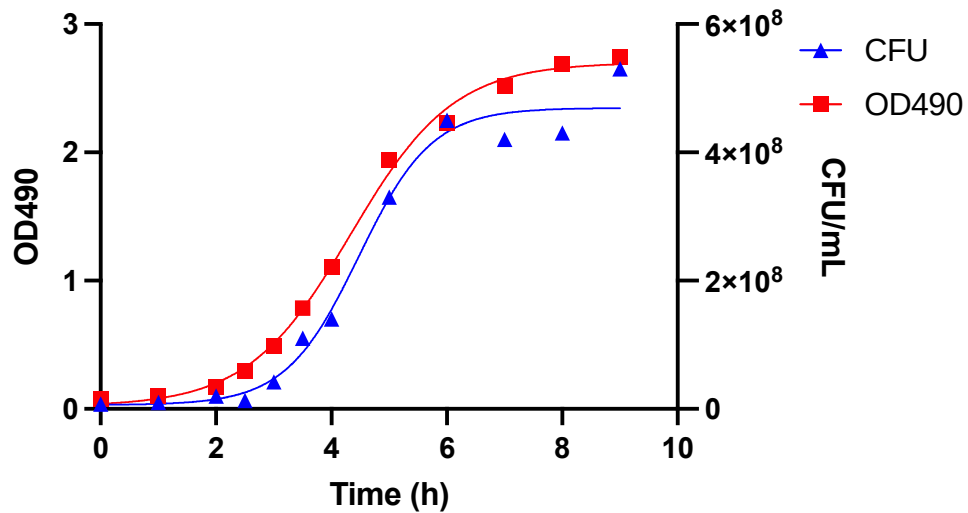


Figure 15. NTHi-398 growth curve.

Measured OD490 and CFU/mL of NTHi-398 over a 9 h period displaying lag, exponential, and stationary phases of growth.

2.5.2 NTHi phagocytosis assays

Measurement of the phagocytosis of pHrodo-conjugated NTHi-398 by eosinophils was performed by resuspending pHrodo particles in DMSO to make a 10 mM solution whilst NTHi-398 was grown to log phase. 2×10^8 /mL bacteria were resuspended in 100 mM sodium bicarbonate buffer. NTHi-398 was added to pHrodo particles to make a 0.5 mM solution and incubated for 45 minutes, protected from light. Bacteria were washed in buffer and centrifuged at $>15\,000 \times g$ for 1 min. Supernatant was aspirated and bacteria resuspended in 1 mL PBS. Wash steps were repeated three times. The NTHi was then plated to confirm viability and CFUs. Once labelled, bacteria were opsonised in 2% pooled human serum for 20 minutes in a 37°C 5% CO₂ incubator. Eosinophils were resuspended in RPMI media at 1×10^6 cells/mL were then incubated with GFP-NTHi-375 at bacteria to cell ratio of 100:1 for 45 mins in a 5% CO₂ incubator. For the ice control, cells were put on ice 20 mins prior to bacterial incubation and maintained on ice for the duration of the

incubation period. Eosinophils were then fixed in 4% PFA for 15 mins at room temperature and kept in the dark at 4°C until analysis on a BD LSR Fortessa.

2.6 ROS measurements

2.6.1 Oxidative burst capacity

Measured using Fc OxyBURST Green assay reagent (Molecular Probes Inc., Eugene, OR) using a FACS-based method for assessment over three minutes or a plate-based method over a twenty-minute period. Fc OxyBURST Green assay reagent consists of bovine serum albumin (BSA) covalently linked to dichlorodihydrofluorescein diacetate (H₂DCF) and complexed with purified rabbit polyclonal anti-BSA IgG antibodies to yield a multivalent particulate immune complex (IC). When these ICs bind to Fc receptors, the non-fluorescent H₂DCF molecules are internalised within the phagosome and subsequently oxidised to green, fluorescent dichlorofluorescein (DCF) (170). Isolated eosinophils or neutrophils ($1 \times 10^6/\text{mL}$) were incubated with 200 ug/mL of ICs in a water bath for 10 min. Incubating the cell suspension for 20 minutes at 4°C prior to the addition of the ICs for 10 min at 4°C was used as a control. ~500 events/second were acquired using a low flow setting continuously for three minutes on a BD LSR Fortessa and detected at an excitation and emission maxima of ~490 nm and ~520 nm, respectively. Eosinophils and neutrophils were also incubated with Fc OxyBURST Green under the same conditions however, cells were then transferred to a 96-well plate with fluorescence measured every 5 minutes on a FLUOstar OPTIMA (BMG Labtech) at 488 nm excitation and 530 nm emission.

2.6.2 Intracellular mitochondrial ROS

Measured in the form of superoxide using MitoSOX red (Thermo Fisher Scientific) on a FACS-based method, isolated neutrophils or eosinophils (5×10^5) were incubated with 1 μ M MitoSOX red in phenol red free RPMI 1640 and stimulated with 10 μ g/mL PMA (MilliporeSigma) for 10 minutes at 37°C. PBS was used to wash the cells and the fluorescence intensity measured on a BD LSR Fortessa.

2.6.3 Extracellular ROS

Measured using the OxyBURST Green H2HFF BSA assay using a fluorometric assay-based method, human primary neutrophils or eosinophils (1×10^6 /mL) were resuspended in KRP reaction buffer (phosphate-buffered saline, pH 7.4, with 1.0 mM Ca^{2+} , 1.5 mM Mg^{2+} , and 5.5 mM glucose; MilliporeSigma) in a 96-well plate. 10 μ g/mL of OxyBURST Green H2HFF BSA was added to each well for 2 minutes at 37°C subsequently with the addition of 10 μ g/mL PMA. The release of extracellular ROS was then measured by continuous fluorescence increase excited at 488 nm and detected at 530 nm over 120 minutes with 15-minute intervals on a FLUOstar OPTIMA microplate reader (BMG Labtech).

2.7 GSH:GSSG ratio

Glutathione is an oxygen scavenger found in eukaryotic cells. Oxidised glutathione (GSSG) is used as an indicator of oxidative stress as glutathione is converted from its reduced form (GSH) to GSSG as it scavenges oxygen, decreasing the ratio of reduced to oxidised. The relative ratios of GSH and GSSG were therefore calculated using the GSH:GSSG-Glo assay (Promega Co, Southampton, UK).

Calculation of the GSH/GSSH ratio was determined through a twofold adjustment for GSSG as one mole of GSH is oxidised to two moles of GSSG.

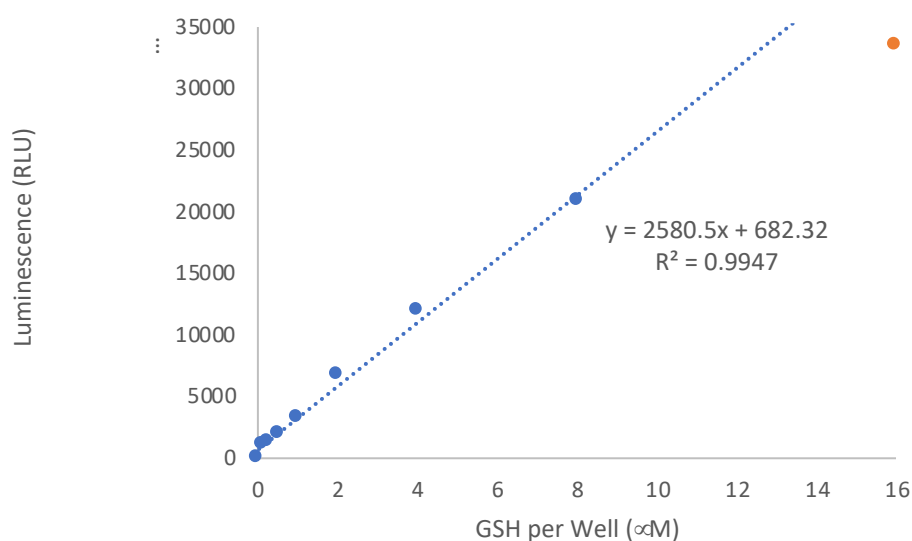


Figure 16. Glutathione standard curve.

A total glutathione standard curve was calculated by serial twofold dilutions of 320 μM glutathione. Standards were plated in triplicate, lysed with Total Glutathione Lysis Reagent and after 30 minutes of incubation at room temperature, Luciferin Generation Reagent was added followed by a brief incubation period and the addition of Luciferin Detection Reagent. Luminescence was measured on a FLUOstar OPTIMA (BMG Labtech, Germany) with the linear portion of the curve (0 – 8 μM GSH) used for calculations. RLU, relative fluorescence units. See table 6 for complete data. The lower limit of quantification (LLQ) for this assay was 391 cells.

Table 6. Total glutathione data

Sample	RLU	μM GSH
Std 1	33331.33	16
Std 2	20776.67	8
Std 3	11765.33	4
Std 4	6582.333	2
Std 5	3177	1
Std 6	1951	0.5
Std 7	1262	0.25
Std 8	909.6667	0.125
Zero	0	0

To determine the appropriate cell density for this assay, 1:2 standard dilutions of eosinophils and neutrophils were generated, and a density of 0.5×10^5 cells chosen for the position in the linear proportion of the curve (see table 7).

Table 7. Cell density data

Cell Number	Eosinophils (RLU)	Neutrophils (RLU)
50000	1598.5	3504
25000	1163.5	1590
12500	777.5	996.5
6250	608	742.5
3125	546.5	623.5
1562.5	463.5	465.5
781.25	586.5	560.5
390.625	509	346.5
Zero	0	0

2.8 Sputum bacterial DNA extraction

Sputum was collected from the ABRA and CORAL trials and stored at -80°C until use. Three DNA extraction kits that utilise different approaches to cell lysis were tested to determine the appropriate extraction kit to use for sputum bacterial DNA extraction: 1) ZymoBIOMICS DNA miniprep kit (Zymo. Irvine. CA. USA); 2) Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany); 3) MP Bio FastDNA spin kit for soil (MP Biomedicals, USA). The Qiagen kit uses an enzyme-based lysis, whereas MP Bio utilises bead beating for physical disruption and Zymo a combination of the two. Five sputum samples from patients with COPD (n=3) or asthma (n=2) were tested for this investigation with each sample divided equally into thirds. DNA yield and A260/A280 ratio was quantified using a NanoDrop 2000 spectrophotometer. Results can be found in the chapter 7.

2.9 Bacterial metagenomic amplicon sequencing

Amplicon generation was conducted at NovoGene using 16S V3-V4 341F-806R specific 470 bp primers to amplify the hypervariable region with Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) and uniquely barcoded. Briefly, 1X loading buffer containing SYBR green (Thermo Scientific) was

mixed with the PCR products and loaded on 2% agarose gel for electrophoresis detection and the products purified with the Qiagen Gel Extraction Kit (Qiagen, Germany).

Library preparation and sequencing was carried out following Illumina protocols for NEBNext Ultra DNA Library Pre-Kit with quality assessed on the Qubit 2.0 fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). The constructed DNA libraries were sequenced on an Illumina NovoSeq 6000 platform to generate 250 bp pair-end reads.

Paired end reads assembly involved assignment of samples from their barcodes and consequential truncation by removal of these barcodes and primer sequence. Fast length adjustment of short reads (FLASH) analysis (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>) was used to merge mate paired end reads at overlapping points when the DNA fragments are shorter than twice the length of reads to produce longer reads that improve assembly by reducing misassembled contigs.

Quality control was carried out by filtering the raw tags produced from the splicing sequence at the assembly step to produce clean tags following the QIIME (V1.7.0, <http://qiime.org/index.html>) protocol. By comparing the clean tags to a reference Gold database (http://drive5.com/uchime/uchime_download.html) using UCHIME Algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html), any chimera sequences were removed to obtain effective tags.

Operational taxonomic unit (OTU) generation was performed by grouping sequences with $\geq 97\%$ similarity to the same OTU using UPARSE (UPARSE v7.0.1001, <http://drive5.com/uparse/>). These sequences could then be compared to

an OTU reference sequence for annotation using the GreenGene Database (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>) and ribosomal database project (RDP) pipeline classifier tools (Version 2.2, <http://sourceforge.net/projects/rdp-classifier/>). Comparisons of different OTUs were made to assess phylogenetic relationships using MUSCLE software (Version 3.8.31, <http://www.drive5.com/muscle/>) to align multiple sequences. Data was normalised by standardising all sample sequence numbers to the sample with the least sequences. Subsequent analyses could then be carried out on this normalised data.

2.10 RNA isolation and cDNA synthesis

Total RNA was isolated from peripheral blood eosinophils as described in 2.3.1 however, prior to cDNA synthesis, RNA was normalised to the lowest concentration of sample RNA as measured on a NanoDrop (Thermo) in RNase free water. Ten microlitres of RNA was used for reverse transcription using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA; catalogue: 4374966) including a 100 nM dNTP mix and 10X RT random primers. Thermal controller conditions consisted of 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes.

2.11 Real-Time Quantitative PCR

For real-time PCR, 4 µL of cDNA template with FAST SYBR green master mix (Applied Biosystems, Foster City, CA; Catalogue: 4385612) with 500 nM of specific primers. Thermal cycling conditions consisted of one cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, and 62°C for 1 minute. Each sample was measured in duplicate. GAPDH was included as an internal control to normalise

for gene expression levels. Results were expressed as relative quantification units (fold change). Relative quantification values were calculated by the $2^{-\Delta\Delta Ct}$ method. The cycle threshold (CT) for the target amplicon and CT for the endogenous control (GAPDH) were determined for each sample. The differences between the average Ct values for the gene of interest were calculated between the average CT value for GAPDH for the ΔCt . The average ΔCt value from the healthy control group was then subtracted from each sample ΔCt to determine the $\Delta\Delta Ct$. To calculate the fold gene expression values, 2 was raised to the power of the negative $\Delta\Delta Ct$ values.

Table. 8 Sequence of primers used in PCR

Gene	GenelD	Forward primer	Reverse primer	Amplicon
GAPDH	2597	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	66
DUOX2	50506	CTGGGTCCATCGGGCAATC	GTCGGCGTAATTGGCTGGTA	144
EPX	8288	GTCCTGCGAGACTGCATAGC	TATAATCTGCGGCCCGAACAA	175
GPX4	2879	GAGGCAAGACCGAAGTAACTAC	CCGAACTGGTTACACGGGAA	100
TXN	7295	GTGAAGCAGATCGAGAGCAAG	CGTGGCTGAGAAGTCAACTACTA	87

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DUOX2, dual oxidase-2; EPX, eosinophil peroxidase; GPX4, glutathione peroxidase-4; TXN, thioredoxin.

2.12 Cell culture

2.12.1 A549

Adenocarcinomic alveolar basal epithelial A549 cells (CCL-185) were isolated from the lung tissue of a 58-year-old Caucasian male with lung cancer and were purchased from the European Collection of Authenticated Cell Cultures (ECACC). A549 cells were cultured in complete growth media: Dulbecco's Modified Eagle's Medium (DMEM) with 15 mg/L phenol red, 4 mM L-glutamine and 4.5 g/L glucose.

Supplemented with heat-inactivated foetal bovine serum (hiFBS, 5%), and 50 mg/mL penicillin-streptomycin. Cells were grown in T-75 culture flasks (Sarstedt Limited), at 37 °C in a humidified incubator (95% air, 5% CO₂).

2.12.2 Subculturing procedure

Cell cultures were maintained at a concentration between 6×10^3 and 1×10^4 viable cells/cm² or once 80% confluency was reached as observed under an inverted microscope. Culture medium was discarded and washed with RT phosphate buffered saline (PBS-/-; 0.01 M, no Ca²⁺ or Mg²⁺). Cells were then detached by adding 2 mL of 0.25% (w/v) Trypsin- 0.53 mM EDTA solution until cell layer is dispersed (5 – 15 minutes). Complete growth media was then added at a volume of 8 mL followed by gentle aspiration and centrifugation at 500 x *g* for 5 minutes. A549s were counted using a haemocytometer and resuspended in complete media at a cell concentration between 6×10^3 and 6×10^4 cell/cm².

2.12.3 HL-60

HL-60 cells are a cell line derived from peripheral blood lymphocytes of a 36-year-old woman suffering from acute promyelocytic leukaemia and were purchased from ECACC. HL-60 cells were cultured in RPMI 1640 with 2 mM glutamine and supplemented with 20% FBS at 37°C in a humidified incubator (95% air, 5% CO₂). Cells were maintained $1 - 9 \times 10^5$ cells/mL for no longer than six weeks as cells may then spontaneously differentiate.

2.12.4 Differentiation procedure

Sodium butyrate (Sigma) was supplemented with complete culture medium to 500 μ M for six days to differentiate into eosinophil-like cells. For neutrophil-like cells, HL-60 cells were cultured in media with 1.3% DMSO for eight days.

2.13 Epithelial permeability assay

Epithelial permeability was performed on a 96 transwell Cell Culture Plate (PET 1.0 μ M) from MilliporeSigma (catalogue: PSRP004R1) with fluorescein isothiocyanate dextran (70 Da; catalogue: FD70-250MG; MilliporeSigma). Briefly, 100 μ L of A549s suspended at 5×10^5 cells/mL in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin were seeded onto the inserts for 24 hours in a 5% tissue culture incubator at 37°C to form a 100% confluent monolayer. Isolated peripheral blood eosinophils were stimulated with 10 ng/mL PMA for 10 minutes prior to coincubation with 5000 cells (1:10 cell-to-cell ratio of eosinophils to A549s) for 24 hours. Following incubation, the inserts were transferred to a new receiver tray with 250 μ L fresh growth media in each well and 75 μ L of 2 mg/mL FITC-dextran at a 1:40 working dilution applied to each insert. The plate was then incubated for 2 hours at RT, protected from light, before removing the insert from the receiver tray. 100 μ L of media containing any permeated FITC-dextran that crossed the monolayer was transferred from each well of the receiver tray to a black 96-well opaque plate for fluorescence measurement on a FLUOstar OMPTIMA (BMG Labtech). For comparison with unstimulated eosinophils, the assay was performed as above without the PMA stimulation step.

2.14 Secreted cytokine quantification by enzyme-linked immunosorbent assay (ELISA)

2.14.1 Sandwich ELISA

Human DuoSet ELISA kits (R&D Systems) were used to quantify IL-4 and IL-8 and human eosinophil peroxidase ELISA (Biomatik) for EPX following the manufacturer's protocol. The lower limit of detection was 31.2 pg/mL for IL-4 and IL-8 and 0.56 ng/mL for EPX, defined as the lowest protein concentration that could be differentiated from zero. TMB (Thermo Fisher Scientific) was used as the substrate and stop solution was 1M (2N) sulphuric acid (H₂SO₄) diluted from an 18M stock (Sigma-Aldrich).

2.14.2 Competitive ELISA

Human DNA damage competitive ELISA kits were used to quantify 8-OhdG (Thermo Fisher Scientific) following the manufacturer's instructions. TMB (Thermo Fisher Scientific) was used as the substrate and stop solution was 1M (2N) sulphuric acid (H₂SO₄) diluted from an 18M stock (Sigma-Aldrich). The lower limit of detection for this ELISA was 50.9 pg/mL.

2.15 Meso Scale Discovery (MSD) platform

U-PLEX Biomarker group 1 human assay was used to quantify IL-1 β , IL-5, IL-13, TNF, eotaxin, eotaxin-2, eotaxin-3, IL-17E/IL-25, IL-33, TSLP following the manufacturer's instructions.

2.16 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.3.0 Software. Data are presented as mean/median/IQR/Range/SEM/SD as appropriate. 2-way ANOVA with Tukey's multiple correction tests when two independent variables were included. T tests were used when a single variable was present. For correlation data the non-parametric Spearman rank test was used. Numbers of biological and technical replicates are specified in individual figure legends.

Chapter 3

RNA-sequencing of peripheral whole blood eosinophils reveals distinct gene expression profiles of eosinophils in COPD during an acute eosinophilic COPD exacerbation compared to stable disease and healthy controls: a methodological study

3 RNA-sequencing of peripheral whole blood eosinophils reveals distinct gene expression profiles of eosinophils in COPD during an acute eosinophilic COPD exacerbation compared to stable disease and healthy controls: a methodological study

3.1 Introduction

High throughput, next-generation sequencing technologies are an increasingly common method to study complex, heterogeneous diseases including chronic obstructive pulmonary disease. Gene expression is dependent on cell, tissue, and disease state and therefore appropriate to study changing states of disease pathophysiology. Quantification or differential expression studies can be utilised to compare gene transcript levels across different diseases, or disease states. The number of reads per sample is often an integral factor to determine the sensitivity of the study, often with higher number of reads required to define all gene isoforms. However, the number of samples is also a consideration when studying human diseases. Poly-A capture technique is best suited for selecting coding transcripts but require a higher quality of input RNA (measured as a higher RNA integrity number [RIN] score) to generate quality sequencing data.

Many previous studies of respiratory diseases have enrolled subjects into broad categories, such as childhood and adult-onset asthma, often avoiding detailed phenotypic definitions of complex disease states. Although this offers the advantage of large population of patients, stringent phenotyping is often lost. This leads to reduced resolution when identifying genes that are relevant to patient subpopulations with potential genetic traits. COPD exacerbations are an important

disease state that may have critical drivers in genes that provide increased sensitivity to environmental factors, for example. As the onset of exacerbations are unpredictable, it is important that the pathways of known biological drivers are explored to identify new possible treatments. Careful characterisation of individual patients makes sample collection increasingly more challenging, especially during acute onset of symptoms which are often treated as quickly as possible to resolve symptoms. Therefore, meticulous study design is required for the acquisition and interpretation of genomic sequencing studies.

Around a third of acute COPD exacerbations are recognised as being eosinophilic in nature and these patients are known to stabilise following treatment with eosinophil depleting therapies. However, why this phenotype is present in these patients is not understood, nor are the drivers that shift the eosinophil into a pathogenic state when many patients are eosinophilic with no acute symptoms. It is vital that any genetic changes are investigated in the eosinophil to better understand what causes these cells to shift into an inflammatory state.

As eosinophils typically circulate in low numbers and are high in RNases, studies into the genome of eosinophils have proved difficult. Here, I aimed to optimise a pipeline for efficient acquisition, isolation, high quality RNA extraction, and sequencing of this enigmatic cell type. I also sought to examine the genome of eosinophils acquired during non-eosinophilic disease state, during an acute eosinophilic exacerbation and from non-eosinophilic healthy controls to elucidate any potential drivers during an eosinophilic crisis event.

3.2 Results

3.2.1 The method of eosinophil enrichment effects the yield of eosinophils from peripheral whole blood

As there are different methods of eosinophil enrichment, it was important to identify which method would be appropriate for use RNA-sequencing experiments. Two commonly utilised methods were chosen: 1. MACS CD16 negative isolation preceded by Ficoll density centrifugation and dextran sedimentation and, 2. MACS Xpress eosinophil isolation kit. The former method was a standard protocol utilised in our lab and had been validated numerous times (171). The latter was a recent method consisting of a cocktail of proprietary antibodies for the negative selection of eosinophils from whole blood. By comparing these two methods I found that both had their benefits. The CD16 negative isolation has the advantage of separating components and cells found in peripheral blood that can be further utilised whereas the latter only produces the eosinophil fraction. In contrast, the major advantage of the MACS Xpress kit is time – taking around 30 minutes per sample, this is much quicker than the alternative method tested which averages around five hours from receiving blood to producing a pure eosinophil population. As COPD exacerbations are unpredictable by nature, this would be a desirable quality for experiments requiring these samples.

To compare each method directly, 60 mL of blood was collected from healthy donors and divided equally. Purity, calculated by trypan blue viability count (fig.17A+B), was comparable, with the Xpress system mean of 92.6% with the CD16 negative selection mean being 90% (fig. 17C). Viability was also similar, with the Xpress mean being 89.2% compared with 93.6% for the CD16 negative isolation of

cells (fig. 17C). I found limited expression of CD11b in these cells in both groups, indicating low activation of this integrin. Whereas CD63, a reported marker of degranulation, averaged 53.2% following Xpress isolation and 12.9% from CD16 negative cells (fig. 17C, $p = 0.01$). As expected, yield of cells varied greatly between donors, but I also found the Xpress method of isolation consistently produced lower yields than CD16 negative isolation (fig. 17D).

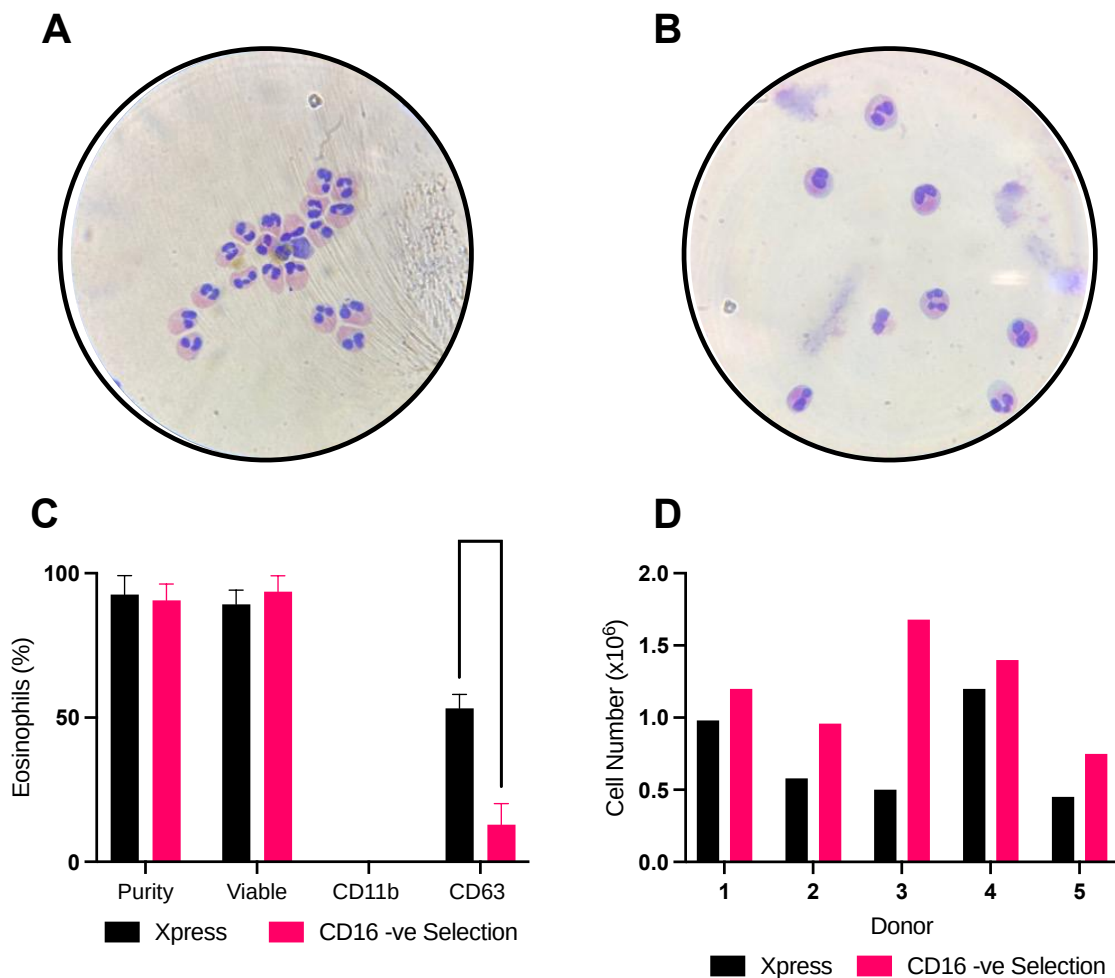


Figure 17. Eosinophil yield is greater with CD16 negative depletion method of isolation. Cytopsin images of eosinophils isolated through (A) MACS Xpress method and (B) eosinophils isolated through CD16 negative depletion. (C) Purity, viability, and expression of CD11b and CD63 surface markers. (D) Yields of cells from 30 mLs of five healthy donor bloods. A and B are representative of numerous experiments. x100 magnification under oil emersion. C and D represent five individual donors following eosinophil enrichment. Unpaired t test in **C** for statistical analysis. Data is mean $\pm$ SEM.

3.2.2 Assessment of eosinophil RNA integrity following enrichment from peripheral blood shows RNA quality is reduced following the MACS Xpress method

An integral component of the chosen method of sequencing was the acquisition of high-quality RNA. Library preparation with poly-A transcript enrichment produces libraries predominantly relating to protein coding regions, making it ideal for gene expression analysis. As the poly-A signal is located at the 3' end of transcripts, degradation would result in bias towards this region, potentially skewing results. I therefore utilised the Agilent TapeStation system for sample quality control of the extracted RNA which provides objective evaluation of RNA degradation with an associated RNA integrity number (RIN^e) scaled from 1 (strongly degraded) to 10 (highly intact). Figure 18 shows the electrophoresis gel image with the associated RNA RIN^e for samples acquired from three healthy donors. Comparing the two isolation methods showed consistently higher intact RNA following CD16 negative enrichment. Mean score for Xpress eosinophil isolation was 6.4 (range: 5.4 – 7.3) compared with 8.2 (range: 7.8 – 8.4) following the CD16 negative isolation procedure ($p = 0.03$). Therefore, eosinophil enrichment by CD16 negative selection was the superior method for isolating highly intact RNA.

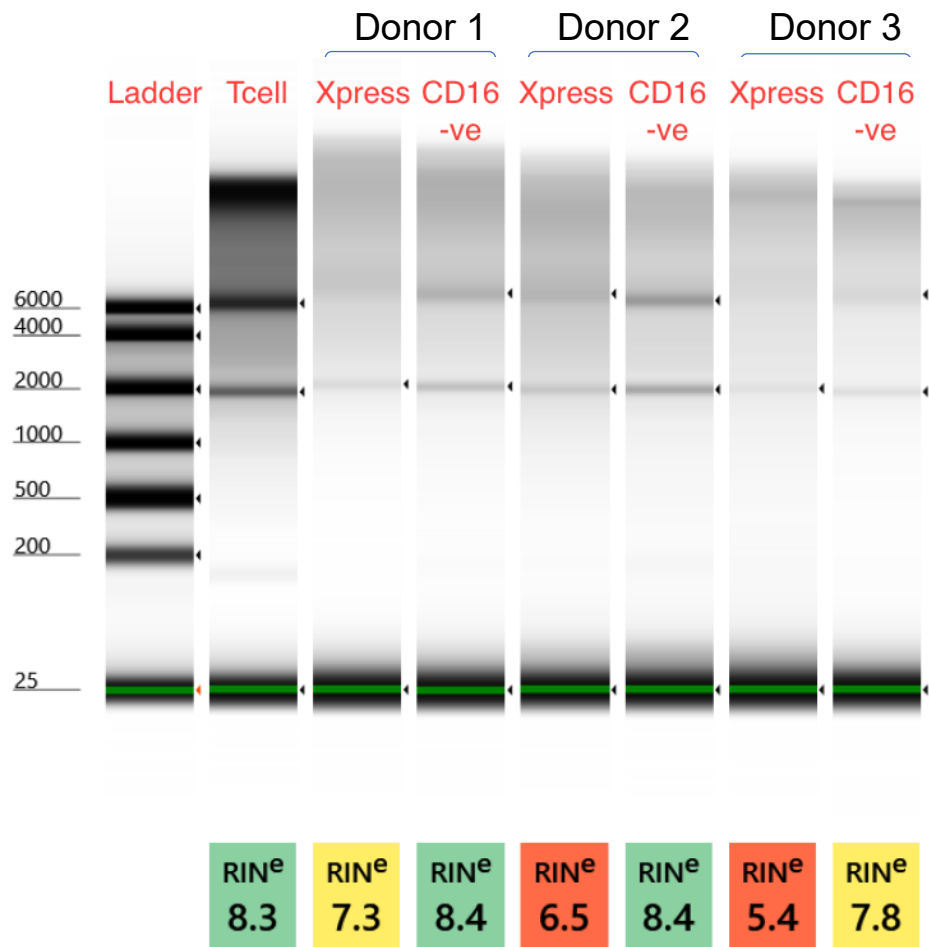


Figure 18. Higher quality RNA is acquired following CD16 negative enrichment.

TapeStation gel image comparing two methods of eosinophil isolation. Values of 8 and above indicate high RNA integrity (green), yellow being intermediate, and red being poor. n = 3; t test for statistical analysis.

3.2.3 Bulk RNA-sequencing of eosinophils from peripheral blood identifies distinct disease specific gene expression profiles

Having shown that the eosinophil isolation procedure can affect the quality of the obtained RNA and that the chosen poly-A capture technique requires high quality RNA, I decided to utilise the Ficoll and CD16 negative selection method to enrich eosinophils from the peripheral blood of donors for the purposes of these experiments.

To study how the gene expression of eosinophils may be affected by disease, and importantly, disease state, I performed RNA-sequencing expression

profiling on eosinophils isolated from the peripheral blood of healthy volunteers, stable non- eosinophilic COPD patients, and COPD patients who were experiencing an acute eosinophilic exacerbation (or crisis). Four donors from each group were selected in this study and their clinical features for these subjects can be found in earlier table 4. I chose stable disease patients to determine if the differences in gene profile were disease specific as healthy volunteers will, under normal circumstances, also not be eosinophilic. Therefore, any differences in gene expression could most likely be attributed to disease specific causes, rather than eosinophilia. As it is commonly accepted that eosinophils are critical in exacerbations of COPD, I wanted to identify if any genes are differentially regulated between those patients that are experiencing an eosinophilic crisis event and those who are not to further understand eosinophil function during these periods as this has not been investigated previously. In this study, eosinophilic events were defined as a blood eosinophil count ≥ 300 cells/ μ L.

The first step in this pipeline was to determine the sequencing depth. I obtained an average of 110.8 million raw sequencing reads per sample (range: 99.7 – 141.6 million reads per sample). Of these reads, an average of 95.5% (range: 94.1 – 96.1%) were aligned to the Homo sapiens (GRCh38/hg38) reference genome. Following the removal of reads with adapters, or low-quality reads, clean reads were produced with a Q20 score $>97\%$ for all samples. Other quality control measurements included identifying the nucleobase quality, the total number of reads and their mapping to the human reference genome (see appendix tables 1 – 4). These measures gave confidence that sequencing and alignment results were found to be of sufficient quality to be included in differential expression analyses.

3.2.4 Distribution of gene expression suggests similarities within groups

Integral to any biological experiment are biological replicates which ensures reliability of the sample selection. Thus, biological replicates should show similarities of gene expression within groups, indicating that the individuals are representative of their group's characteristics. Commonly used methods of estimating gene expression levels includes calculating the expected number of fragments per kilobase of transcript sequence (FPKM). By performing PCA on the gene expression profiles, and therefore the FPKM of each individual, the gene variables can be reduced, and principal components extracted so any differences between the samples can be identified. By assessing the variability, any patterns from the RNA-seq dataset can therefore be identified. Figure 19A shows that healthy individuals tended to be dispersed away from patients with COPD whereas disease state appeared to have less effect on the variability of the samples, appearing to cluster more closely. This would suggest that gene expression of eosinophils during an exacerbation are more like the stable disease state than to eosinophils from healthy volunteers, as there are less intergroup differences. Additionally, this may mean that there may be certain genes that predispose COPD patients to an eosinophilic phenotype and eosinophil targeting therapies may prove fruitful in treating this disease state.

In addition to PCA, the correlation of gene expression levels (FPKM) can be calculated between the samples to display any similarities amongst the individuals. 50.9 pg/mL To estimate if any differences exist between samples, Pearson correlation is commonly established. Correlation of gene expression between individuals can also be used to infer the extent of the differences between groups, with the closer

the value to 1, the higher the similarity the samples have and less differentially expressed the genes are. An R^2 value greater than 0.8 is accepted to have a strong similarity. The matrix in figure 19B shows that although there is strong correlation between most of the individual samples, an R^2 value generally closer to 0.9 was found within groups compared with outside groups. Again, this would suggest that there is a closer expression pattern within the predefined groups. The distribution of gene expression levels among samples and the associated FPKM can be found in appendix table 4 and figure S2. In conclusion, the gene expression patterns in this dataset would suggest that disease samples are more similar than healthy samples based on PCA clustering and correlation analysis.

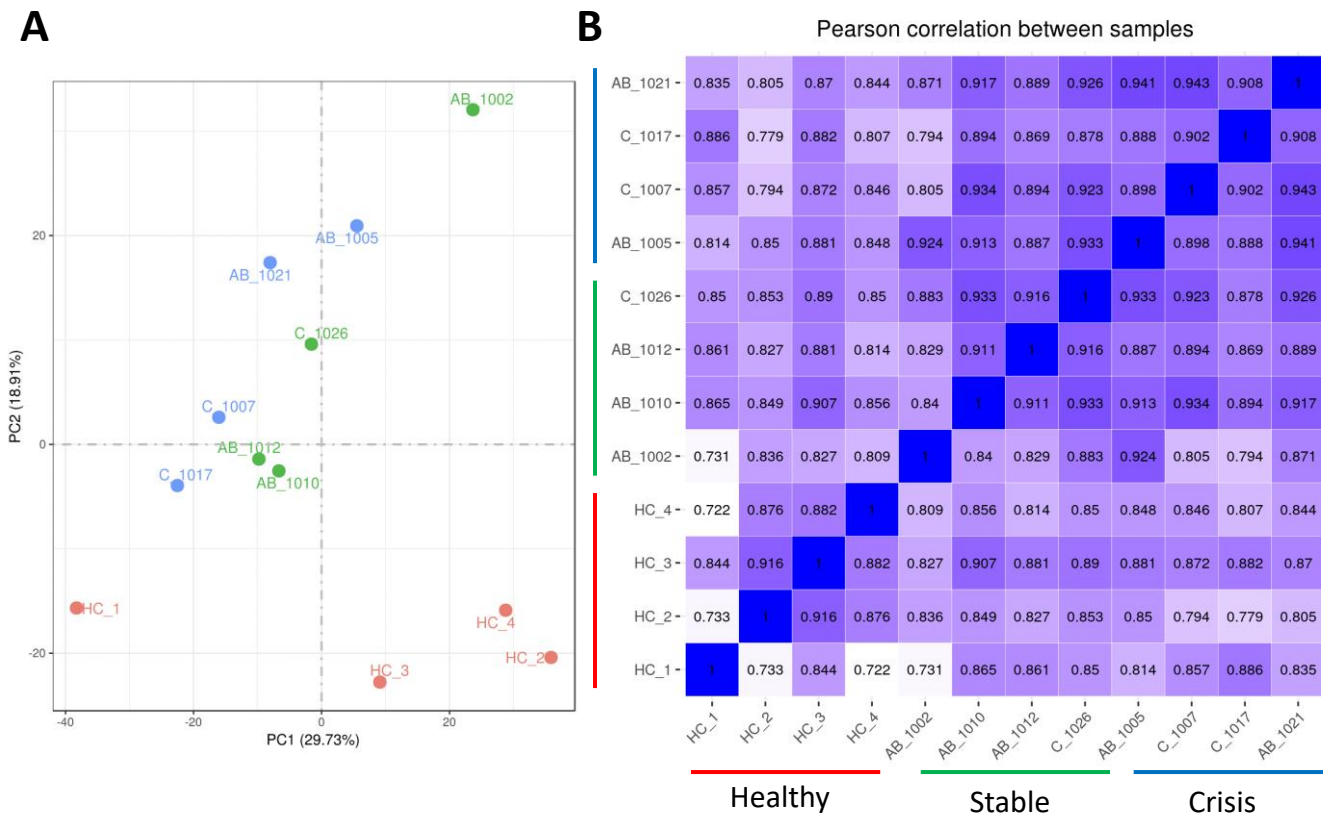


Figure 19. Gene expression patterns are more similar between disease states than healthy volunteers.

(A) PCA plot reveals that healthy donor samples cluster separately from patients with COPD. Red dots for healthy donors, green for COPD non-eosinophilic stable disease state, and blue for eosinophilic crisis events. $n = 4$ / group. (B) Pearson's r matrix displays a strong positive correlation within groups. PCA, principal component analysis; R^2 , square of Pearson correlation coefficient (R).

3.2.5 Eosinophil crisis events induce the biggest changes in gene expression compared to healthy controls

Following quantification of gene expression, statistical analysis was carried out to determine which genes were significantly differentially expressed between groups. To correct for differences in the sequencing depth, the raw read count was normalised followed by the negative binomial distribution calculated to test the probability of the hypothesis that there are no systematic differences between the average read count values between groups for a given gene. To control for the number false positive results that will inevitably accumulate due to comparisons performed on numerous genes, the p-value is calibrated into a p-adjusted value (padj) after correcting for the false discovery rate by the Benjamini-Hochberg approach. The standard threshold for screening differentially expressed genes is typically set to a log₂ fold-change of greater than 1 and a padj ≤ 0.05. A full list of significant genes with their associated fold changes can be found in appendix tables 5 – 10.

Overall, there were 48 genes that were significantly upregulated between crisis and stable groups whereas 74 were significantly downregulated (total = 122; fig. 20A). Upregulated genes included cell adhesion molecules *ICAM5* and *ALCAM* and the immune suppressor of damage-associated molecular patterns (DAMPs) *CD24*. Downregulated genes of interest included the neutrophil chemoattractant *CXCL8* (IL-8) and *CHI3L1*, a gene involved in promoting IL-8 production.

There were 50 genes significantly upregulated between stable and healthy groups with 117 genes significantly downregulated (total = 167; fig. 20B). *RNASE3*, the gene that codes for eosinophil cationic protein, was upregulated along with

RNASE2, which codes for eosinophil derived neurotoxin. The matrix metalloprotease gene *MMP9* was also upregulated between stable and healthy groups. Pro-inflammatory genes often associated with microbial defence were downregulated and included: *IL6*, *IL1 β* , and *CXCL2*.

Finally, there were 395 genes significantly upregulated between crisis and healthy control groups, with 574 genes significantly downregulated (total = 969; fig 20C). Upregulated genes involved in the production of hydrogen peroxide included *DUOX2* and *DUOXA2*. Consequently, antioxidant genes included *TXN* and *PRDX5* were also upregulated. Genes related to eosinophil granule proteins included *EPX*, *RNASE2/3*, and *CLC*. Several genes related to mitochondrial function included *COX* genes, *SDHAF2*, *ATP5MF*, and *HIGDIA*. *MMP15* was also upregulated. Significantly downregulated genes included those involved in antipathogen response: *CXCL8*, *CXCL2*, *IL1 β* , *TNF*, *CHI3L1*, *TLR6*, *IL6ST*.

The Venn diagram found in figure 20D displays the read number of co-expressed and exclusively expressed genes found in each group. In total there were 10873 overlapping genes between all groups, 243 between crisis and healthy, 236 between crisis and stable, and 722 co-expressed genes between stable and healthy.

Validation of gene expression was confirmed by quantitative real time PCR on the following genes of interest: *Duox2*, *Txn*, *Gpx4*, (found in chapter 4, figure 29) and *Epx* (found in chapter 5, figure 36). These genes were chosen due to their involvement in ROS activities and relation to eosinophil activation as a possible mechanism that could drive acute symptoms experienced in eosinophilic exacerbations. Further details relating to ROS mechanisms are discussed in chapter four along with series of assays that explore the mechanisms involved in

ROS generation. IL-8 levels were also quantified between neutrophilic and eosinophilic patients in chapter five, figure 33.

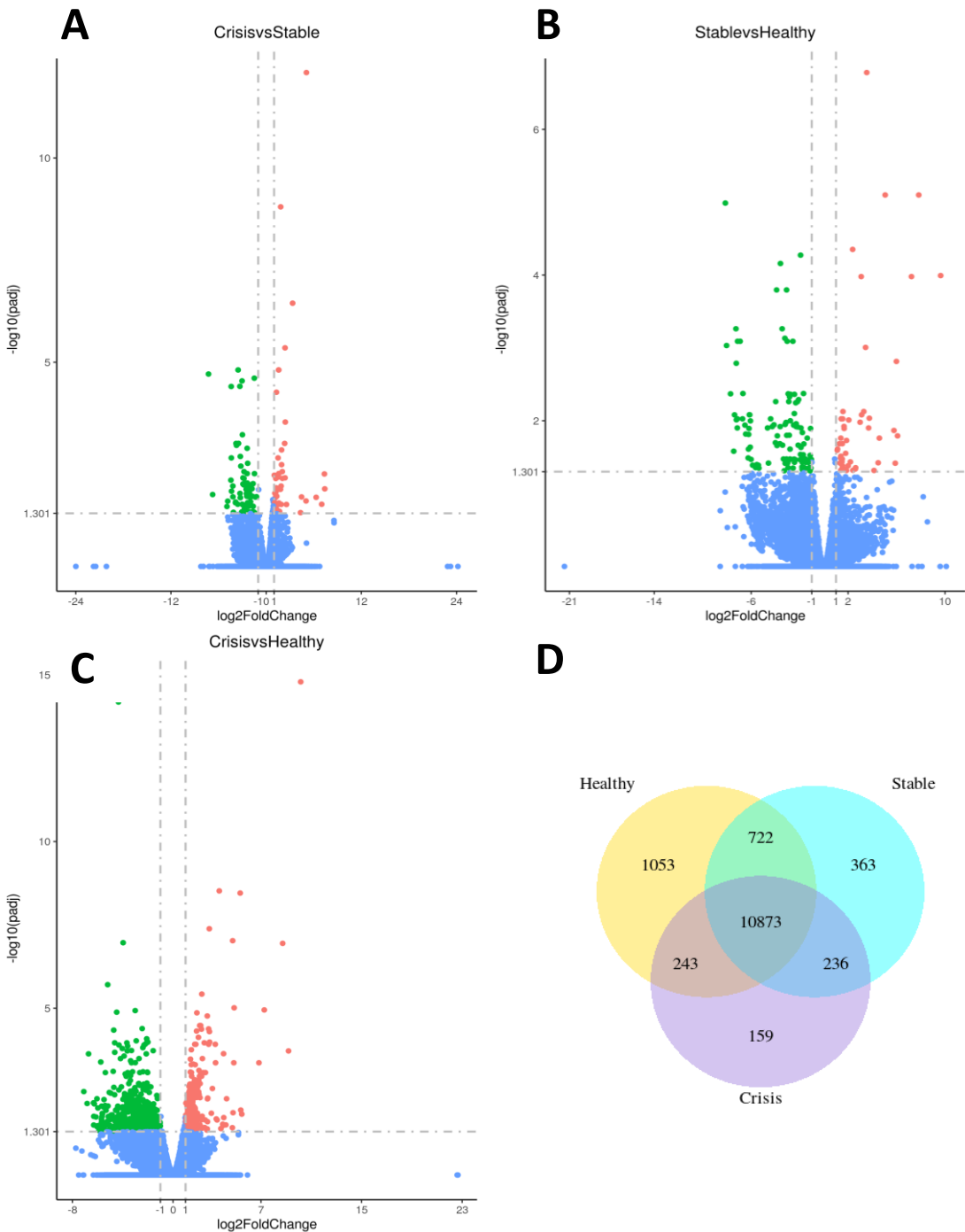


Figure 20. Genes are differentially expressed between groups of healthy volunteers and disease state of COPD.

(A-C) Volcano plots displaying overall gene-based differential expression results between the sample groups (each dot corresponds to a gene). The negative log (base 2) of the fold change for difference in expression is shown on the x-axis with the statistical significance of the differences on the y-axis corresponding to the negative log (base 10) of p-values. Red dots identify significantly up regulated genes with green representing down. Genes that are not significantly differentially expressed are in blue. (D) Venn diagram showing the read number of co-expressed and exclusively expressed genes.

To visualise the gene expression values across each sample, heatmaps were generated. As can be seen from figure 21A, individual sample trends indicated similar patterns within each group, with healthy volunteers clustering on the right-hand side of the panel, stable in the middle and crisis on the left. The top left quadrant shows a cluster of upregulated genes (red) in the crisis individuals which contrasts with the top right quadrant showing decreased expression (green) in the healthy volunteers. Stable disease individuals show an intermediary expression profile. Below the left-hand increased expression quadrant, most genes appear down regulated which again contrasts with the healthy group showing mostly upregulated genes. The stable group is mainly downregulated, with few genes showing upregulation.

Figure 21B shows the expression profile average for each group, displaying where genes are up- and down-regulated more clearly. Crisis and healthy expression patterns tended to show an inverted trend whereas the stable group displayed an expression pattern more aligned to the crisis group but with lower expression.

In summary, eosinophilic crisis events had the most differentially expressed genes when compared to healthy volunteers and the lowest number of differentially expressed genes were found between crisis and stable groups. This indicates that the activation status of eosinophils is most similar between disease states.

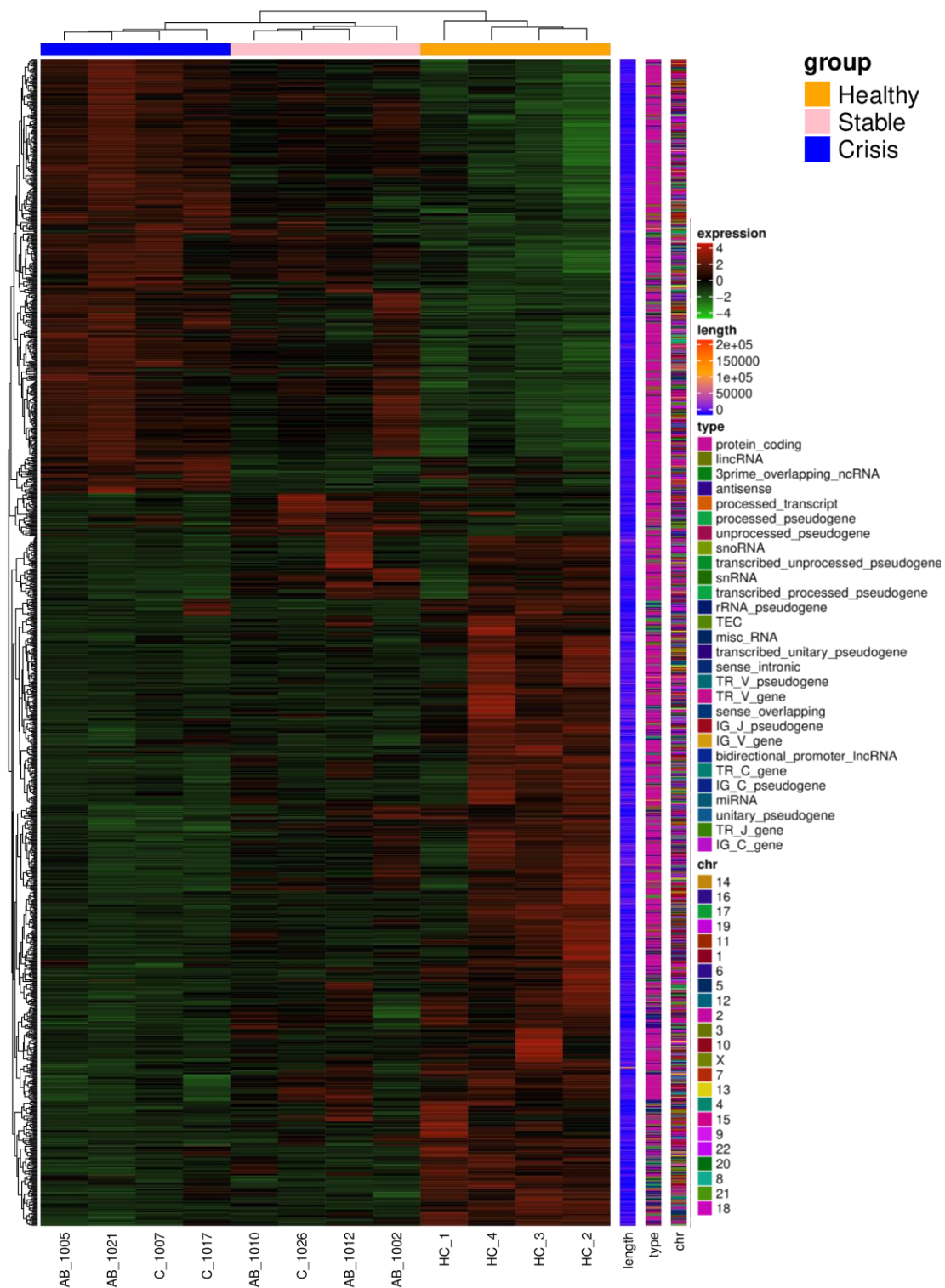


Figure 21. (A) Gene cluster analysis heatmap displays individual differences in gene expression between donors.

Genes clustered using the $\log_2(\text{FPKM}+1)$ value. Heatmap is coloured according to mean normalised expression with red indicating genes with high expression levels and green indicating genes with low expression. False discovery rate (FDR) < 0.05 for all comparisons. The chromosome to which each gene belongs, the gene length, and the biological type of each gene can also be found to the right of the cluster analysis.

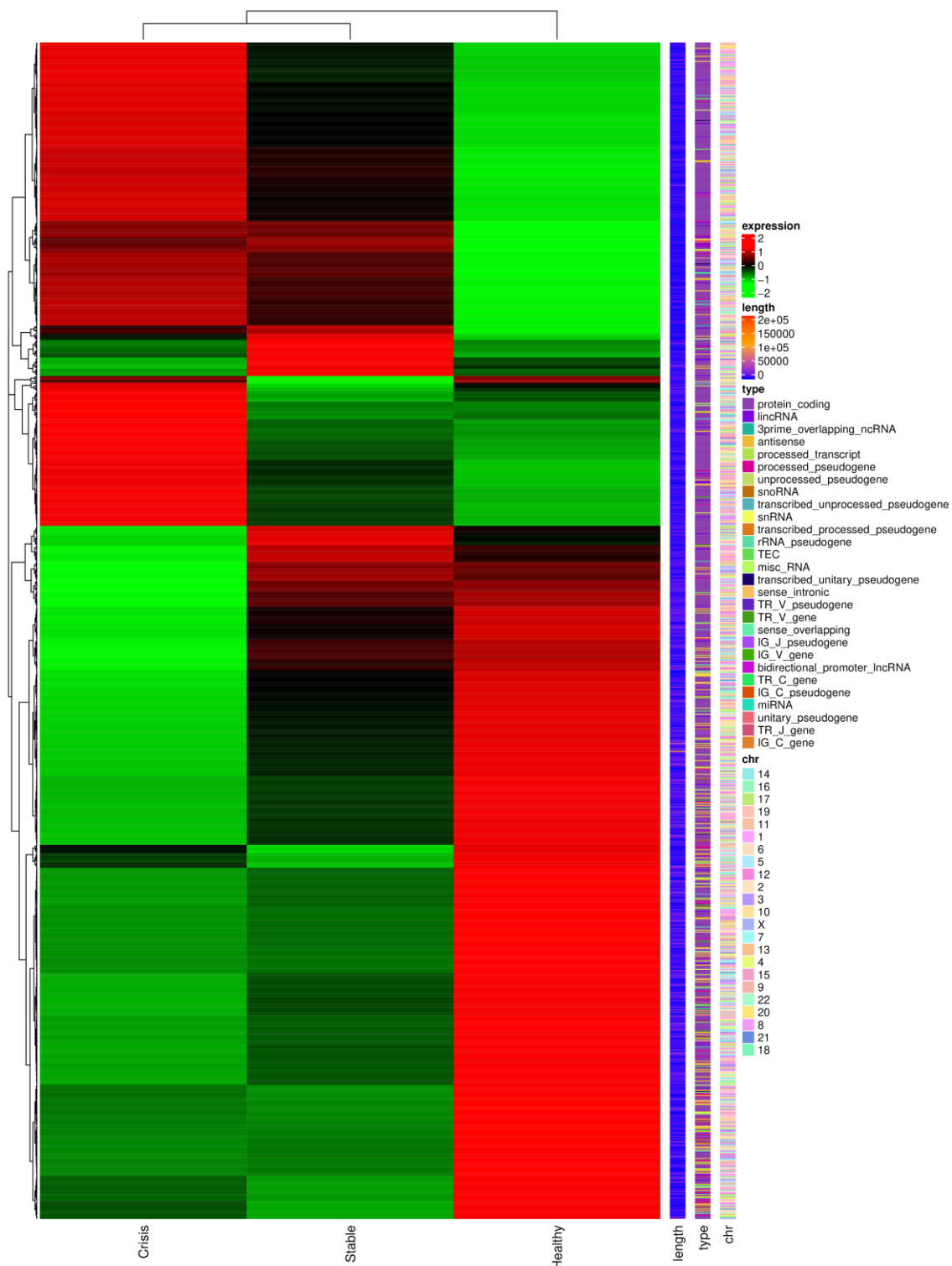


Figure 22. (B) Gene heatmap clustering by group identifies differences in gene expression are related to disease and whether the patient is exacerbating.

Genes clustered using the $\log_2(\text{FPKM}+1)$ value. Heatmap is coloured according to mean normalised expression with red indicating genes with high expression levels and green indicating genes with low expression. False discovery rate (FDR) < 0.05 for all comparisons. The chromosome to which each gene belongs, the gene length, and the biological type of each gene can also be found to the right of the cluster analysis.

3.2.6 Gene ontology enrichment analysis reveals differential gene expression of mitochondrial and hydrogen peroxide metabolic pathways between disease and healthy groups

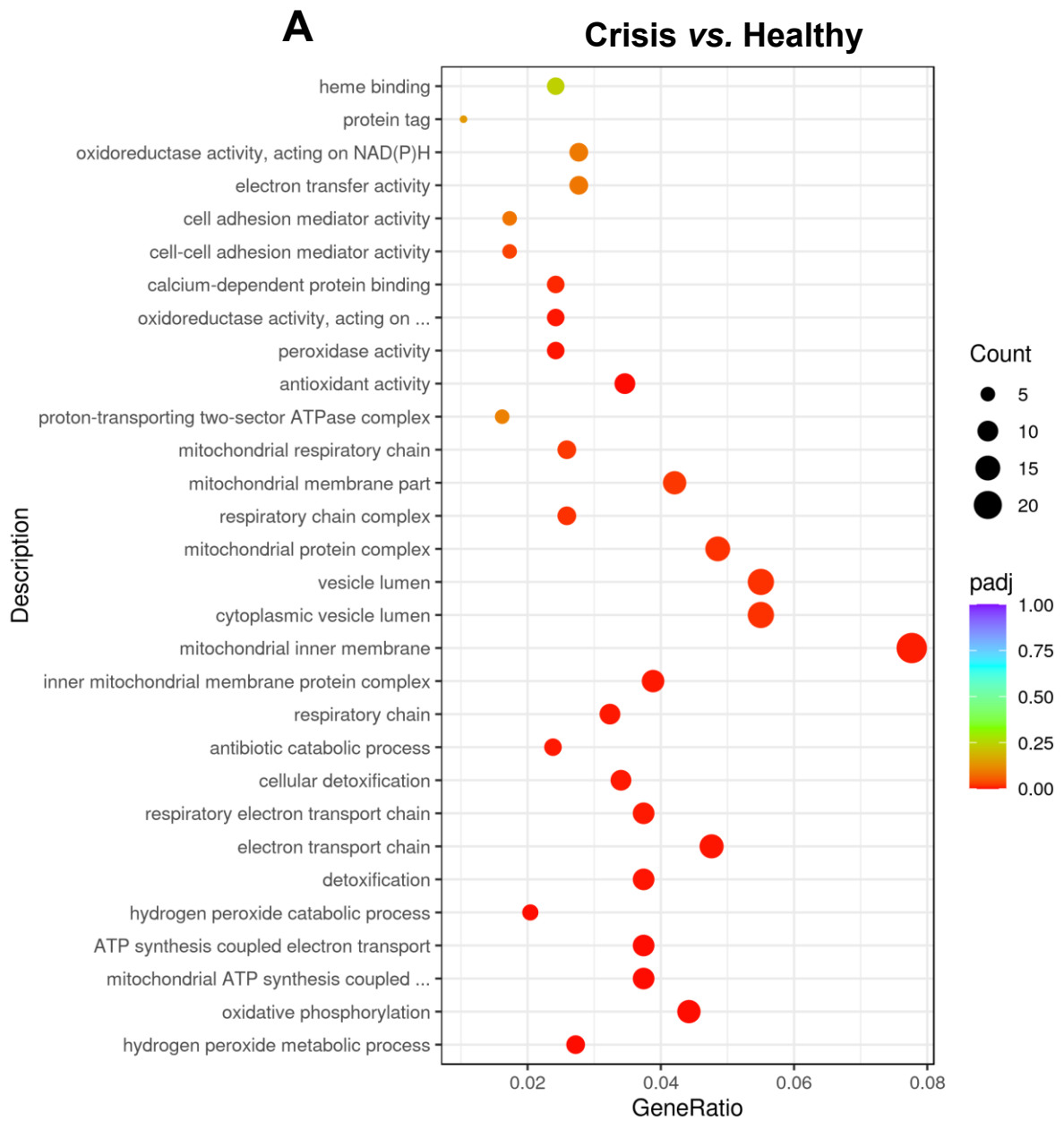
Gene ontology enrichment is a computational model of biological systems and the largest source of information of the functions of genes. This bioinformatics classification system utilises three classes to represent the biological domain: molecular function (MF), cellular component (CC), and biological process (BP). The scatter plots in figures 23 and 24 show the top 30 GO terms impacted between each group (ten in each category). The gene names and GOIDs that make up the significant terms can be found in appendix tables 11 – 15.

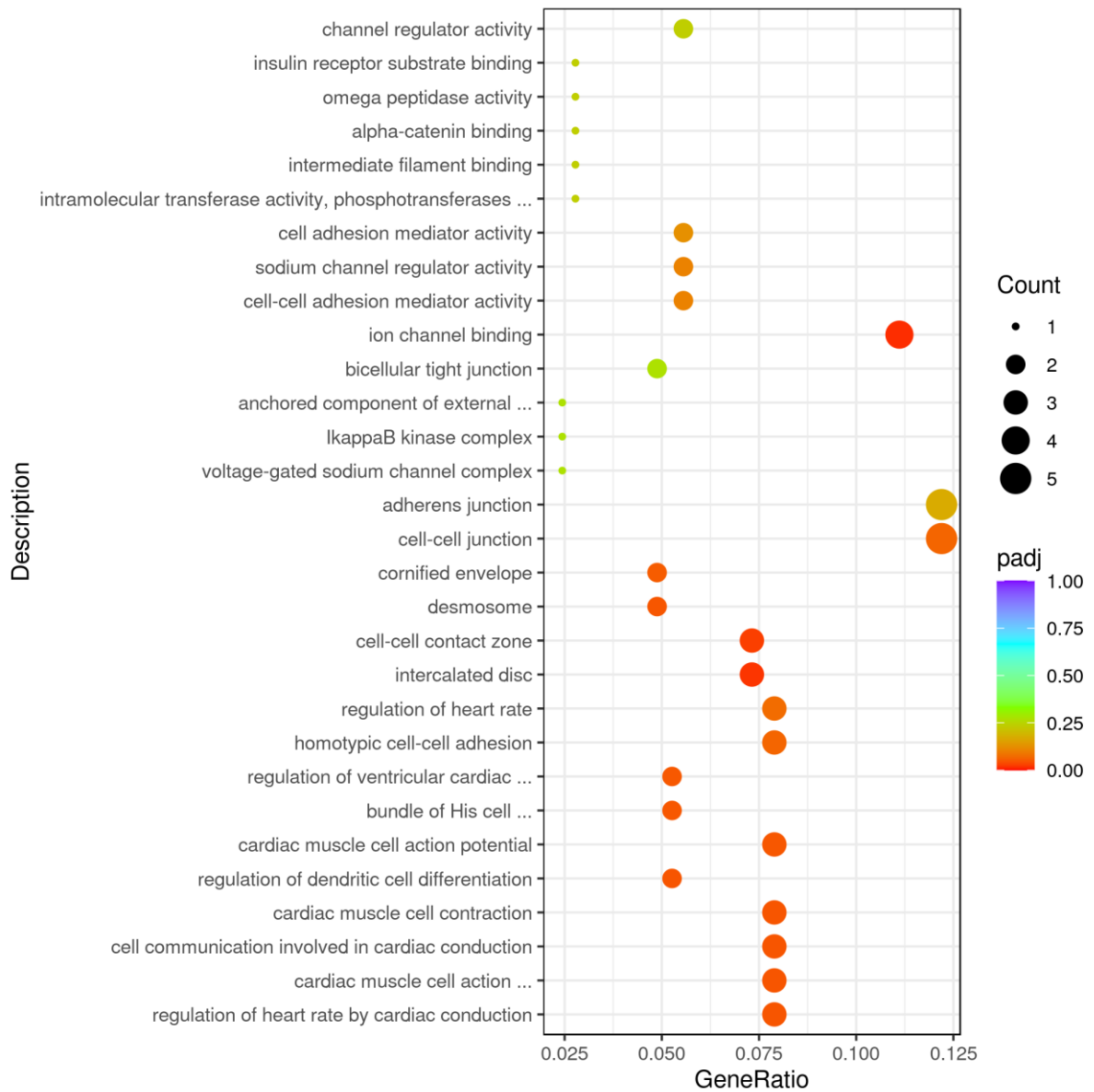
Top clusters in the crisis vs. healthy comparison group showed various biological processes, cellular components, and molecular functions were related to mitochondria function and the respiratory transport chain and included *SDHAF2*, *COX5A/B/6B*, *NDUF*, *ATP5MF*, *HIGDIA*, and *IDO1*. Biological processes related to hydrogen peroxide metabolism included *HBA2*, *DUOX2*, *DUOXA2*, *EPX*, *PRDX5* and molecular functions for antioxidant processes *PRDX5*, *GSR*, *TXN*, *MT2A*, *HBA1/2*, *ALOX5AP*, *SELENOW* (fig. 23A). The major upregulated terms for crisis vs. stable were biological processes related to cardiac functions (fig. 23B) contained *DDSC2*, *PKP2*, and *SCN4B*. Upregulated in the stable vs. healthy group, there were clusters relating to antioxidant activity but also neutrophil activation (fig. 23C). In addition to this analysis being from a purified population of eosinophils, further investigation showed genes that are common eosinophil markers, such as RNase2 (eosinophil derived neurotoxin) and RNase3 (eosinophil cationic protein), present

in this cluster. This likely represents eosinophils and not neutrophils as genes in this group showed specificity to granulocyte activation.

Downregulated GO terms in the crisis vs. healthy groups related to transcription factor activity including *KLF4*, *FOS*, *JUN*, *RORA*, *SOX4*, and *CLOCK* (fig. 24A). No GO terms were found to be significantly downregulated between crisis and stable groups (fig. 24B). Inflammatory cytokine production, mostly relating to IL-6, IL-1 β , and epidermal growth factor (EGF) was a top term found to be downregulated between stable and healthy groups (fig. 24C).

In summary, GO enrichment analysis was utilised to highlight groups of genes that may work together to elicit a biological response in an unbiased approach. Major terms that could be related to eosinophil contributions to the acute symptoms experienced during an eosinophilic exacerbation included ROS generation. Consequently, antioxidant systems were also upregulated in response. Eosinophils during stable disease also displayed signs of activation when compared to healthy volunteers.



B**Crisis vs. Stable**

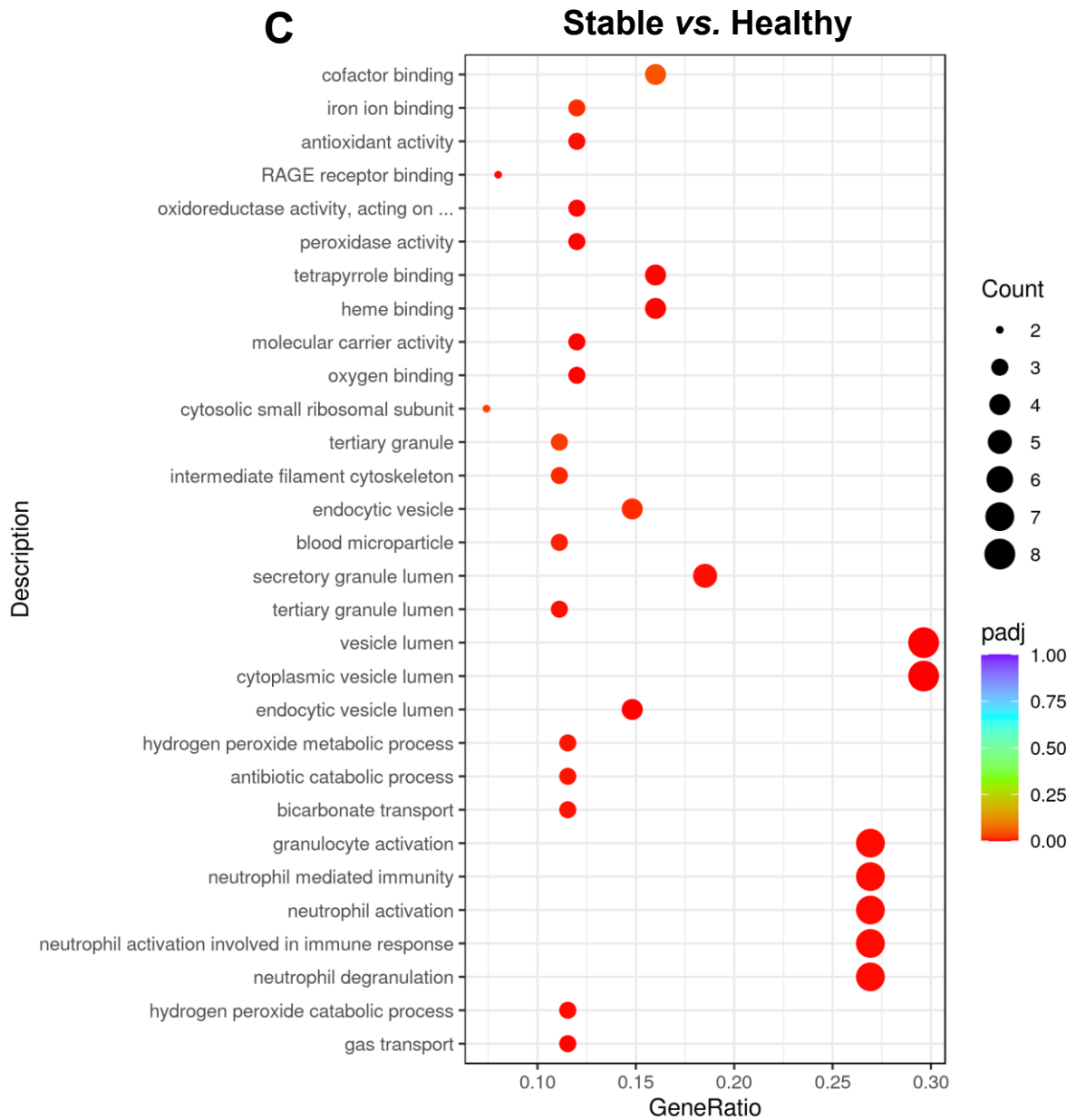
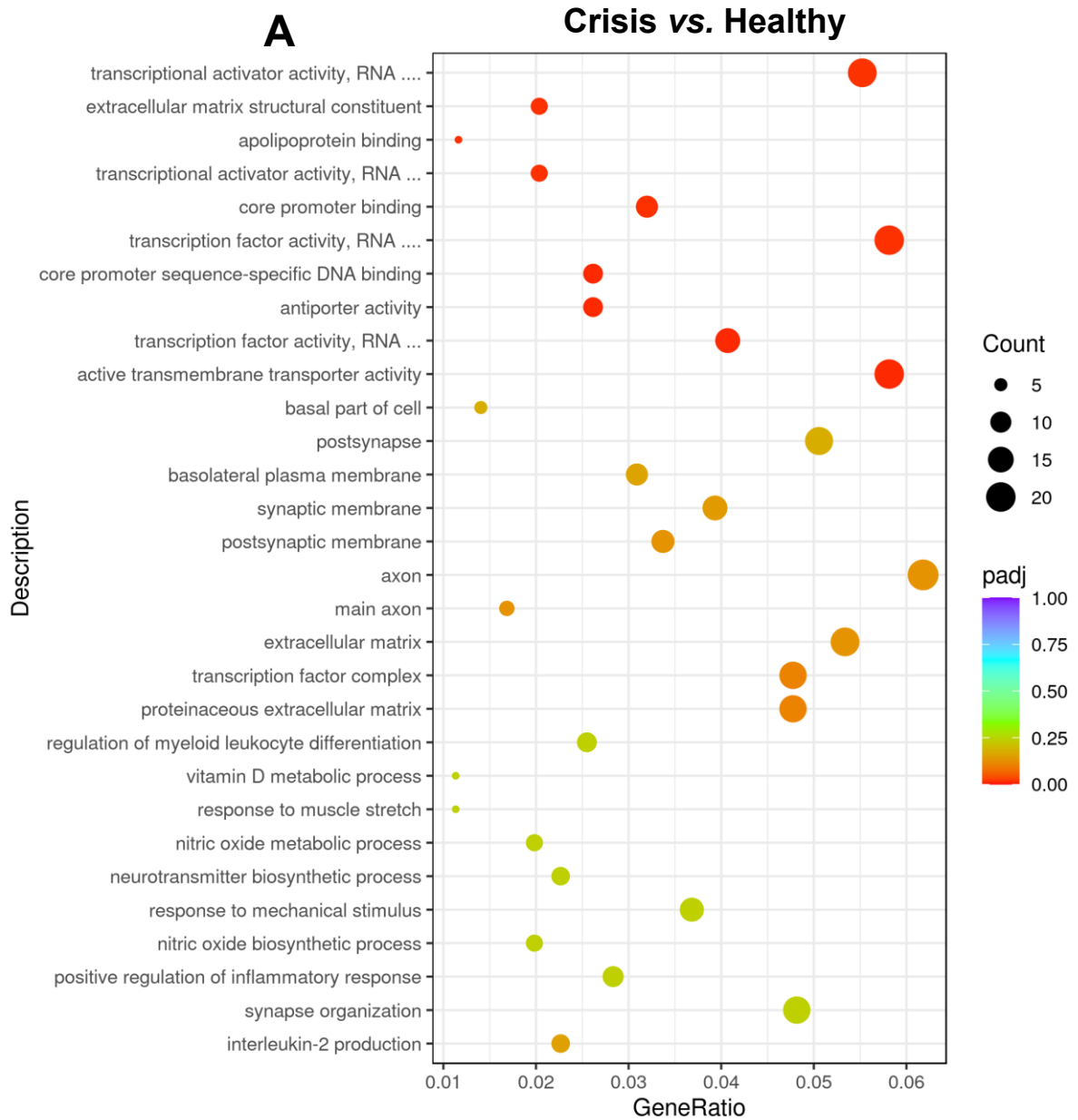
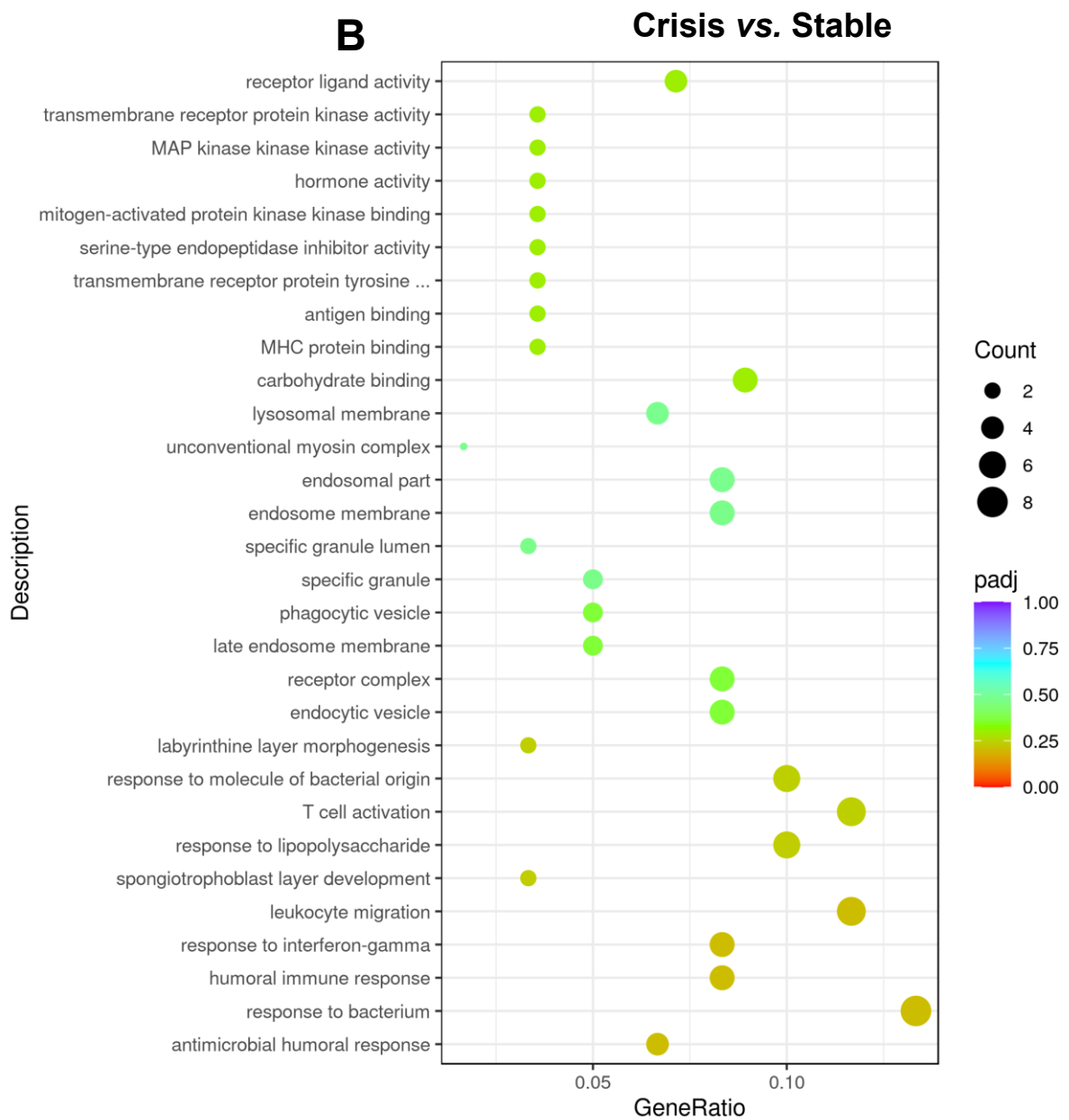


Figure 23. Scatter plot of gene ontology (GO) pathway enrichment of upregulated genes between each group.

(A) crisis vs. healthy (B) crisis vs. stable and (C) stable vs. healthy. The x-axis is the ratio of the number of differentially expressed genes linked with the GO term to the total number of differential genes. Y-axis is the GO pathway term. The dot represents the number of genes annotated to a specific GO term. The colour represents the p-adjusted significance, with red being most significant (0) through to purple being least significant (1).





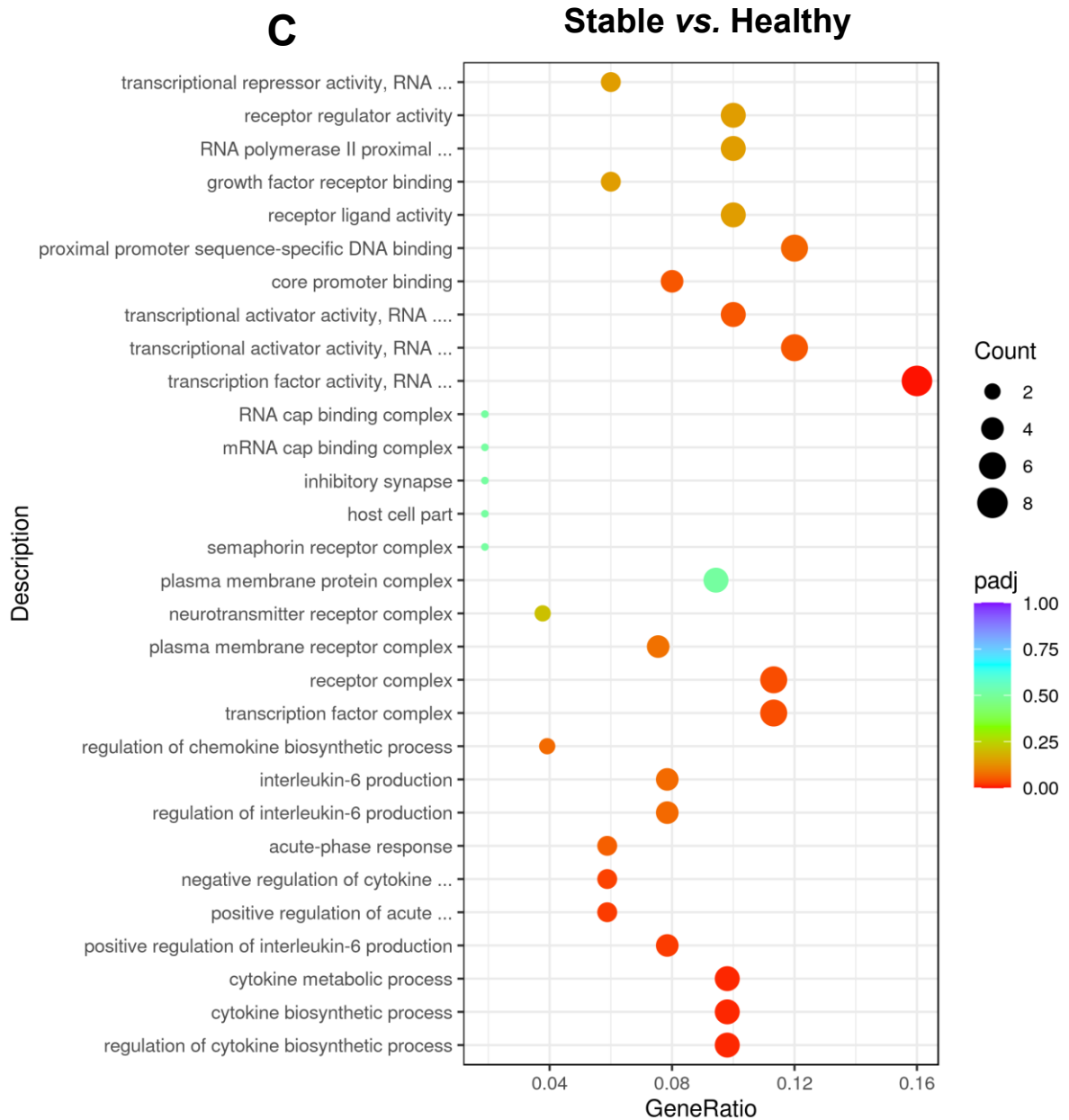


Figure 24. Scatter plot of gene ontology (GO) pathway enrichment of down regulated genes between each group.

(A) crisis vs. healthy (B) crisis vs. stable and (C) stable vs. healthy. The x-axis is the ratio of the number of differentially expressed genes linked with the GO term to the total number of differential genes. Y-axis is the GO pathway term. The dot represents the number of genes annotated to a specific GO term. The colour represents the p-adjusted significance, with red being most significant (0) through to purple being least significant (1).

3.8 Summary of results

In this chapter, I have outlined an optimised pipeline suitable for eosinophil enrichment from whole blood along with efficient extraction of high-quality RNA for the purposes of RNA-seq. I found that the method of eosinophil isolation can impact the yield and the surface marker expression but, importantly for sequencing experiments, the RNA integrity. Although a much longer and more technically demanding process, Ficoll density centrifugation followed by dextran sedimentation and CD16 negative microbead isolation produced consistently higher RNA integrity scores than the MACS Xpress system.

Following selection of COPD patients based on their clinical traits, I acquired blood from healthy volunteers, stable disease patients and, for the first time, patients at the point of an acute eosinophilic exacerbation. Following quantification of gene expression, I found that healthy volunteers had profiles that were dissimilar to those of disease patients, indicating that patients shared similarities in gene expression. Subsequent statistical analysis of the expression data produced a total of 1139 differentially expressed genes between all groups, with distinct gene expression profiles in each. Biggest differences in gene expression were found between the healthy volunteers and patients undergoing an eosinophilic crisis event, with 969 genes differentially expressed, whereas the crisis and stable groups showed the smallest number of differentially expressed genes of 122.

Gene ontology enrichment analysis was utilised to classify genes into functional properties, identifying biological processes related to groups of genes. Major groups of interest involved mitochondrial pathways that were upregulated between crisis and healthy individuals and hydrogen peroxide metabolism. These pathways related to reactive oxygen species responses, proving pertinent for further

mechanistic investigations in the following chapters to elucidate how eosinophils may drive exacerbations in patients with COPD.

3.9 Discussion

It is widely accepted that eosinophils are critical drivers of disease in multiple airways diseases however, the underlying mechanisms involved in their contributions remain elusive (172,173). Given that eosinophils can exist in various forms of activation (174–177), and more recent evidence to suggest that eosinophils may not only be destructive effector cells but may be implicated in homeostatic functions (106,160), investigating the transcriptome would prove vital to our understanding of this enigmatic cell type. Next generation sequencing technologies have revolutionised our understanding of the genome in the context of cell, tissue, and disease state. Despite these advances, sequencing the genome of the eosinophil has proved difficult. As of writing, there is only one human eosinophil transcriptomic project uploaded to the European Bioinformatics Institute database and this is a microarray experiment, limiting the potential detection of differentially expressed genes. This is the first of its kind transcriptomic experiment to sequence eosinophils in depth and compare the expression profiles of healthy volunteers to patients with COPD in different states of disease.

As there are various methods of eosinophil isolation (178), I compared two common approaches to determine if there were any discrepancies that may influence our choice of procedure. One well established technique involved layering blood on Ficoll for density gradient centrifugation followed by sedimentation in high molecular weight dextran to produce a mixed granulocyte population. Neutrophils highly express the CD16 surface marker (179) which allows for negative selection

of eosinophils using CD16 antibody-coated magnetic beads once run through a magnetic column. The MACS Xpress application utilised a cocktail of proprietary antibodies for the negative selection of eosinophils and allowed for antibodies to be added directly to whole blood. This dramatically reduced the time requirement from around five hours per sample for isolation of a pure population to below one hour. This was an important consideration in the planning of future experiments as exacerbations are unpredictable in nature, a quick procedure would be beneficial for unexpected visits. This reason also prevented the use of fluorescent associated cell sorting (FACS) to isolate eosinophils as the cell sorter in our facility had restricted access and booking the machine with limited notice of an exacerbation visit was not possible. The desired assays following isolation would also influence the choice of procedure, as antibody cocktails prevent the use of all but the negatively selected population, prohibiting other cell types from use in other assays. Other factors that were considered when comparing these techniques included purity of the population, yield of isolated cells, activation of cells, and quality of the acquired RNA.

I found that the purity along with the viability and expression of the surface marker CD11b remained consistent across both techniques. However, there were notable differences in the activation marker CD63, and a lower yield of eosinophils acquired following MACS Xpress selection. Both these parameters are important considerations for subsequent experiments. Highly activated cells may respond to conditions in assays differently, confounding results and leading to incorrect conclusions. A low cell number may also hinder experiments, limiting the number of biological replicates in a cell type that is already relatively low in abundance. Of paramount importance for sequencing experiments was the acquisition of high-

quality RNA. When these isolation techniques were compared head-to-head by comparing cells isolated from the same donors, the RNA integrity score was consistently lower in the Xpress system, making samples unlikely to pass quality control checks and suitable for sequencing. Taking all these factors into consideration, I decided to follow the CD16 negative selection protocol for the purposes of eosinophil RNA sequencing and consistency in future experiments.

As eosinophilia is reported in around a third of acute exacerbations of COPD (180), I explored the transcriptomic profile of peripheral blood eosinophils during non-eosinophilic stable disease state, eosinophilic exacerbations (blood eosinophil count ≥ 300 cells/ μ L) and healthy volunteers ($n = 4$ / group), with the hopes of unravelling which genes may actively contribute towards active eosinophil state and therefore drive exacerbations. Once patients had been recruited to the acute exacerbations treated with Benralizumab (ABRA) study, their white cell count was taken, and the patient stratified into either the eosinophilic or non-eosinophilic group.

Using bulk RNA sequencing, I generated data that advances our understanding of eosinophils in health and disease. Indeed, these data support the hypothesis that eosinophils do exist in different states of activation. Through PCA and correlation coefficient analysis, I found that samples clustered closer in disease states and healthy volunteers tended to cluster away from patient samples, indicating that the eosinophil gene expression is affected by disease.

Moreover, differential gene expression analysis indicated that 1139 genes were differentially expressed between all groups, with the most differentially expressed genes found between patients experiencing an eosinophilic exacerbation and healthy volunteers and the smallest number of differentially

expressed genes found between the eosinophilic exacerbators and stable disease patients. There were also more overlapping genes between the healthy and stable groups than the crisis groups combined. From these data, it can be inferred that the state of eosinophil activation is affected by the disease state, or in other terms, the severity of the disease is the stimulus that triggers a particular activation response in the eosinophil.

Decreases in *IL1 β* , *TNF*, *CXCL8*, *CXCL2*, *IL15*, *TLR6*, and *IL6ST* gene expression between eosinophilic exacerbations and healthy volunteers support previous evidence that eosinophilic exacerbations predict a lower risk for bacterial infection. These genes are often associated with a proinflammatory response characteristic of pathogenic infection. Indeed, during bacterial exacerbations, blood eosinophil counts are significantly lower compared with stable state, presenting an inverse relationship between bacterial infection and blood eosinophil count (181–183). Rather, bacterial infection of potentially pathogenic microorganisms in COPD is associated with an airway neutrophilic profile, an increase in blood neutrophil count and poor responsiveness to corticosteroids. Higher sputum neutrophil percentage and lower absolute eosinophil sputum counts are both associated with higher sputum bacterial counts and may account for the variability of the eosinophil count also seen at stable state (184–186).

Higher sputum IL-1 β levels have previously been associated with increased risk of exacerbation in COPD (187). Transgenic mice expressing inducible human IL-1 β in the lung epithelium showed elevated inflammation, enlargement of distal airspaces, mucus metaplasia, and airway thickening and fibrosis. IL-1 β was also found to heighten the production of neutrophil chemoattractant proteins including CXCL2 and CXCL8 (188). Additionally, an airway IL-1 β and systemic IL-6 axis has

been proposed as a potential inflammatory pathway linking exacerbations (187). Taken in context, it could be suggested that a reduction of inflammatory cytokines that are associated with neutrophil recruitment into the airways creates a microenvironment that preferentially recruits eosinophils during an eosinophilic exacerbation. However, as these genes are only associated with eosinophil expression, analysis of other IL-1 β producing airways cells, including macrophages, lymphocytes, and epithelial cells, would also need to be measured at the gene and protein level.

Tumour necrosis factor (TNF) is another proinflammatory mediator thought to have a role in the pathogenesis of COPD and found to be elevated systemically in stable disease patients when compared to healthy controls. Sustained systemic inflammation is related to accelerated decline in lung function and found to be increased during exacerbations (189). These factors justified the trial of the anti-TNF antibody infliximab in the treatment of moderate to severe COPD. Surprisingly, infliximab demonstrated a lack of clinical effect, this was in contrast with other chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (190). It appears that no single inflammatory cytokine is directly responsible for the pathogenesis of COPD and studies should incorporate multiple inflammatory mediators to determine if there are any patterns of inflammation that can be treated with combinational therapies. Nevertheless, it was interesting to observe a decrease in gene expression of TNF during an eosinophilic exacerbation particularly in combination with other classical proinflammatory genes. It may be that eosinophils do not contribute towards airway inflammation directly through inflammatory mediator release, rather they respond to the local tissue milieu which

orchestrates the recruitment of eosinophils. Multiplex cytokine analysis platforms would be useful in determining whether this is indeed the case.

As genes do not operate in isolation, but work in tandem to exert biological effects, pathway enrichment analysis can be used to investigate the expression of groups functionally related genes. Gene ontology enrichment analysis for biological processes returned 15 significantly enriched gene sets between crisis and healthy groups. Modules of upregulated gene sets included hydrogen peroxide metabolic processes, cellular detoxification, and respiratory electron transportation, amongst other similar processes. Oxidative stress is a known feature of COPD pathogenesis and results from various activated structural and inflammatory cells in the airways and a reduction of antioxidants. Systemic oxidative stress has also been found to increase during acute exacerbations (191). Downstream functional effects are linked with amplified inflammatory responses and bronchoconstriction. Eosinophils have also been shown to cause direct tissue damage in allergic inflammation (192). Taken in context, I hypothesised that these related gene sets may be contributing to reactive oxygen species responses during eosinophilic exacerbations, and I therefore conducted detailed mechanistic assessments of ROS production and antioxidant activity during an eosinophilic exacerbation (see chapter four).

Genes of interest that were upregulated in this group included *DUOX2* and its associated maturation factor *DUOXA2*. Dual oxidase-2 is a member of the NADPH oxidase family responsible for the generation of hydrogen peroxide (193). Dual oxidase knockout mice have also been shown to have decreased influx of neutrophils in the bronchoalveolar lavage (BAL) fluid and in lung sections even though the circulating numbers of neutrophils remained the same in the peripheral blood. DUOX activity in the airways is regulated by IL-4 and IL-13 in the airways

and implicated in features of allergic asthma including mucus cell hyperplasia and airway hyperresponsiveness. Th2 cytokines, along with IL-6, were also lower in the BAL fluid of knockout mice. Interestingly, the levels of eotaxin were also reduced (194). Together, these findings may suggest an DUOX selective role for eosinophil recruitment into the airways further amplified by eosinophil activation during eosinophil exacerbations.

The eosinophil specific myeloperoxidase EPX is a crucial cytotoxic granule protein and a co-substrate of H_2O_2 . When activated eosinophils degranulate to release EPX which has direct killing effect on extracellular pathogens. However, prolonged detrimental effects are also reported in the airways which manifest as bronchial remodelling (195). EPX is also used as a marker of eosinophil activation in airways diseases as nasal and pharyngeal EPX levels directly correlate with asthma severity and eosinophil percentage in induced sputum (196).

RAC2 is translocated from the cytosol of activated phagocytic cells to their membrane during the production of respiratory bursts by NADPH oxidase and is crucial in the release of superoxide species following stimulation (197). Collectively, these genes support a role for elevated ROS generation during an eosinophilic exacerbation. Oxidative stress as the result of activated cells in the airways is already reported as a critical driver of COPD pathogenesis with a wide range of inflammatory effects (82). Although direct measurements of local ROS production in the lower airways are difficult to measure real-time due to the short-lived nature of ROS, markers of ROS-induced damage are measurable in exhaled breath condensate. These markers include H_2O_2 , isoprostanes, malondialdehyde, nitrosative stress markers, and antioxidants such as glutathione (198).

The normal production of ROS is countered by antioxidant mechanisms, which are also found to be upregulated in eosinophilic exacerbations. *TXN*, the gene for thioredoxin was included in the antioxidant activity gene ontology antioxidant activity node. This is a central antioxidant system by functioning as an electron donor and cofactor for reductive enzymes. Thioredoxin therefore can work in tandem with enzymes such as peroxiredoxins including *PRDX5*, which was also upregulated in eosinophilic exacerbations, to scavenge hydrogen peroxide (199). This would indicate that the higher production of H_2O_2 has resulted in the increase of enzymes that would counter the detrimental effects on the eosinophil. Of interest, it would be advantageous to measure the production of similar antioxidant enzymes in the epithelial airways to determine if they also scavenge eosinophil generate ROS.

Glutathione is an important enzyme involved in maintaining a healthy cellular state through detoxification of ROS. In its reduced thiol state, glutathione is conjugated to oxidised substrates to resist oxidative stress (200). Glutathione-disulphide reductase is responsible for maintaining the conversion of glutathione in the reduced form and its gene, *GSR*, was found to be upregulated in the eosinophilic exacerbation group compared to healthy volunteers (201). This may indicate that glutathione reductase is increased in the cell to compensate for the increase ROS that is being produced by eosinophils. Measurements of the ratio of oxidised to reduced glutathione during an exacerbation would identify if this is the case. *SELENOW* is the gene that codes for a selenoprotein found to be regulated in the cell by glutathione and works in conjunction, protecting the cell from oxidative stress (202,203). This gene was also upregulated and may have similar roles in ROS detoxification during eosinophilic exacerbations.

The most well-established genetic risk factor for COPD is the Mendelian inherited disorder alpha-1 antitrypsin deficiency however, only up to 5% of people diagnosed with COPD carry this genotype (204). Most studies of the genetic determinants of COPD have focused on genome-wide association studies (GWAS) and identifying genetic variants of COPD but with relatively little success. This is mainly related to the requirement of large numbers of COPD subjects to identify differences in a complex and heterogeneous disease that exists in various phenotypes and co-morbidities (205). It is vital that future studies of COPD focus on extensively phenotyping patients before enrolment in genomic studies. There are multiple measurable disease endotypes that present in COPD patients and clearly grouping a cohort before identifying potential biological drivers of pathogenesis relevant to each patient will not provide discriminatory genetic identifiers. This will aid in understanding the underlying biological mechanisms of COPD in relation to the disease heterogeneity.

Previous investigators have turned to animal model comparisons with COPD GWAS studies to identify overlapping genes (206). However, murine emphysema is blunt model of a multifaceted disease which led to the identification of only eight overlapping genes. These included *HHIP*, *MMP1*, *MMP12*, *FAM13A*, *IREB2*, *AGER*, *SFTPD*, and *FBLN5*. These genes are related to Wnt and hedgehog signalling (207), mitochondrial iron regulation (208), protease balance (209), surfactant protein production (210), and extracellular matrix repair (211). Data from the eosinophilic exacerbation differential gene set analysis did show upregulation of *MMP15* in comparison with healthy controls, implicating eosinophils in the breakdown of the airway extracellular matrix. *FAM13A* promotes β -catenin degradation, a molecule involved in the promotion of airway repair. Although

FAM13A was not differentially regulated in any of these gene sets, *GSKIP* was upregulated, also in the eosinophilic exacerbation group. This gene shares a similar function to *FAM13A* by promoting β -catenin degradation therefore limiting airway repair thus, indicating a pathogenic rather than a homeostatic function.

This is the first genetic study to utilise a known biological driver of COPD pathogenesis to contextualise potential genetic contributions into biological mechanisms. It is evident that eosinophils exist in various states of activation relating to disease state. This also complements other evidence that eosinophils display heterogeneity and are not a purely defined population of cells. Indeed, eosinophils possess a vast arsenal of chemokines, cytokines, receptors, and various effector functions, allowing them to exist in numerous tissues (212,213). Although they possess the ability to contribute to pathogenic functions, it is plausible that they can carry out immunoregulatory or homeostatic roles. Future biomarkers of eosinophil activation may prove useful in deciphering eosinophil contributions to disease. This would be particularly beneficial for predicting response to biologic therapies that not only target eosinophils directly, but other aspects of the immune system involved in eosinophil localisation and response. An additional patient cohort that could be included in future analyses would be the eosinophilic but stable COPD cohort, as the current comparison does not account for differences in stable eosinophilic patients and those who are presenting with an eosinophilic exacerbation. This would aid in understanding which genes may be specific to driving eosinophilic COPD exacerbations.

Ultimately, the contribution of eosinophils to disease is highly dependent on their development in the bone marrow, their recruitment to, and their communication with the tissue niche in question, which leads to the activation state that determines

the final response. This may also be a determining factor in why subsets of patients respond positively (or conversely are unresponsive) to eosinophil targeting therapies regardless of the cut-off threshold used to determine treatment allocation. Combinations of therapies may therefore prove more fruitful in treating eosinophil related diseases and further support the requirement for biomarkers of eosinophil activation. Accurately phenotyping eosinophil subsets will be paramount in providing clues to this problem. Next generation sequencing technologies such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), single-cell RNA-seq, spatial transcriptomics, and mass cytometry are all platforms that could provide detailed phenotyping at the single cell level and providing predictions into the type of response exhibited by the eosinophil. Patient phenotyping would also aid in understanding the genetic drivers that are specifically relevant to the biological disease processes that underly the pathophysiology of COPD. Additional investigations into other phenotypes of COPD may also identify other distinguishing gene signatures. This may also provide insight into which genes make patients vulnerable to traits. Furthermore, this was a relatively small study and larger powered studies may yield more insights into gene expression profiles. Finally, the healthy controls in this study were not age matched, being half the age of their study counterparts. As aging is known to give rise to changes in both lung function and immune system functions, it would be particularly important to assess these changes with healthy age matched comparators.

Chapter 4

Rapid onset and excessive eosinophil-derived oxidative stress is critical to eosinophilic exacerbations of COPD

4 Rapid onset and excessive eosinophil-derived oxidative stress is critical to eosinophilic exacerbations of COPD

4.1 Introduction

Increases in systemic and localised airway inflammation are associated with COPD exacerbation events. While patients frequently experience similar episodes of worsening symptoms, the inflammatory profiles can vary between patients and likely reflect the aetiology of the acute event. For instance, 55% of exacerbation events are associated with airway neutrophilia while around 30% of exacerbations are recognised to be eosinophilic (14,91,93,214–216). While these two cell types are activated via release of different immune mechanisms, both have the capacity to contribute to antipathogen activities through various mechanisms including release of cytotoxic granules, extracellular DNA traps, and reactive oxygen species (ROS) (144,146,149,152,217–221).

ROS include a group of partially reduced oxygen metabolites that are highly reactive (75). They are generated as a by-product of aerobic respiration through the electron transport chain (ETC) in mitochondria and NADPH oxidases but also directly through oxidative bursts by phagocytic cells (71–73,222,223). At low concentrations they contribute to complex signalling functions and are an essential component of innate immune function (223). Either through an increase in ROS production or a decrease in antioxidant capacity, oxidative stress can occur (71). Resulting damage to DNA and protein oxidation is deleterious to cells with excessive prolonged ROS exposure thought central to inflammatory disease progression (74,75,77,224). Due to the destructive capabilities of these ROS-

producing cells, their homeostasis is tightly regulated to prevent tissue damage and endothelial dysfunction. It is proposed in COPD exacerbation events that ROS release contributes to uncontrolled inflammation and increased symptoms (225).

Neutrophils are highly abundant leukocytes and a crucial component of the innate immune system characterised by dynamic nuclear architecture (23,92). Sharing a common lineage, eosinophils circulate at much lower levels and comprise between 1-5% of circulating leukocytes with bilobed nuclear morphology (132,226). Both leukocyte types have the capacity to contribute to antipathogen activities through various mechanisms including release of cytotoxic granules, extracellular DNA traps, and ROS (144,146,149,152,217–221). Due to the destructive capabilities of these cells, their homeostasis is tightly regulated to prevent tissue damage and endothelial dysfunction.

Commonly associated with allergy and parasitic defence, there is growing evidence that eosinophils contribute to a much broader range of physiological functions than previously thought (227). Indeed, a high blood eosinophil count increases the risk of COPD exacerbations but is also advocated as a biomarker to direct systemic corticosteroid therapy towards patients who are most likely to benefit, thus reducing treatment failures (104,186,228,229).

Following the identification of genes pertinent to ROS activity in the gene set analysis from chapter 3, this chapter aims to explore the mechanisms that may contribute towards eosinophilic exacerbation onset. Differentially regulated genes will be validated in combination with measurements into eosinophil capabilities to produce ROS in disease states and healthy controls. Known mechanisms of ROS generation will be investigated to identify sources of ROS in the cell. This will be compared to the neutrophil, a more widely studied cell that shares similar lineage

to extrapolate potential functions and capabilities. Vital to ROS production are ROS scavenging functions to prevent detrimental effects to the cell and surrounding microenvironment. Therefore, antioxidant systems will also be measured to understand their contributions to prevent cell damage.

4.2 Results

4.2.1 Eosinophilic exacerbation events are associated with increased extracellular ROS

Based on findings from the transcriptional analysis, I investigated the *ex vivo* release of eosinophil ROS from stable COPD or at exacerbation (see previous clinical characteristics table 4). In unstimulated conditions, ROS production from patients at exacerbation increased at a more rapid rate than compared to stable COPD (fig. 25A). Eosinophils displayed a two-fold increase in the rate of change from 62.9 FI/min during stable state to 129.3 FI/min during an exacerbation. To compare the rate of ROS production further, neutrophils, another ROS-producing cell which contribute to other non-eosinophilic COPD exacerbation events were assessed from the same donors. Rapid ROS release was confined only to eosinophils during eosinophilic exacerbations, as unstimulated neutrophils did not show a significant difference at the two-hour time point between stable state (FI = 34187) and eosinophilic exacerbation (FI = 35741; fig. 25A-B).

Furthermore, eosinophils produced more ROS than neutrophils under the same conditions. Eosinophils from healthy donors showed a 9807 FI increase compared to neutrophils ($p = 0.004$), stable eosinophils had 4152 FI increase ($p = 0.7$), and exacerbation-derived eosinophils had 12808 FI increase when compared

to neutrophils ($p = 0.0002$; fig. 24B). This lack of increased neutrophilic response was not a consequence of any immunomodulation at the exacerbation event as when stimulated with PMA *ex vivo*, neutrophils and eosinophils both increased ROS production (fig. 25C+D). These data suggest that during eosinophilic exacerbations, eosinophils generate ROS more rapidly and to a similar extent to neutrophils following stimulation.

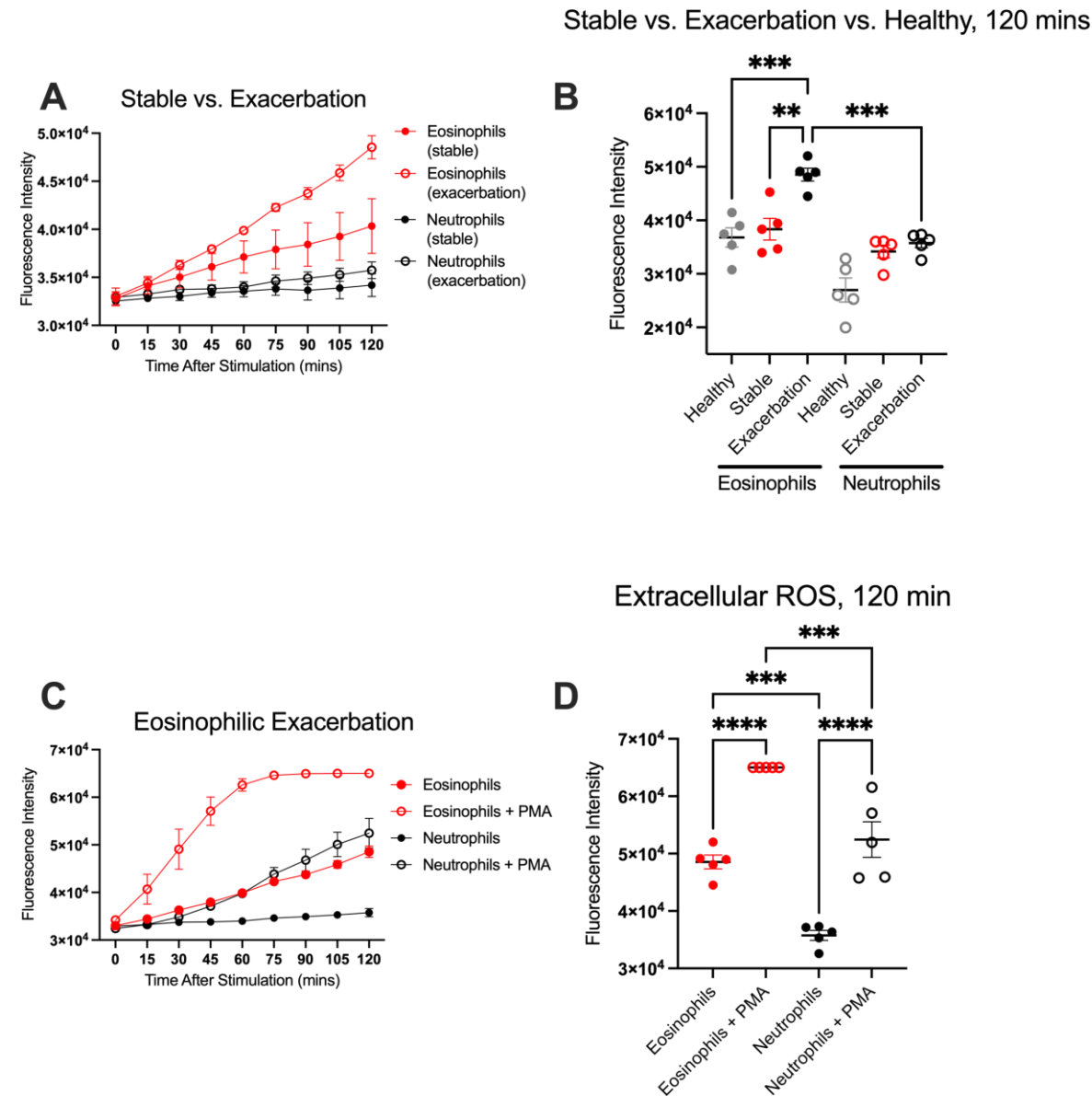


Figure 25. Eosinophils produce significantly more extracellular ROS during eosinophilic crisis.

Extracellular ROS was measured using OxyBURST Green H_2HFF BSA. **(A)** Comparison of unstimulated cells from eosinophilic exacerbations and from stable state over two hours. **(B)** Extracellular ROS production at 120 minutes comparing unstimulated cells from healthy, stable disease state, and eosinophilic exacerbating donors. **(C)** Eosinophils or neutrophils were isolated

from peripheral blood during an eosinophilic exacerbation with or without 20 ng/mL PMA over two hours. **(D)** Extracellular ROS production at 120 minutes with or without PMA stimulation. Data show mean $\pm$ SEM, n = 5 / group. 2way ANOVA was used for statistical analysis in **B** and **D**. *P $\leq$ 0.05, **P $\leq$ 0.01.

To explore whether increases in ROS production were a consequence of changes in basal state production during cell processing, I also investigated extracellular ROS generation in COPD compared to healthy controls. Notably, both eosinophils and neutrophils had a higher resting ROS production in disease state than healthy equivalents: stable eosinophils = 32779.9 FI vs. healthy = 18971.78 FI ($p = <0.0001$; fig. 26A+B). Overall, healthy and COPD eosinophils both reached the maximum ROS output in 60 minutes following stimulation. However, unstimulated eosinophils remained at a higher basal rate of 38339 FI units that required 120 minutes for healthy unstimulated eosinophils to achieve: 36794.5 (fig. 26C+D). This may suggest that eosinophils isolated from COPD patients are primed to produce ROS in exacerbation events.

Upon PMA stimulation, there was no difference in the total amount of ROS generated in stable COPD (FI = 59660) compared to healthy (FI = 65000) in eosinophils ($p = 0.63$; fig. 26E+F). Overall, during exacerbation eosinophils produce more ROS more quickly than neutrophils.

To note, following co-incubation of isolated cells and NTHi (MOI = 100:1), eosinophils and neutrophils produced extracellular ROS to a similar extent (eosinophils = 60502 FI vs. 56387 FI; $p = 0.95$; fig 26G).

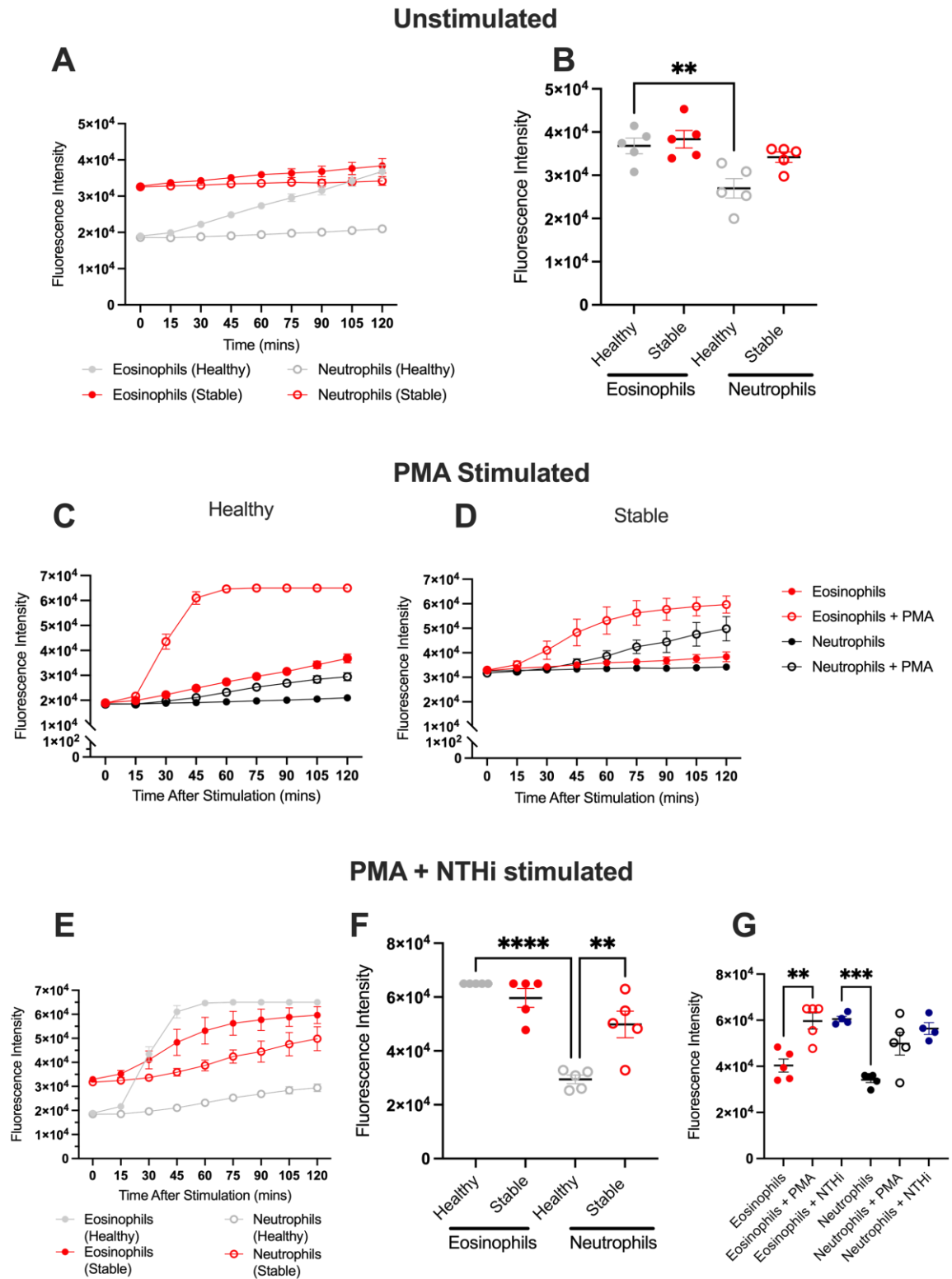


Figure 26. COPD eosinophils have a higher resting state and increased production of extracellular ROS than neutrophils.

Extracellular ROS was measured using OxyBURST Green H₂HFF BSA. Eosinophils or neutrophils were isolated from peripheral blood and incubated with or without 20 ng/mL PMA over two hours. **(A)** Comparison of unstimulated cells from healthy and stable state over two hours. **(B)** Extracellular ROS production at 120 minutes comparing unstimulated cells from healthy and stable disease state. **(C)** Extracellular ROS production of cells isolated from healthy donors and following PMA stimulation. **(D)** Extracellular ROS production of cells isolated from stable disease patients stimulated with PMA and unstimulated. **(E)** Comparison of PMA stimulated cells from healthy donors and stable disease state patients. **(F)** Extracellular ROS production at 120 minutes comparing PMA stimulated cells from healthy and stable disease state. **(G)** Comparison of stable state PMA stimulated cells and cells co-incubated with NTHi after 120 minutes during stable disease state. Data show mean $\pm$ SEM, n = 4 – 5 / group. 2way ANOVA was used for statistical analysis. *P $\leq$ 0.05, **P $\leq$ 0.01.

4.2.2 Eosinophils are capable of respiratory bursts

The intracellular oxidative burst capacity is integral to professional phagocytic cells, including eosinophils and neutrophils, to eliminate pathogens. To note, when intracellular ROS production is complexed with non-fluorescent H₂DCF molecules within the phagosome, oxidised fluorescent dichlorodihydrofluorescein (DCF) is released to provide a sensitive and exclusive internal measure of ROS. Detailed methods can be found in section 2.6.1. In this experiment, as shown by detectable fluorescence, I showed that not only were eosinophils capable of producing a respiratory burst, but they possessed a significantly higher oxidative capacity in comparison to neutrophils over a three-minute interval: 1.59×10^5 vs. 1.22×10^5 AUC (p = <0.0002; fig. 27A). This difference was reduced following this timepoint and was equivalent to neutrophils up to 20 minutes (eosinophils 4.98×10^4 vs. 4.85×10^4 FI units (mean difference = 1112; 95% CI = -802.3 to 3026; fig. 27B) suggesting, as observed in extracellular ROS production, eosinophils have a quicker response (fig. 27C).

Cells isolated from stable non-eosinophilic COPD patients' (0.5×10^6) showed that eosinophils had an elevated basal level of ROS production at 37 °C (1.5×10^3 MFI) in comparison to neutrophils from the same donors (8.5×10^2 MFI;

p = 0.001) which displayed a significant increase upon stimulation with the PKC activator PMA to 2.3×10^3 MFI (p = <0.0001) whereas neutrophils did not (p = 0.39; fig. 27D). Moreover, eosinophils produced mitochondrial ROS continuously over a one-hour period only upon priming with interleukin (IL) 5 and CCL11 (eotaxin-1) prior to incubation with NTHi (fig. 27E). Here, I showed that primed eosinophils could produce intracellular ROS quicker than neutrophils and were potent producers of mitochondrial ROS.

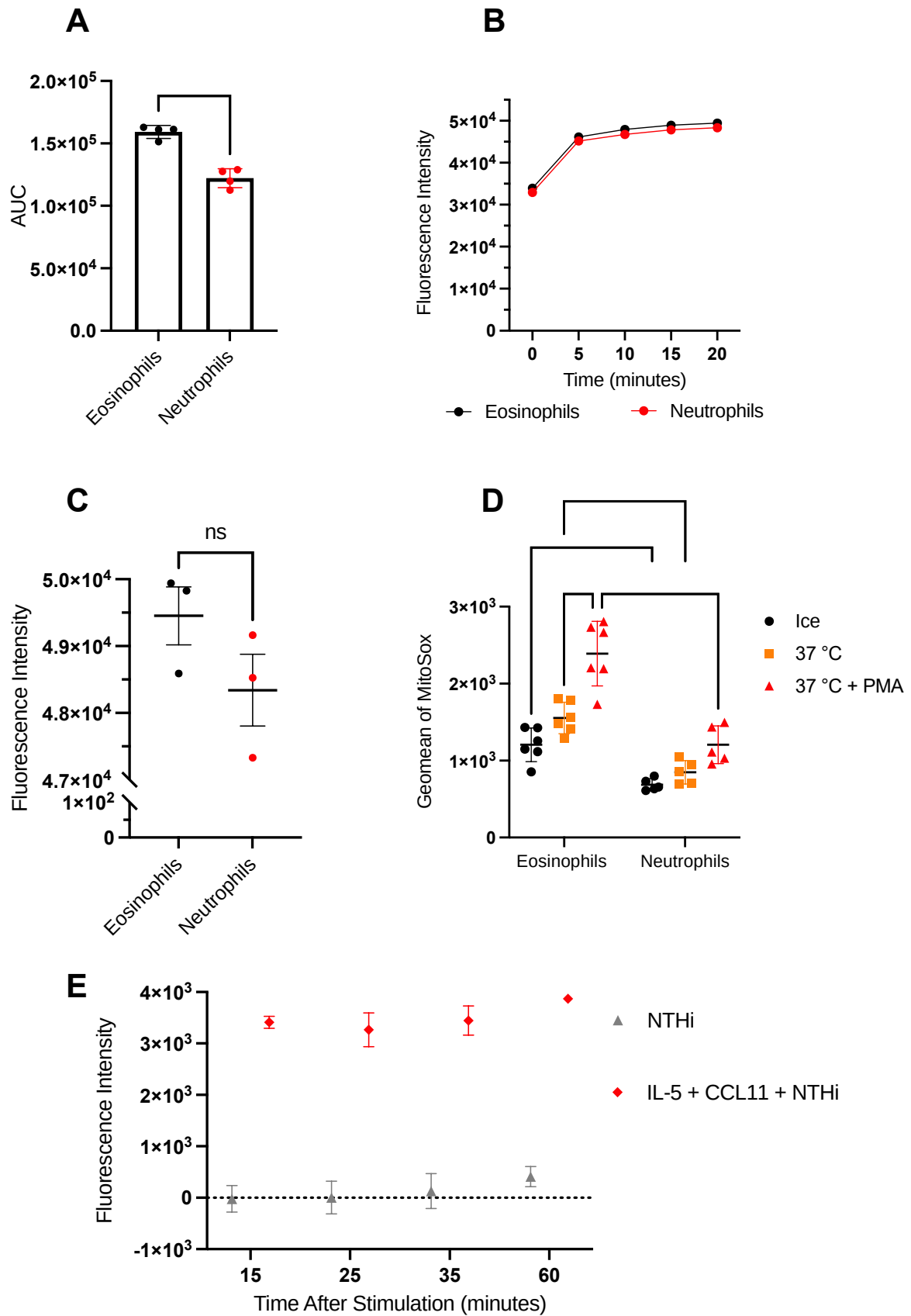


Figure 27. Superoxide is released within the phagovacuole of immune complex-stimulated eosinophils.

Eosinophils produce more mitochondrial ROS than neutrophils upon PMA stimulation and require priming to produce mitochondrial ROS in response to NTHi. Respiratory burst capacity of eosinophils and neutrophils was measured with Fc OxyBURST® Green assay reagent. **(A)** Area under the curve over three minutes for eosinophils and neutrophils. Using the kinetics feature in FlowJo, mean fluorescence intensity was plotted over time and the 4°C control subtracted to normalise for background fluorescence. **(B)** Oxidative burst measured at 5 minutes intervals following addition of 200 µg/mL Fc OxyBURST Green reagent. **(C)** Shows the fluorescence intensity at 20 minutes. **(D)** Mitochondrial ROS measurement for eosinophils and neutrophils kept on ice, at 37°C, and stimulation with 20 ng/mL PMA at 37°C. **(E)** Mitochondrial ROS production following co-incubation with NTHi alone and in combination with IL-5 and CCL11 **(F)**. Data show mean ± SEM, n = 3 in **A**, **B** and **E**; n = 4 in **C**; n = 6 for eosinophils and n = 5 neutrophils in **E**. t test for statistical analysis in **A** and **C**, 2way ANOVA in **D**. *P ≤ 0.05, **P ≤ 0.01, ns: not significant.

4.2.3 Eosinophils are capable of phagocytosing NTHi

Additionally, I investigated mitochondrial ROS production in the context of bacterial infection, the most common trigger for exacerbation events in COPD. Following the finding that eosinophils produce mitochondrial specific ROS in response to NTHi, I investigated whether eosinophils isolated from the peripheral blood of stable non-eosinophilic COPD patients could also phagocytose bacteria. As eosinophils typically have high autofluorescence and pre-conjugated pHrodo particles are not provided with relevant respiratory pathogens, I opted to label live cultured NTHi with the pHrodo red particle labelling kit (detailed methods can be found in section 2.5.2). These fluorophores are non-fluorescent in a basic environment but provide a sensitive signal once the stained bacteria enter the phagosome and the pH decreases (detailed methods found in methods section 2.5.2). Here I show that the eosinophils can indeed phagocytose immune complexes, as shown in figures 28A – C, but also fluorescently labelled bioparticles conjugated with non-typable NTHi (fig. 28A). I found that eosinophils can dose-dependently phagocytose NTHi (fig. 28B), a finding that was further increased following opsonisation of NTHi by incubating bacteria with pooled serum from stable disease patients (n = 10), although this finding was not significant (p = 0.23; fig. 28C). Figure 28D shows an

example plot where phagocytosis is inhibited by incubating eosinophils on ice prior to incubation with NTHi-conjugated pHrodo red particles.

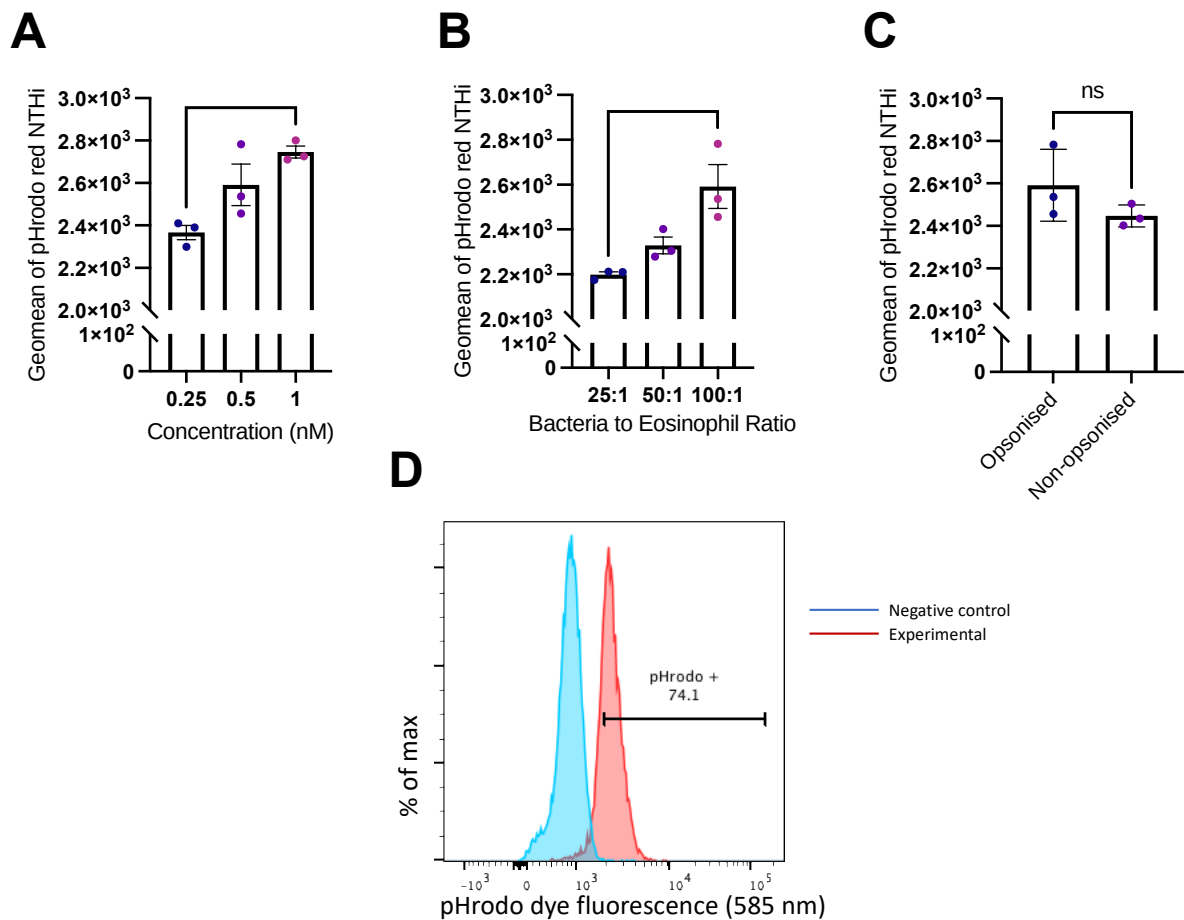


Figure 28. Eosinophils are capable of phagocytosing NTHi.

(A) Phagocytosis of live NTHi with increasing concentration of pHrodo red conjugate dye from 0.25 nM to 1 nM at a bacterium to cell ratio of 100:1 (B) Phagocytosis of NTHi at 25, 50, and 100 bacteria to 1 eosinophil ratio at a concentration of 0.5 nM. (C) Phagocytosis of human serum opsonised and non-opsonised NTHi. (D) Histogram overlay showing an example fluorescence separation between a negative control sample of inhibited phagocytosis and cells that have phagocytosed the NTHi-conjugated pHrodo particles. Data show mean ± SEM, n = 3. One-way ANOVA in A and B; t test for statistical analysis in C.

4.2.4 Eosinophils have reduced ability to scavenge ROS

To understand mechanisms contributing to increased ROS production in eosinophils, I next assessed components associated with antioxidant activity that were differentially regulated from the bulk sequencing analysis. As compared to

neutrophils, eosinophils had less glutathione content (mean difference = 0.76; $p = <0.0001$; fig. 29A). Moreover, neutrophils showed a two-fold increased ratio of oxidised to reduced glutathione ratio during stable disease state: 4.6 in neutrophils and 2.4 in eosinophils (fig. 29B), however this was not statistically significant (mean difference = 2.2; $p = 0.08$). Glutathione total levels also remained the same during an eosinophilic exacerbation and at a stable disease state (fig. 29C). This data suggests that the glutathione contribution in sequestering ROS in eosinophils is lower than that in neutrophils and may contribute to the increased ROS expression observed at crisis. Eosinophils and neutrophils were isolated from the peripheral blood of non-eosinophilic stable COPD and eosinophilic exacerbation COPD from the same donors.

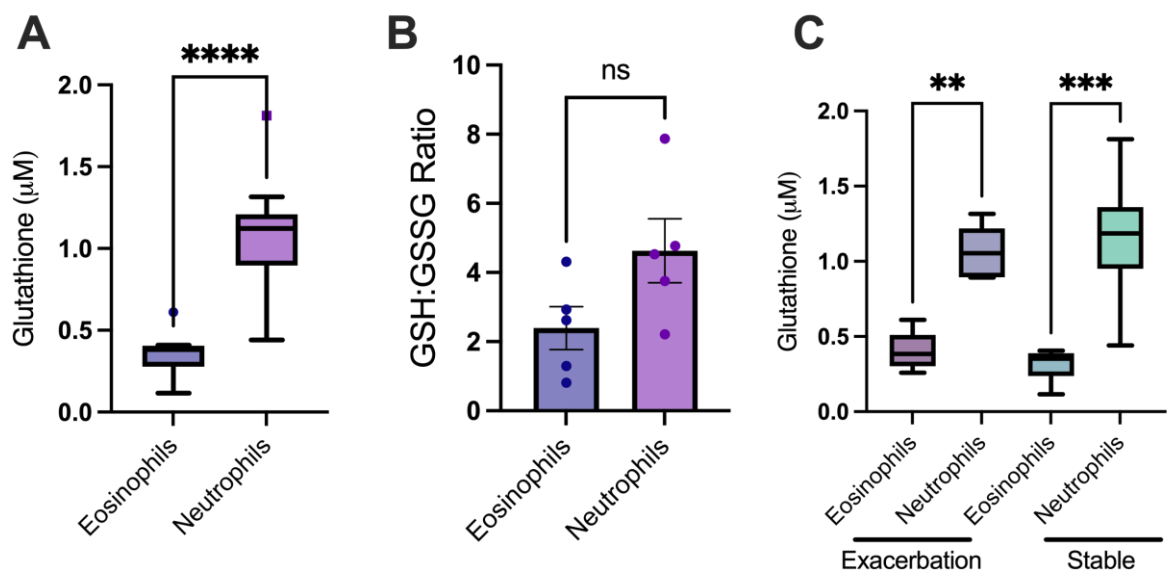


Figure 29. Eosinophils have reduced glutathione compared to neutrophils.

(A) Total amount of glutathione in 0.5×10^6 eosinophils and neutrophils. (B) The relative ratio of reduced (GSH) to oxidized (GSSG) glutathione in eosinophils and neutrophils. (C) Total glutathione during an eosinophilic exacerbation and at stable disease state. Data are representative of 11 independent experiments in A and 5 in B and C. Mann-Whitney test used for statistical analysis. * $P \leq 0.05$, ** $P \leq 0.01$, ns = not significant.

4.2.5 Expression of redox systems are increased during an eosinophilic exacerbation

Sequences of primers used in this study can be found in methods table 7 along with detailed methods in section 2.11 and clinical characteristics table 4. COPD patients were generally much older and had impaired lung function compared with healthy controls. Of the measured gene expressions between non-eosinophilic stable and eosinophilic exacerbation, thioredoxin and dual oxidase 2 were significantly upregulated: 1.7 (range 0.9 – 3.17) vs. 0.7 (range 0.06 – 1.2) $p = 0.04$ and 4.12 (range 1.12 – 7.39) vs. 0.72 (range 0.09 – 2.06) $p = 0.03$, respectively. However, glutathione peroxidase 4 was not: 1.45 (range 0.78 – 2.64) vs. 0.75 (range 0.07 – 1.15) $p = 0.1$ (fig. 30).

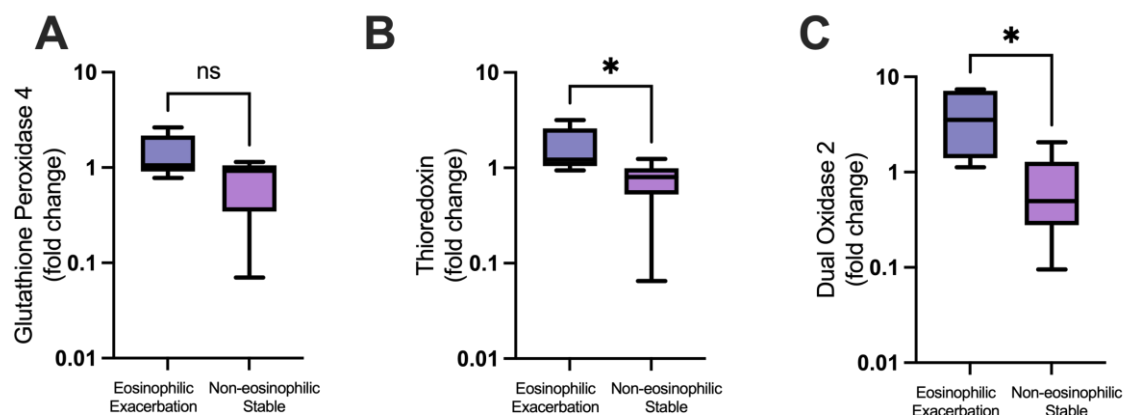


Figure 30. Glutathione peroxidase 4, thioredoxin, and dual oxidase 2 are upregulated during an eosinophilic exacerbation.

Box plots of the expression of glutathione peroxidase 4 (A), thioredoxin (B), and dual oxidase 2 (C). $n = 5$. * $P \leq 0.05$, ns: not significant.

4.2.6 The DNA damage marker 8-OHdG does not increase during eosinophilic exacerbations

When free radicals meet DNA or RNA, guanine can become oxidised and is detectable as 8-hydroxy-2'- deoxyguanosine from DNA and 8-hydroxyguanosine from RNA. I aimed to measure these species in sputum and serum to determine if these levels increase during an eosinophilic exacerbation compared with non-eosinophilic stable state. Figure 31A shows the sputum eosinophil count of the patient samples used in this study and included five eosinophilic patients (sputum eosinophil range: 11 – 89%) and five non-eosinophilic patients (sputum eosinophil range: 0 – 1.2%). Figures 31B and 31C show that there was no significant difference between 8-OHdG levels of eosinophilic exacerbators and stable non-eosinophils from the sputum (2277 vs. 4756, respectively; $p = 0.015$) and serum (9918 vs. 11470, respectively; $p = 0.57$).

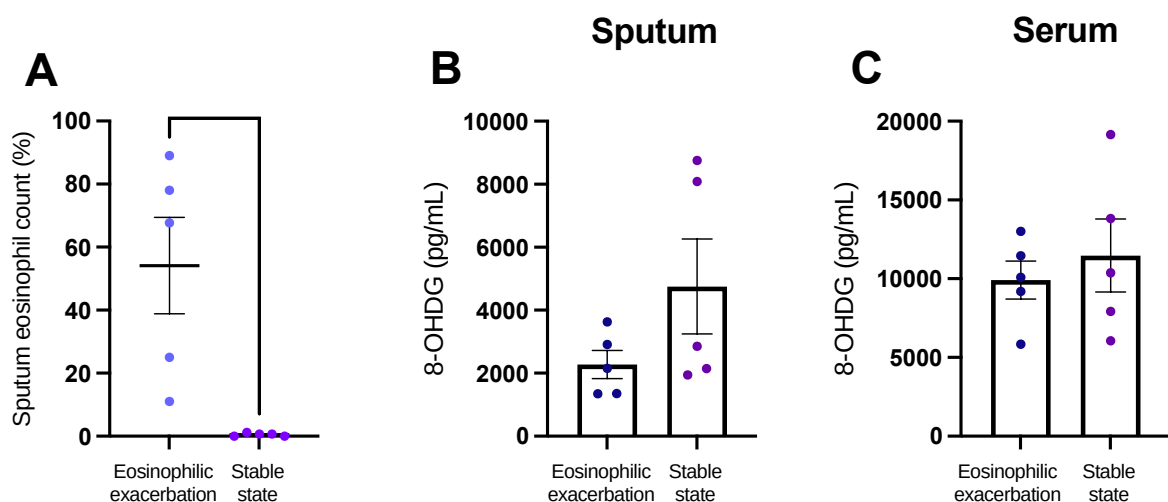


Figure 31. 8-OHdG levels are similar during an eosinophilic exacerbation and non-eosinophilic stable state.

(A) Scatter plots presenting differential sputum eosinophil count for patients undergoing an eosinophilic exacerbation and who are not eosinophilic during stable state. (B) Sputum 8-OHdG levels (C) Serum 8-OHdG levels. Data show mean $\pm$ SEM, $n = 5$. Mann-Whitney test was used in A and t test was used in B for statistical analysis. ** $P \leq 0.01$.

4.2.7 Eosinophils disrupt the barrier integrity of epithelial cells

As per section 4.2 and 4.3, I have shown that during an exacerbation event, eosinophils produce intracellular and extracellular ROS more rapidly than neutrophils. To understand the consequences of this increased production I co-cultured isolated eosinophils from the peripheral blood of stable disease patients with a A549 epithelial monolayers and assessed barrier integrity (fig. 32A-D). A549 culture protocols can be found in methods section 2.12.1 along with detailed procedures on detecting barrier integrity.

Following 2 h incubation with FITC-dextran and no epithelial monolayer formation, fluorescence intensity reached maximum intensity at 6.5×10^4 , implying that the FITC-dextran was able to completely permeate through the membrane (fig. 32D+H). This effect was halted when the A549 monolayer was established (fig. 32E+H). Upon incubation with eosinophils, permeability was increased as FITC-dextran solution leaked through the monolayer to be detected in a receiver well. Permeability increased from 1.8×10^4 FI with a monolayer alone (fig. 32E+I) to 2.0×10^4 FI upon incubation with eosinophils ($p = 0.006$; fig. 32F+I). This hyperpermeability was heightened upon eosinophil stimulation with PMA to 2.1×10^4 FI ($p = 0.03$; fig. 32G+I). These data suggest that increased eosinophilic ROS is sufficient for impaired barrier integrity and may impact epithelial damage following previous assays that showed PMA to be a stimulator of eosinophil ROS release.

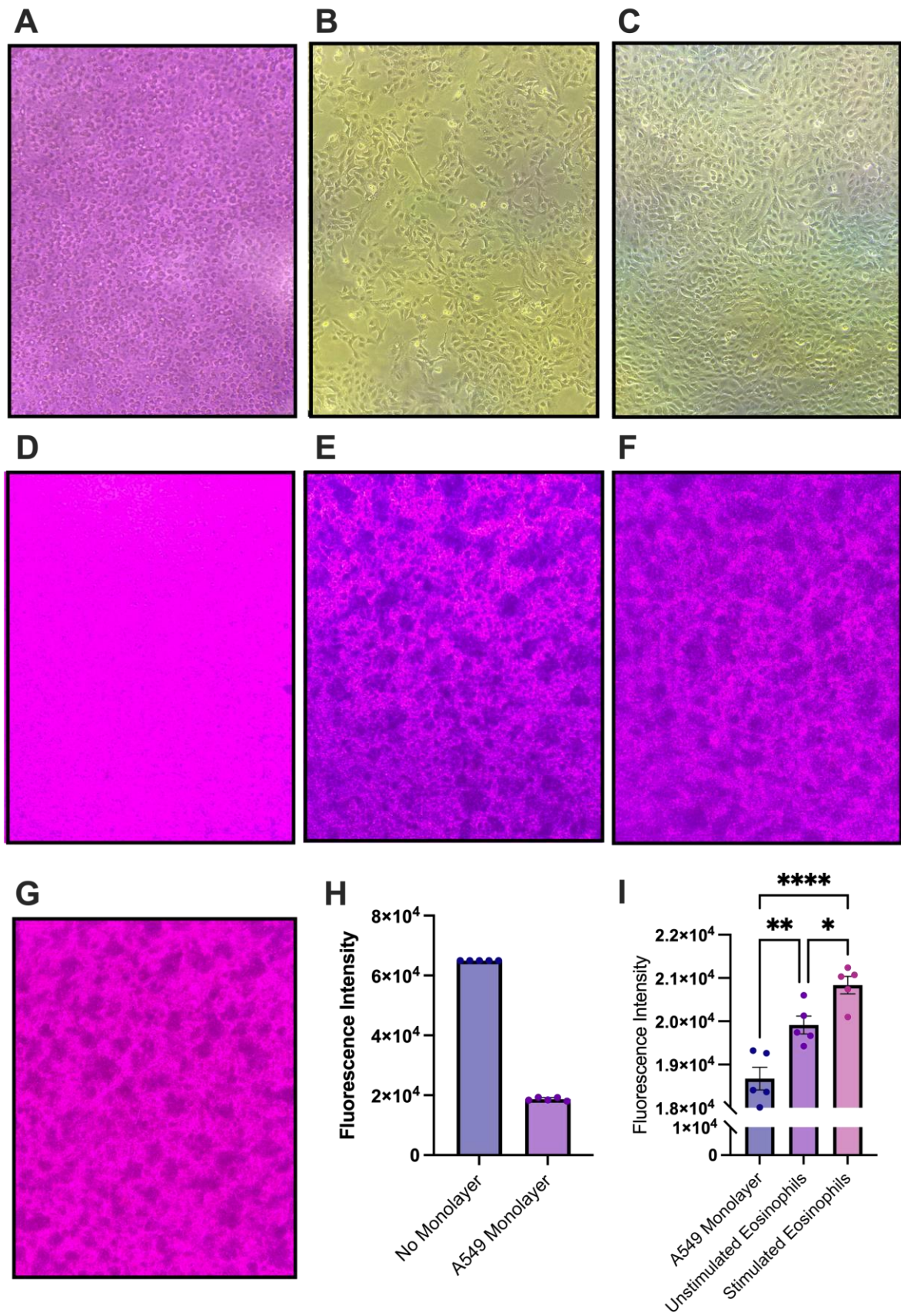


Figure 32. Eosinophils can disrupt epithelial barrier integrity resulting in hyperpermeability.

(A) Freshly seeded A549 cells. **(B)** A549 cells following 5 h incubation. **(C)** A549 cells after 24 h incubation as a confluent monolayer. Crystal violet (0.1%) staining of: **(D)** insert with no monolayer **(E)** A549 monolayer **(F)** A549 monolayer with unstimulated eosinophils **(G)** A549 monolayer with stimulated eosinophils. **(H)** Fluorescence intensity with and without A549 monolayer. **(F)** Fluorescence intensity following 24 h incubation with unstimulated or 10 ng/mL PMA-stimulated eosinophils at a 1:10 cell ratio. Magnifications shown at x10. One representative from 5 independent experiments shown in **D – G**. Data show mean $\pm$ SEM, $n = 5$ in **E**. One-way ANOVA used in **E**. * $P \leq 0.05$, ** $P \leq 0.01$.

4.3 Summary of results

In this study, I found that eosinophils not only had a higher capacity for ROS production in comparison to neutrophils, but eosinophils isolated from COPD patients undergoing eosinophilic exacerbations generated even higher levels of extracellular ROS, possibly contributing towards epithelial damage as shown when stimulated eosinophils were incubated with an epithelial cell line monolayer. Dual oxidase 2 had increased expression during eosinophilic exacerbations and may be critical in ROS production during exacerbations that are eosinophilic in nature. Eosinophils also possessed significantly less glutathione than neutrophils, an enzyme essential in scavenging ROS and may reduce the capacity of eosinophils to breakdown excess ROS. These findings provide insight into the mechanisms by which eosinophils contribute to exacerbations of patients with COPD and provide a new approach in the treatment of eosinophilic exacerbations through the utilisation of antioxidant compounds.

4.10 Discussion

Following the discoveries in the differential gene set analysis, I explored mechanisms of eosinophil reactive oxygen species generation and associated antioxidant activities *in vitro* and *ex vivo* following isolation of cells from exacerbating patients. As ROS are produced in response to various stimuli and have functions inclusive of signalling to antipathogen defence, I sought to understand the requirement of ROS generation in COPD disease states.

Although there is evidence that implicates oxidative stress in the pathogenesis of COPD, and increases in oxidative stress detected during acute exacerbations, eosinophil contributions have gone relatively unexplored (77,230–233). Previously, increased reactive nitrogen species (RNS) have been reported in COPD and asthma patients when compared to healthy controls, with biomarkers of RNS production also significantly increased in eosinophils from asthma patients. Airway obstruction was also significantly correlated with the amount of nitrotyrosine in this same study (234). However, this was a small study of ten COPD subjects and patients were not stratified by eosinophilia or neutrophilia, which may have underestimated the contributions of eosinophils in COPD. To my knowledge, this is the first study to explore eosinophil specific mechanisms of ROS generation and their contributions to the pathogenesis of COPD.

Firstly, I aimed to validate findings at the gene level that eosinophilic exacerbations are linked to increased ROS production. Indeed, I found a strong relationship between increased ROS production during these events, when compared with healthy volunteers and stable patients, suggestive of eosinophils being in a heightened state of activation. When measured over a two-hour time

course, I found that ROS production from exacerbating cells was much more rapid when compared with stable patients, also supporting this view.

Interestingly, the extracellular ROS production of neutrophils acquired from the same donors remained relatively constant over the two-hour period, suggesting that the stimulus was specific to an eosinophil response. I next sought to artificially stimulate cells through phorbol-12-myristate-13-acetate (PMA), a well-known NADPH-oxidase dependent pathway inducer of ROS (235). Again, I found there was an intensified and rapid response in eosinophils whereas the response was much more gradual in neutrophils. I found that eosinophils from healthy donors had a lower basal rate of ROS production but upon stimulation generated ROS rapidly, possibly indicative of erroneous response to stimuli displayed in disease, likely the result of insensitivity to ongoing stimuli and constant production of ROS not present in healthy individuals.

Of note, I also measured cellular responses to NTHi, a common potential pathogenic microorganism in COPD exacerbations, and found that eosinophils could produce extracellular ROS with similar potency to neutrophils. This finding was intriguing, as although eosinophils do possess antibacterial responses, neutrophilic inflammation is associated with bacterial infection (25). This is in combination with data reported in the previous chapter to suggest that genes associated with bacterial response are downregulated during eosinophilic exacerbations indicating that eosinophilic exacerbations are not associated with pathogenic bacterial infection. It is likely that neutrophils have evolved to play a more specialised role in bacterial defence as they are much more abundant in circulation and therefore capable of producing a rapid and efficient response to

pathogens, potentially relegating eosinophils to a bystander position during infection (236).

Following the thread of eosinophil antipathogen responses, I explored the ability of eosinophils to carry out phagocytosis. An integral innate immune function, phagocytosis is carried out by various immune cells, such as macrophages and neutrophils, and requires the production of an internal respiratory burst once the pathogen has been engulfed by the cell. Using a sensitive immune complex reagent that will only fluoresce following Fc mediated phagocytosis and entry into the phagovacuole, I found that eosinophils were indeed capable of this function. As eosinophils are commonly thought to have evolved to carry out anti-parasitic functions through their major effector function of degranulation, it was interesting to find that their capability to phagocytose was similar to that of neutrophils (237).

Indeed, the oxidative burst function appeared maximal at three minutes, being significantly higher than that of neutrophils. I also explored the production of mitochondrial specific ROS production which appeared to have a higher basal level of production when compared to neutrophils but also at 37°C and upon PMA stimulation. This function may be in part related to eosinophils ability to produce eosinophil extracellular traps (EETs). Like neutrophil extracellular traps (NETs), eosinophils can expel their mitochondrial DNA and granule proteins in a ROS-dependent manner in a process that can bind and kill pathogens without causing the death of the cell. This data supports the findings of Porter *et al.*, 2018, where eosinophils were found to have a higher rate of mitochondrial respiration, maximum respiratory capacity, greater metabolic flexibility, including the ability to use other mechanisms of oxidation than neutrophils (238). These capabilities would allow

eosinophils to produce more ROS upon stimulation, allowing eosinophils to respond to a more diverse range of functions including homeostasis and immunomodulation.

Using pHrodo-red conjugate dye, I stained NTHi to investigate whether eosinophils can phagocytose live bacteria, rather than artificially constructed immune complexes. By testing various concentrations of dye and bacterial to cell ratios, I found that eosinophils were capable of phagocytosing NTHi in a dose dependent manner, indicating that eosinophils have the capacity to contribute towards host defence through phagocytosis. It is likely that this ability is often overlooked in the airway considering the abundance of professional phagocytic cells, including macrophages that are found to make up around 94% of the leukocyte population in airways (239). It was also intriguing that eosinophils can carry out this function considering their association with the defence of larger multicellular pathogens and the comparative abundance of other phagocytic cells, but it could be related to the ancestral developmental lineage that neutrophils and eosinophils share and is now a redundant function. Alternatively, macrophages from COPD patients have been reported to be defective in phagocytosis and related to altered mitochondrial function due to increased oxidative stress (73). Therefore, it may be beneficial to have multiple cell types capable of similar functions in environments where certain cells may be more prone to malfunction in micro-niches, like alternate metabolic pathways reported in Belchamber *et al.*, 2019, so innate functions can continue (73).

One study has compared eosinophil and neutrophil phagocytic functions *in vitro* in response to *S. aureus*. This study found that although eosinophils were able to carry out phagocytosis, this capability was less active than that of neutrophils. However, there are certain limitations of this study that are addressed in the

experiments of this thesis. Firstly, bacteria were stained with propidium iodide (PI) to measure bacteria engulfment by the cell as a measure of fluorescence staining of DNA. Although a useful indicator, pHrodo dye is much more sensitive than PI as the stained bacteria will only fluoresce once the bacteria has entered the acidic environment of the phagosome. Bacteria that are stained with PI may also not be engulfed but stick to the surface of the cell membrane and register a positive signal in the by flow cytometry (240). The bacteria in my investigations were also live rather than heat-killed which may also cause discrepancies. Hydrogen peroxide production was used as a measure of phagocytic activity by 2',7'-dichlorofluorescein diacetate (DCF) and showed no significant difference in production. The authors of this study incorrectly reported that DCF is solely a measure of H_2O_2 and relate this activity to the phagovacuole, when DCF is de-esterified also in the cytosol to its fluorescent product upon oxidation by a range of species. Therefore, DCF cannot be used as a direct measure of H_2O_2 (241). Additionally, I found that priming eosinophils with CCL11 and IL-5 effected their response to NTHi therefore it may be incorrect to assume that the *in vitro* activities observed here are entirely reflective of the microenvironment.

As eosinophils evidently have a strong ability to produce ROS, I explored their ability to scavenge ROS. As excessive production can be deleterious to cells (199), I examined the glutathione content of eosinophils and neutrophils. Glutathione is an abundant ROS scavenger produced in the liver and present in virtually all human cells. I measured the total amount of glutathione along with the ratio of oxidised to reduced glutathione in eosinophils and neutrophils. Here I found that neutrophils contained significantly more of this enzyme than eosinophils, a surprising finding considering the high production of ROS by eosinophils would

imply the requirement for antioxidant enzymes to prevent cell damage from excess ROS. I also found that the ratio of oxidised to reduced glutathione was increased in neutrophils compared with eosinophils, indicating that more ROS is scavenged by neutrophils. It appears that ROS is more tightly contained by neutrophils, preventing excessive release from the cell. Of note, I did not find any differences in amount of glutathione at exacerbation in either cell type, so although there is more ROS produced by eosinophils during crisis events, ROS are not more tightly regulated, allowing eosinophils to release larger amounts of ROS.

To validate my findings from the RNA-seq dataset, I aimed to measure the expression of genes that were found to be upregulated in the eosinophilic exacerbation group: *Txn* and *Duox2*, and a gene that showed no significant difference: *Gpx4*. These genes were chosen for their integral involvement in regulating oxidative stress. I found that *Txn*, an alternative enzyme involved in maintaining a reducing environment, was upregulated when compared with non-eosinophilic stable state patients. Thioredoxin systems are namely involved in the NADPH + H⁺ and peroxidase pathways to protect cells from the detrimental effects of ROS, both pathways of which were upregulated in the gene ontology enrichment analysis (199). It may be that thioredoxin is of particular importance in maintaining this antioxidant system, as *Gpx4* was found not to be significantly upregulated, also in support of our measurements of glutathione in eosinophils during exacerbations.

Duox2 is the gene that codes for the dual oxidase 2 enzyme responsible for the generation of hydrogen peroxide (242). This gene was found to be upregulated in the eosinophilic exacerbation group and is likely the major source of ROS generation found during crisis events. Expression of DUOX in the epithelia of the lung and the regulated release of hydrogen peroxide has also been associated with

normal lung function (193). As the epithelium of the lung is continuously exposed to external infectious particles, all sections of the airways must present defence mechanisms to prevent invasion from potentially pathogenic sources. Hydrogen peroxide is reportedly secreted from the airway epithelium into the surface liquid lining where it is converted by lactoperoxidase (LPO) into bactericidal OSCN^- (243). Other closely related peroxidases that convert H_2O_2 into OSCN^- include myeloperoxidase and eosinophil peroxidase (244). Therefore, degranulation of eosinophils may contribute towards maintaining surface lining constituents of the airways. Of note, El-Chemaly *et al.*, reported that glutathione peroxidase only plays a minor role in H_2O_2 scavenging and may explain why I did not see any changes in expression or change in the ratio of oxidised to reduced glutathione during exacerbations. This study recruited eight healthy non-smokers to acquire tracheal secretions for investigations into peroxidase activity and hydrogen peroxide scavenging. It would be therefore useful to replicate a similar study to determine the effects of airways diseases in comparison (245). Previous studies have highlighted that H_2O_2 is generated continuously in low amounts in healthy lungs however, during inflammatory disease states, including COPD, the concentration of H_2O_2 detected in exhaled breath is significantly increased (246–249). The deleterious effects experienced by patients undergoing exacerbations may well be associated with the heightened release of ROS by eosinophils during their influx into the lung, contributing to inflammation and aggravating the lung tissue (250).

Following the findings in this chapter of excessive ROS production during eosinophilic exacerbations, I sought a biomarker to detect DNA damage from the resulting ROS release. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a reported ubiquitous marker of oxidative stress (76). When DNA is repaired by exonucleases,

oxidised 8-OHdG is excreted, as such, I measured the 8-OHdG levels in serum and sputum to determine its utility as a biomarker of eosinophil derived oxidative damage during eosinophilic exacerbations (251,252). I did not find a difference in the amount of detectable 8-OHdG in either sputum supernatant or serum when comparing eosinophilic exacerbators or stable disease patients. However, as 8-OHdG is secreted ubiquitously and not necessarily the result of ROS damage, it may be that this marker is not specific to the type of ROS damage I was hoping to measure, and other markers may prove more fruitful.

To measure the effects of ROS damage directly, I developed an epithelial model system by culturing A549 monolayers in an insert and assessed the permeability of the tight junctions following incubation and stimulation with eosinophils. I found that stimulating eosinophils through previous mechanisms I had shown to produce ROS did increase the hyperpermeability of the monolayer, allowing more FITC-dextran to be detected in the receiver well. These findings support a functional role for eosinophils contributing towards detrimental effects within the airways during an eosinophilic exacerbation. I suggest that activated eosinophil influx produces excessive ROS release that consequently intensifies inflammation within the lungs and aggravates the tissue lining. This may contribute to the symptoms experienced by COPD patients during an exacerbation and explain why eosinophil depleting drugs resolve the experienced symptoms.

This chapter has utilised the findings from the RNA-seq dataset and explored possible mechanisms of eosinophil responses in the airways. Through detailed measurements of ROS production and a model co-culture system, I have provided a holistic explanation of how activated eosinophils may contribute towards disease pathology. Transcriptomics of peripheral blood eosinophils isolated from patients

during an eosinophilic crisis event identified upregulation of several genes involved in ROS homeostasis. Many of these genes were related to mitochondrial complex formation that contribute to the generation of mitochondrial specific ROS. It has previously been reported that there are only 5-6 mitochondria per neutrophil whereas the number of mitochondria per eosinophils is much higher at around 24-36 (253) – coinciding with our findings that eosinophils have the capacity to produce much more mitochondrial superoxide than neutrophils and that DUOX2 NADPH family oxidase, involved in the generation of H_2O_2 , was also upregulated. The increased capability for ROS production by eosinophils defined eosinophilic crisis and therefore approaches such as blocking DUOX pathways may prove effective in reducing exacerbations. Exacerbations of COPD are driven by the acute worsening of symptoms experienced by patients. To date, the mechanisms contributing to eosinophilic crisis events are not known. Here, I showed a rapid and increased production of ROS by activated eosinophils during a crisis highlighting a plausible mechanism whereby eosinophils directly contribute towards the acute symptoms experienced by patients. I have shown that not only are eosinophils producing excess ROS during resting state, but that they also produce ROS much more rapidly upon stimulation, which may reflect the inflamed milieu within the airways.

Chapter 5

Characterising eosinophilic inflammation in the context of COPD exacerbations

5 Characterising eosinophilic inflammation in the context of COPD exacerbations

5.1 Introduction

Eosinophils are used as a biomarker to phenotype airways disease, in addition to being a potential indicator for avoiding treatment failures by guiding anti-inflammatory therapies and predicting the risk of exacerbations. This has led to the development of monoclonal antibodies directed at reducing the eosinophil count in severe asthma. Targeted therapies have also improved our understanding of type 2 inflammatory pathways and how eosinophils are recruited by blocking specific pathways to prevent undesirable responses. Eosinophilic inflammation itself requires further understanding to interpret the pathways involved that drive the pathogenic role of eosinophils in COPD.

The success of eosinophil depleting biologics in asthma was expected to be replicated in COPD (109,254–257). However, as Benralizumab failed to reach its primary endpoint in COPD, it is apparent that our understanding of eosinophil related pathologies requires further development. It is now apparent that other aspects may be at play that drive pathogenic eosinophil responses other than a general threshold for eosinophils; and identifiers of eosinophil activation or inflammatory subtypes would be advantageous in not only understanding the relationship of eosinophils and disease but also developing further therapies.

This study aimed to appropriately phenotype eosinophilic inflammation in patients with COPD to understand the interplay between eosinophils and exacerbations and importantly why some individuals may be eosinophilic but do not

require intervention. This will aid in utilising biomarkers to their full potential in an era of personalised medicine.

5.2 Results

5.2.1 Heightened eosinophilia is associated with eosinophilic exacerbations in airways disease

Eosinophil blood counts taken from 21 stable state, treatment naïve patients and during eosinophilic exacerbations showed an increase in eosinophilia, which was also reflected in sputum from 14 stable and 17 exacerbation patients. Paired samples were acquired from patients with airways disease during recruitment at stable state and when patients presented with an exacerbation. I found that there was a significant increase in eosinophil count from an average of $0.50 \times 10^9/\text{L}$ to $0.83 \times 10^9/\text{L}$ [(p = <0.0001) fig. 33A]. Spontaneous sputum samples were also collected from patients when possible. Unpaired samples showed a similar trend with a significant increase in the median value of the stable state eosinophil cell differential from 8.5% to 47% during an eosinophilic exacerbation (p = 0.0021; fig. 33B).

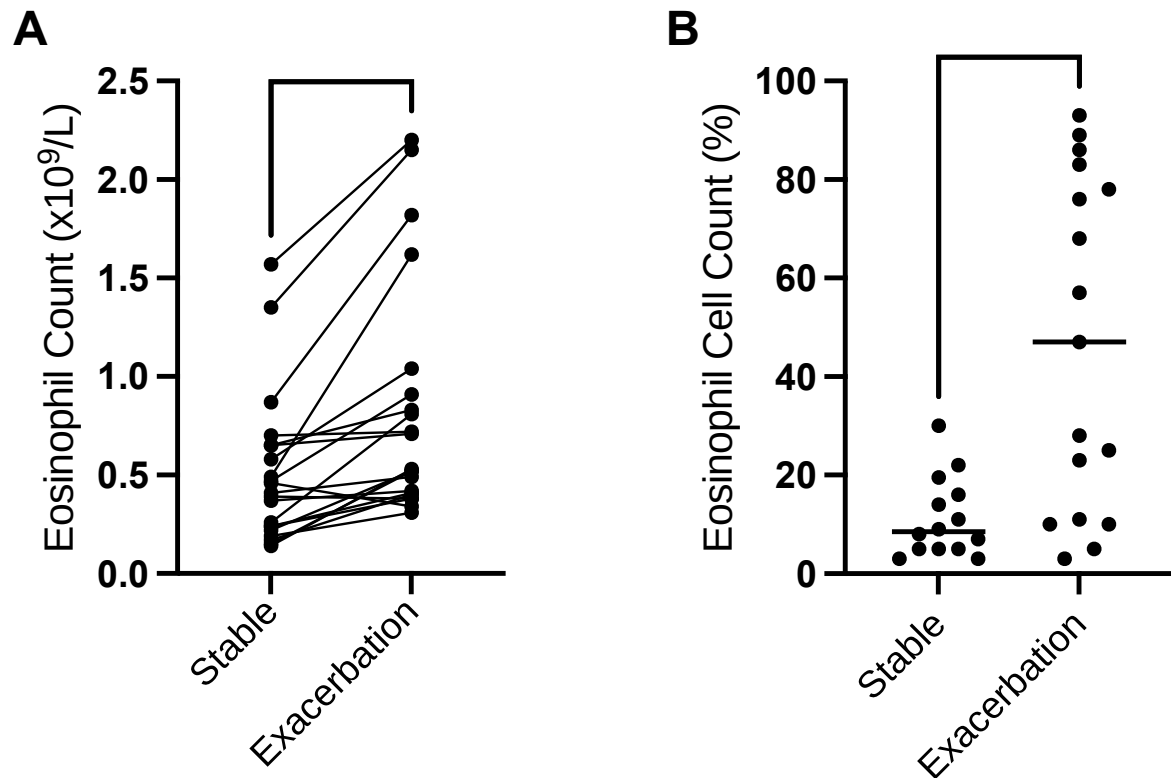


Figure 33. Eosinophilic exacerbations are associated with heightened eosinophilia.

(A) shows the eosinophil blood count taken at stable state and eosinophilic exacerbation time point from the same patient. (B) shows the percentage eosinophil cell count from spontaneous sputum samples acquired at eosinophilic stable state and eosinophilic exacerbation. Paired samples in A include $n = 21$, B includes $n = 14$ at stable state and $n = 17$ at exacerbation. Wilcoxon test was used in A and Mann-Whitney test used in B for statistical significance. * $P \leq 0.05$, ** $P \leq 0.01$.

5.2.2 IL-8 levels are similar in the lung and blood compartments of COPD patients regardless of stratification by neutrophilia and eosinophilia

Interleukin (IL) -8 is a key pro-inflammatory mediator involved in the recruitment and activation of neutrophils. Sputum eosinophilia was described as $>3\%$ eosinophils of non-squamous cells and sputum neutrophilia was classified as $>61\%$ neutrophils of non-squamous cells (209). As neutrophilia is an important clinical phenotype of COPD, I measured the IL-8 levels in serum and sputum to distinguish neutrophilic from eosinophilic phenotypes. Figure 34A shows the sputum cell counts that

represent patients classified as neutrophilic whereas figure 34B displays the cell counts for eosinophilic patients. I found that there was no difference in the IL-8 concentration used to classify these two phenotypes in either the sputum (fig. 34C), neutrophilic: 534.1 pg/mL vs. eosinophilic: 489.2 pg/mL ($p = 0.49$), or serum (fig. 34D), 21.00 pg/mL neutrophilic vs. 21.59 pg/mL eosinophilic ($p = 0.84$).

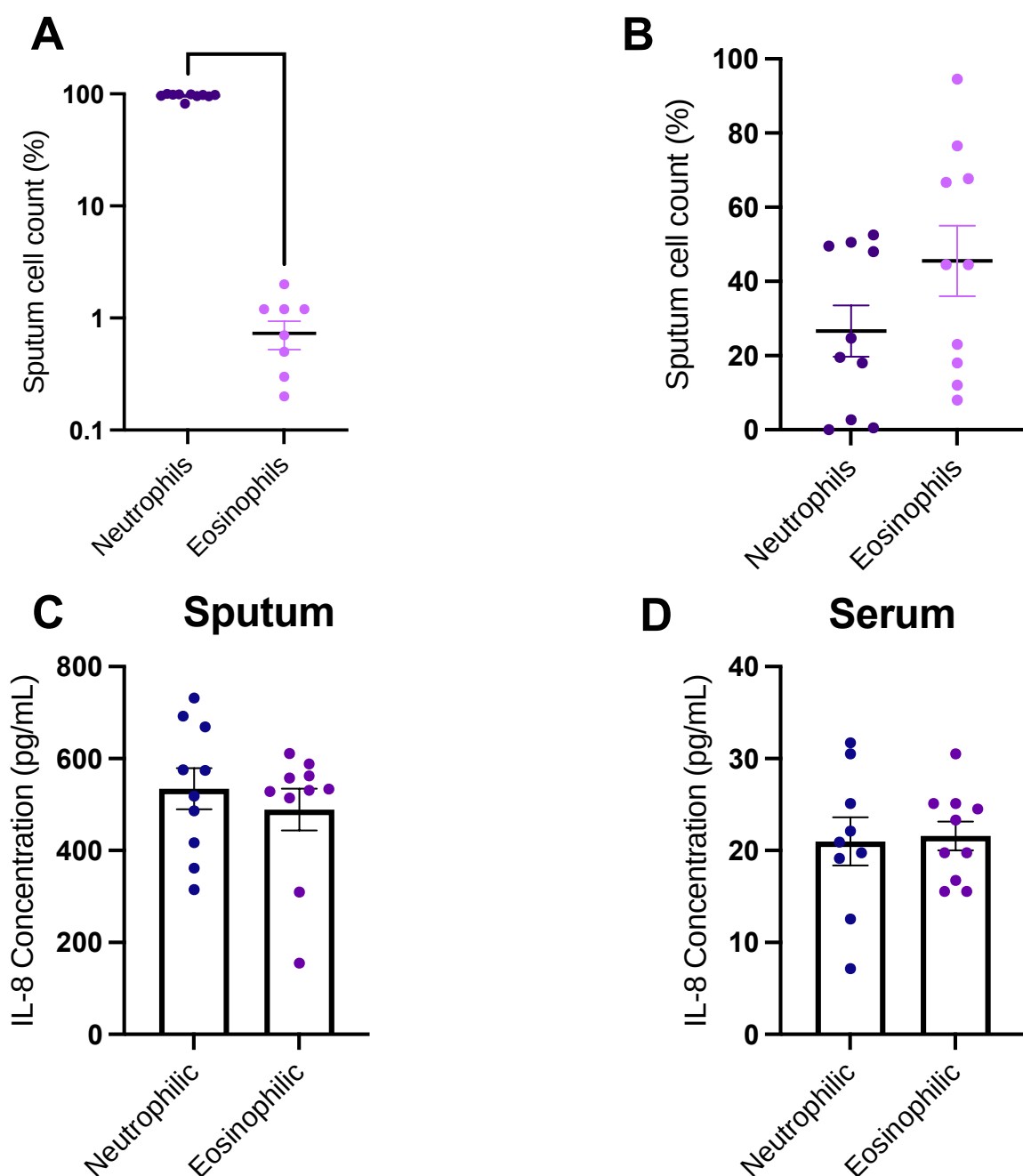


Figure 34. IL-8 levels in serum and sputum are similar in patients with COPD regardless of neutrophilia and eosinophilia.

Scatter plots presenting differential sputum neutrophil and eosinophil counts for COPD subjects in the neutrophilic (**A**) and eosinophilic (**B**) cohorts. Box plots comparing IL-8 levels in (**C**) sputum and (**D**) serum in neutrophilic and eosinophilic patients with COPD. Data show mean $\pm$ SEM, $n = 10$. Mann-Whitney test (**A**); unpaired t test (**B**) and (**C**). * $P \leq 0.05$, ** $P \leq 0.01$.

5.2.3 Eosinophilic exacerbations present with increased IL-5 in the lung and therapies used in the treatment of exacerbations eliminate IL-13 in the blood compartment

There is unmet need for reliable biomarkers indicative of eosinophil driven symptom exacerbations that would correctly identify patients responsive to eosinophil directed therapies. Furthermore, a clearer understanding of how these therapies resolve symptoms may aid in future targeted drug development. Therefore, I measured the cytokine levels of ten cytokines related to eosinophil recruitment and activation at stable disease state, during an eosinophilic exacerbation and following treatment.

Sputum mediator profiling from COPD patients during an eosinophilic exacerbation showed increased levels of IL-5 when compared to stable state [mean fold difference (95% CI): 82.61, $p = <0.0001$] and one week following treatment of either Benralizumab or prednisolone [mean fold difference (95% CI): 86.92, $p = <0.0001$ (fig. 35A)]. No differences in stable state vs. treatment were detected for CCL11 (eotaxin-1), CCL24 (eotaxin-2), CCL26 (eotaxin-3), TNF- α , TSLP, or IL-1 β (fig. 35A + B). Undetectable sputum cytokines included: IL-17E/IL-25, IL-33, and IL-13. In serum, IL-13 was undetectable following treatment whereas it was detectable during both stable state and eosinophilic exacerbation [geometric mean = 4.18 and 6.68, respectively (fig.35B)]. All other mediators showed no detectable differences between conditions. Mediators that were undetectable in serum included: IL-1 β , IL-33, IL-17E/IL-25.

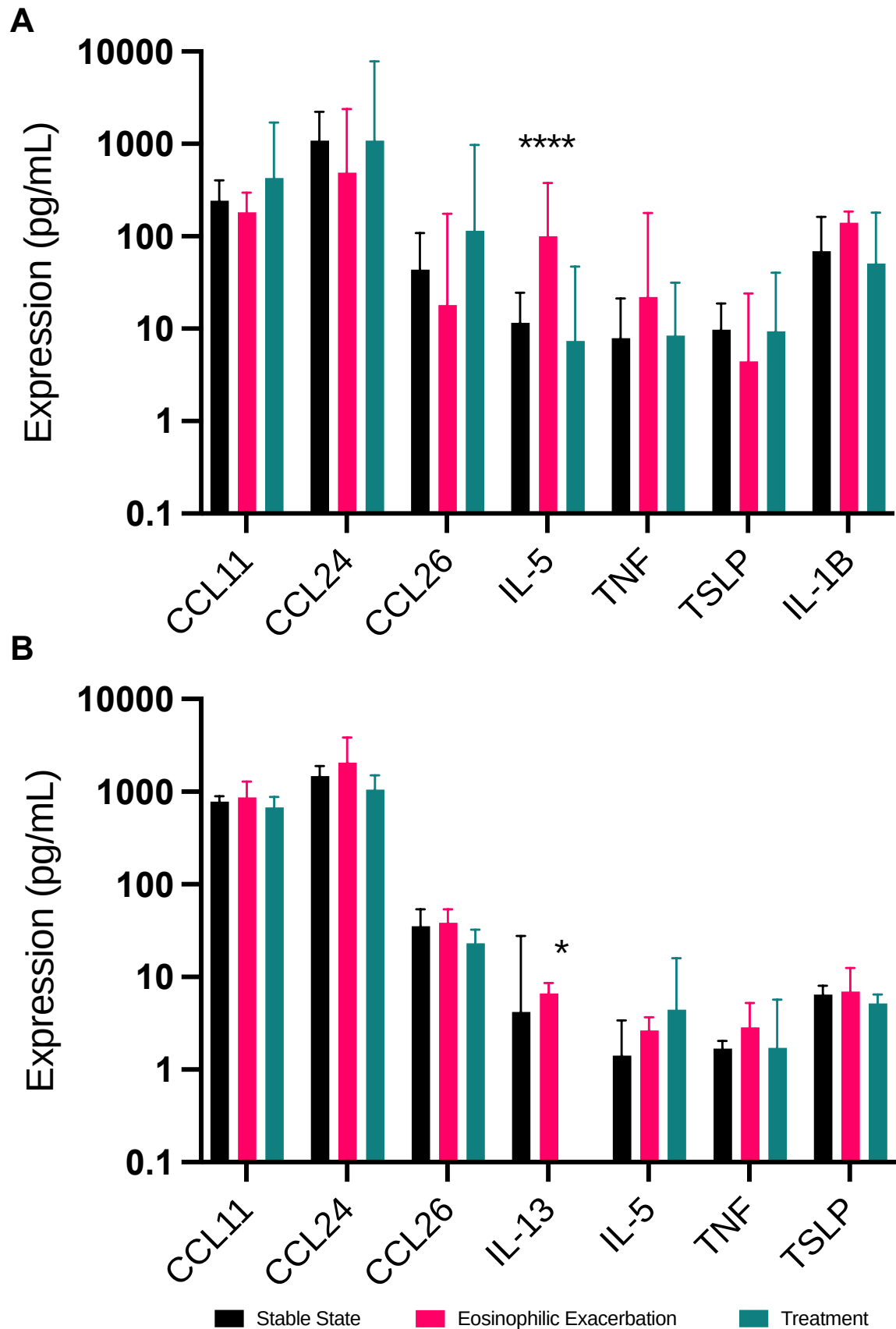


Figure 35. Eosinophilic exacerbations are associated with increased IL-5 in sputum and treatments directed at eosinophilic exacerbations eliminate IL-13 in serum. Bar charts representing measured sputum (A) and serum (B) cytokines and chemokines in COPD subjects during stable state, eosinophilic exacerbations, and one week following treatment. n = 8.

Mediators that are marked by an asterisk are significantly different ($p < 0.05$). Horizontal and error bars are set at the geometric mean and 95% CI.

I compared eosinophilic and non-eosinophilic COPD patients between stable state and exacerbations across the time course of the study treatment, from treatment naïve to treatment, as shown in Figure 36. Further subgroup analysis by stratifying patients based on their disease status revealed that non-eosinophilic stable and treatment groups had similar cytokine expression profiles (fig. 36A-D). In the serum of patients, IL-13 was undetectable in non-eosinophilic or treatment groups however, eosinophilic groups showed a similar expression: mean difference 85.2, 95%CI 1.96 – 385.6 (stable eosinophilic) vs. 2.74 – 451.4, $p = 0.48$ (fig. 36A), whilst in the sputum there was no evidence of differential expression (fig. 36B). There was little difference between mediators whether this was in non-eosinophilic stable state or treatment (fig 36C+D) or eosinophilic stable state and eosinophilic exacerbation following treatment in serum or sputum (fig. 36E+F). IL-1 β levels were significantly upregulated in the sputum during an exacerbation (fig. 36F). This was interesting because the IL-1 β gene was downregulated in eosinophilic exacerbation vs. healthy groups in the differential gene set analysis (appendix table 10). I was unable to validate these findings in healthy donors as sputum inductions were not possible.

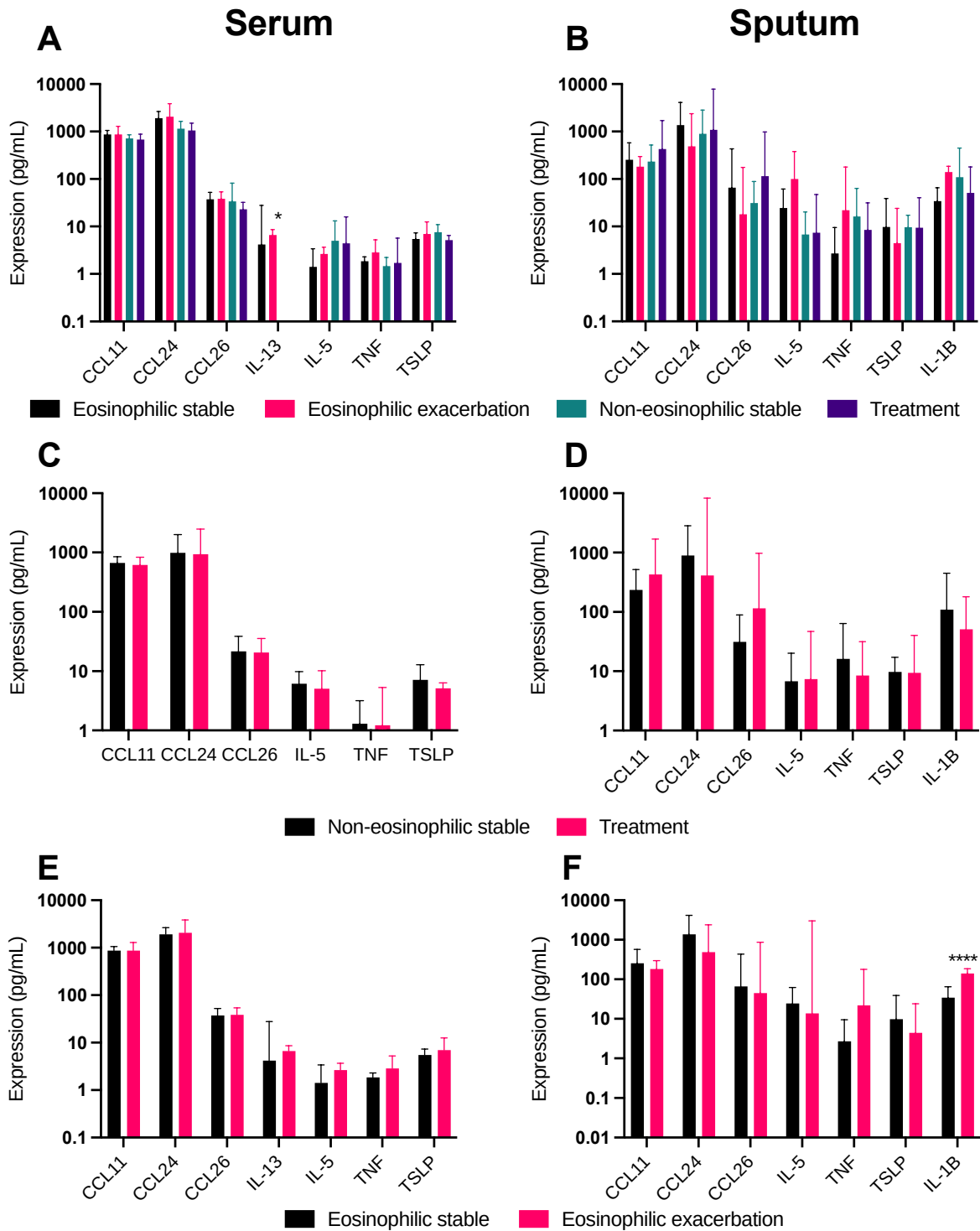


Figure 36. Subgroup analysis reveals non-eosinophilic and treatment groups have depleted IL-13 and IL-5 in serum; IL-1B is upregulated in eosinophilic exacerbations.

Left panels (A, C, E) show serum levels, right panels (B, D, F) show sputum. Top row includes eosinophilic stable, eosinophilic exacerbation, non-eosinophilic stable, and treatment groups. Middle panel shows non-eosinophilic and treatment. Bottom panel shows eosinophilic stable and eosinophilic exacerbation groups. Mediators that are marked by an asterisk are significantly different ($p < 0.05$). Horizontal and error bars are set at the geometric mean and 95% CI.

5.2.4 Eosinophil peroxidase levels are not increased during an eosinophilic crisis event

EPX is a highly toxic granule and specific to eosinophil degranulation making it a marker of eosinophil activation. Consequently, I compared the EPX concentration in sputum and serum samples between COPD patients undergoing an eosinophilic exacerbation and patients that were eosinophilic during stable disease state to determine if the release of granule proteins could also be contributing factor to lung inflammation during eosinophilic crises (fig. 37A). I found that there was no detectable difference in sputum: exacerbation = 30.53 ng/mL vs. stable = 17.27 ng/mL ($p = 0.33$) or serum: exacerbation = 6315 ng/mL vs. 6294 ng/mL ($p = 0.98$). EPX concentration between these two eosinophilic groups (fig. 37B and C). I also found that there was no significant correlation between sputum eosinophil count and sputum EPX concentration [$r = 0.27$; $p = 0.45$; fig. 37D+E]. This was further supported by qPCR which indicated that EPX gene expression was not increased between eosinophilic exacerbators and patients who were not eosinophilic at stable disease state ($p = 0.3$; fig. 37F). Detection of the eosinophil specific granule protein EPX in sputum and serum samples obtained from COPD patients undergoing eosinophilic exacerbations did not suggest that there was increased degranulation when compared to stable disease state or it's use as a biomarker for eosinophil activation in the ELISA platform.

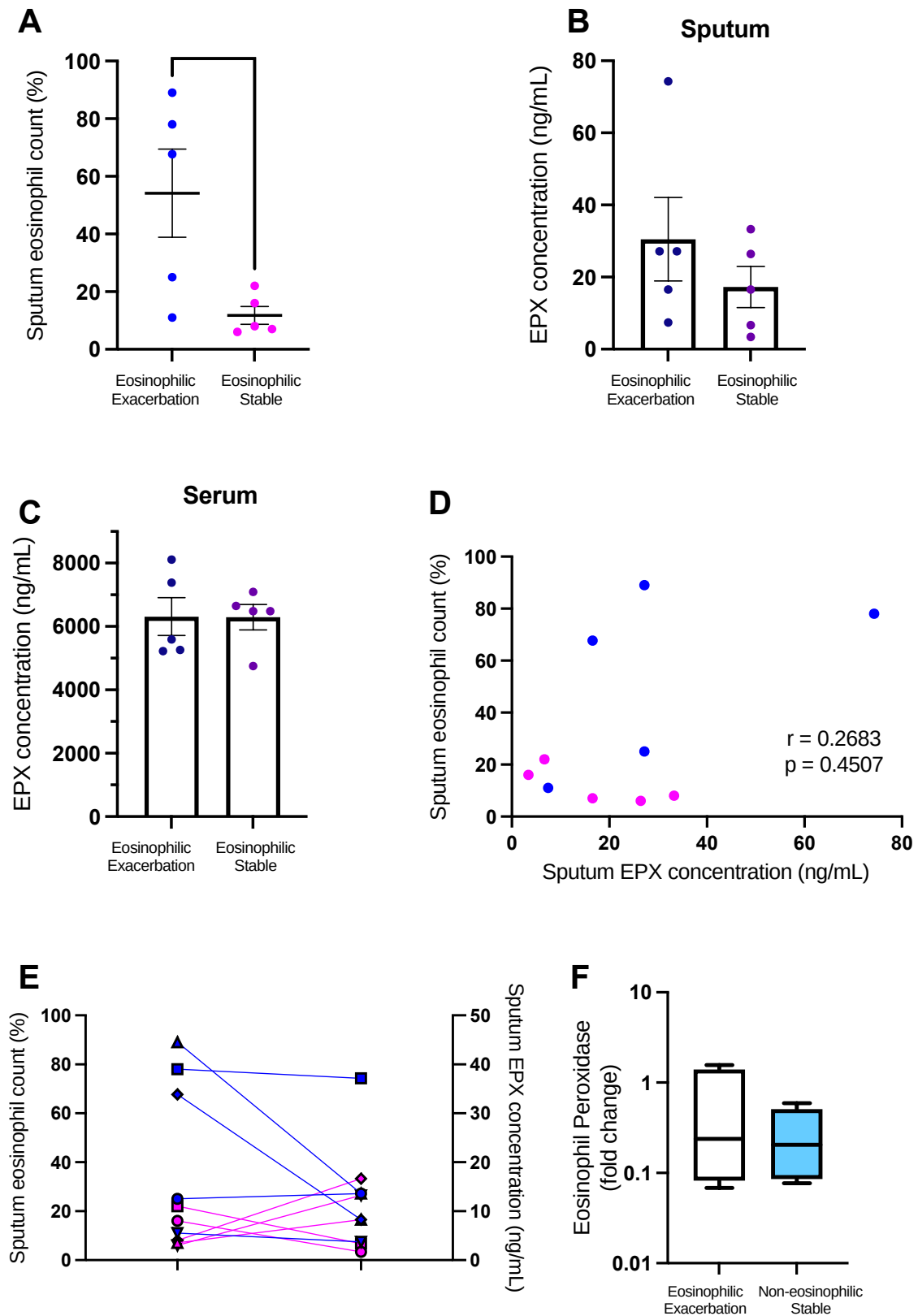


Figure 37. Serum and sputum eosinophil peroxidase (EPX) levels in patients with COPD undergoing an eosinophilic exacerbation are comparable with eosinophilic patients at stable state.

(A) Scatter plots presenting differential sputum eosinophil count for patients undergoing an eosinophilic exacerbation and who are eosinophilic at stable state **(B)** EPX concentration shown

against percentage sputum eosinophil count **(C)** EPX concentration in sputum **(D)** EPX concentration in serum **(E)** percentage sputum eosinophil count shown with the corresponding EPX concentration **(F)** correlation of percentage sputum eosinophil count against sputum EPX concentration. Data show mean $\pm$ SEM, n = 5. t test was used in **A**, **C**, **D** and Spearman's rank correlation in **F** were used for statistical analysis. *P $\leq$ 0.05. EPX, eosinophil peroxidase.

5.2.5 Eosinophil regulatory or inflammatory subtypes are not increased during exacerbations

Regulatory and inflammatory eosinophil subsets were first reported in asthma patients by Mesnil *et al.*, 2017 based on eosinophil CD62L and IL-3 receptor expression. I hypothesised that upregulation of the inflammatory subtype could be responsible for driving eosinophilic exacerbations of COPD. Eosinophil subpopulations in whole blood were identified by the gating strategy outlined in figure 38A in airways disease patients at stable state and during an eosinophilic exacerbation.

Briefly, granulocyte populations were first gated based on high forward and side scatter, followed by siglec-8 high populations for eosinophils and neutrophil exclusion for CD16 high. Percentage of siglec-8 positive cells that were IL-3 receptor low and CD62L positive, identified by Mesnil as regulatory eosinophils (rEOS), and siglec-8 positive cells that were CD62L low and IL-3 receptor high, recognised as inflammatory eosinophils (iEOS), are shown in figure 38B where 2.2% of blood eosinophils would be classified as rEOS whereas 10% would be iEOS. Eosinophilic exacerbations are highlighted in magenta and show indiscriminate distribution in both inflammatory and regulatory groups. Representative flow cytometric histograms of CD62L and IL-3 receptor expression in the gated populations are shown in figure 38C.

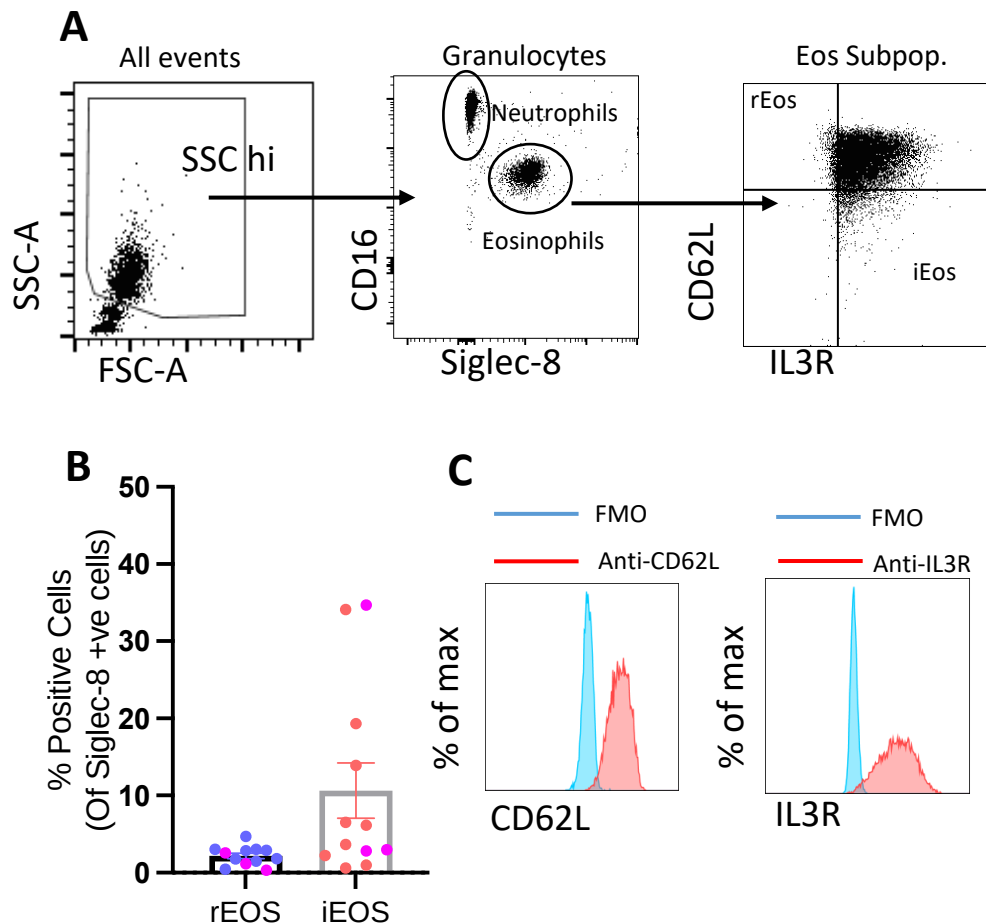


Figure 38. Identification of eosinophil subtypes (rEOS/iEOS) in human peripheral blood display no distinct populations.

(A) Representative flow plots identifying eosinophil subtypes based on Siglec-8, CD62L, and IL-3 receptor (IL3R) surface marker expression in peripheral blood. **(B)** Percentage of cells that are positive for the proposed subtypes as outlines in **(A)**. **(C)** Representative plots showing FMO and antibody overlay of CD62L and IL-3 receptor. Data shown in **A** and **C** are representative of 1 of 12 independent experiments. Purple and red dots are stable state, pink is exacerbation. Data shown represent the mean $\pm$ SEM as well as individual values. Each dot represents a single individual analysed ($n = 12$). FMO, fluorescence minus 1; FSC, forward scatter; SSC, side scatter.

5.2.6 Expression of CD63 and the IL-3 receptor are increased on eosinophils found in sputum compared to whole blood

As the expression of cell surface markers are known to change following migration into different tissues, I aimed to characterise the expression profile in the lung and blood compartments. This would be vital in understanding which makers may be useful in identifying activation responses in different tissue milieu and disease

states. CD63 and CD11b are both markers reported to be upregulated during eosinophil activation and may aid in identifying eosinophil subsets. Figure 39A shows a representative flow cytometric staining histograms in blood with the additional marker staining for sputum in figure 39C. The sputum gating strategy outlined in figure 39B included LIVE/DEAD and CD45 inclusion to differentiate debris, with siglec-8 used to identify eosinophils. When measured by fold change compared to MFI (fig. 39D), I found that IL-3 expression was increased two-fold from 2.2 in the blood to 4.3 in sputum ($p = 0.03$). The reported activation marker for degranulation, CD63, was also increased from 4.6 in the blood to 12.9 in sputum ($p = 0.03$). Other surface markers in this panel included in CD62L and CD11b, however, neither of these markers were significantly upregulated when compared to fold change of the FMO.

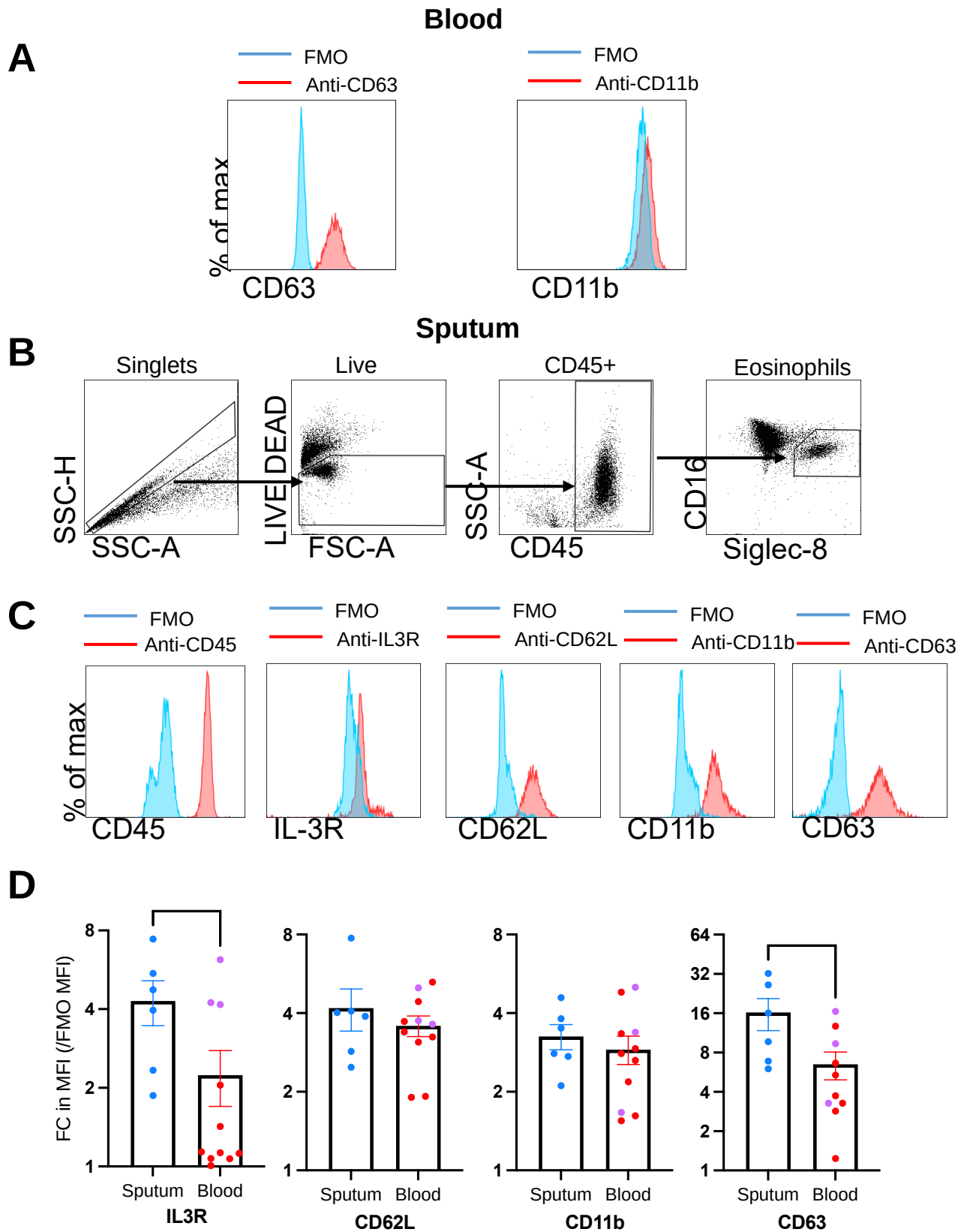


Figure 39. IL-3 receptor and CD63 are upregulated in sputum compared to whole blood.

Following the gating strategy outlined in Fig. 6A, eosinophils were identified in peripheral whole blood of patients with COPD. Representative plots showing FMO and antibody overlay for CD63, and CD11b in blood **(A)**. Sputum gating strategy for identifying eosinophils **(B)** with the representative FMO and antibody overlay for CD45, IL-3 receptor, CD62L, CD11b, and CD63 in sputum. Representative plots showing FMO and antibody overlay for CD45, IL-3 receptor, CD62L, CD11b, and CD63 in sputum **(C)**. Quantification of IL-3 receptor, CD62L, CD11b, and CD63 expression levels on the surface of eosinophils, expressed as the FC increase in MFI as compared with the FMO MFI. Data shown represent the mean $\pm$ SEM as well as individual values. Each dot represents a single individual analysed (n = 5-11/group). Blue circles identify sputum, red circles for stable state blood samples, pink for eosinophilic exacerbator blood samples. Mann-Whitney test for statistical significance in **D**. *P < 0.05. FMO, fluorescence minus 1; FSC, forward scatter; SSC, side scatter. FC, fold change.

5.2.7 Eosinophilic exacerbations display increased surface marker expression in whole blood

When stratified by eosinophilic exacerbations, all surface markers showed increased expression by fold change in whole blood (fig. 40). However, the IL-3 receptor was the only surface marker to show a statistically significant increase from a fold change of 1.3 at stable state to 4.9 during an exacerbation (p = 0.01). Unfortunately, due to the Covid-19 pandemic, I was unable to acquire sputum samples during this phase of the study and only a small number of blood samples were taken from patients who were eosinophilic exacerbators.

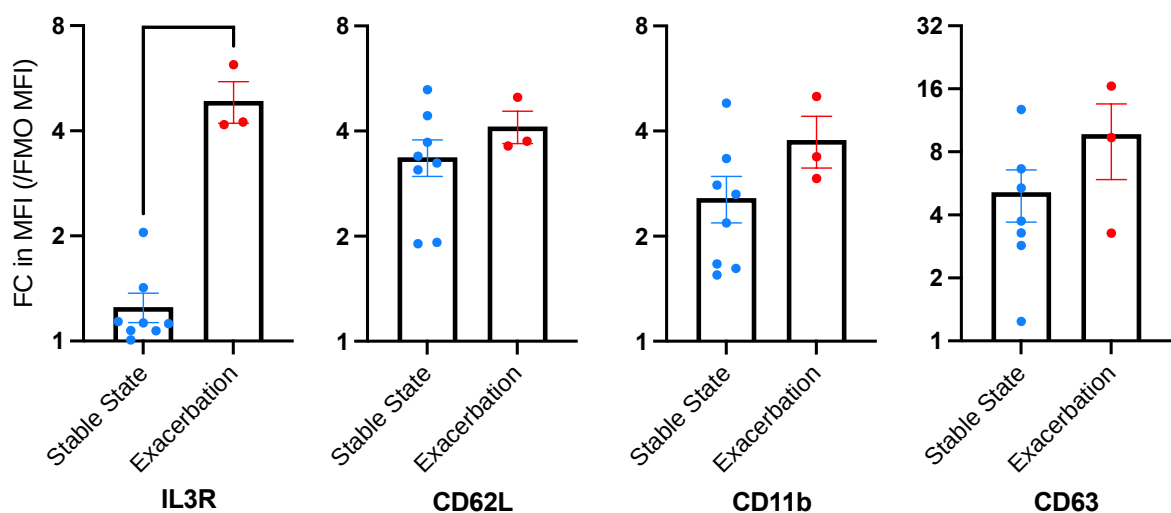


Figure 40. Eosinophil surface markers are upregulated in the blood during an exacerbation. Quantification of IL-3 receptor, CD62L, CD11b, and CD63 expression levels on the surface of eosinophils, expressed as the FC increase in MFI as compared with the FMO MFI in the peripheral

whole blood of COPD patients at stable state and during an eosinophilic exacerbation. Data shown represent the mean $\pm$ SEM as well as individual values. Each dot represents a single individual analysed (n = 3-8/group). *P <0.05. FMO, fluorescence minus 1; FSC, forward scatter; SSC, side scatter. FC, fold change.

5.3 Summary of results

Eosinophilic inflammation is a prominent form of inflammation found in airways diseases although, the mechanisms surrounding the role of the eosinophil and the drivers of eosinophilic inflammation remain elusive in COPD. Here I sought to characterise eosinophilic exacerbations of COPD and the distinguishing factors between those patients that present with acute symptoms and those who do not. Interestingly, I found that IL-8 was similarly expressed in patients regardless of their stratification of neutrophilia and eosinophilia, even though IL-8 is a key driver of neutrophilic inflammation and activation. Further profiling of cytokines closely associated with drivers of eosinophilia indicated that eosinophilic exacerbations were associated with increased IL-5 in the lung, supporting the use of IL-5 biologics, such as Mepolizumab, in the treatment of eosinophilic airway inflammation. Additionally, I found that IL-13 was not detected in patients one week following therapy. IL-13 is associated with allergic inflammation and therefore also strongly implicated in eosinophil recruitment. Further stratification of patients undergoing eosinophilic crisis events and stable but eosinophilic patients displayed an increase in IL-1 β expression in the sputum, possibly implicating a role for a pro-inflammatory antipathogen response. I also found no difference in cytokine profiles between non-eosinophilic, treatment naïve patients and patients following one week of treatment.

Degranulation is a major effector response in eosinophils and therefore I investigated whether not only ROS release, but also release of eosinophil specific granules could contribute to inflammation within the airways. Eosinophil peroxidase

is the only granule protein secreted specific to eosinophils therefore I measured the levels in serum and sputum samples as a sensitive marker of eosinophil degranulation. By stratifying eosinophilic exacerbators and patients that were eosinophilic but not showing any symptoms of acute inflammation, I did not find any differences in both expression of eosinophil peroxidase or secretion, strongly implicating ROS as the major mechanism of eosinophil induced acute symptoms.

In 2017, Mesnil *et al.*, reported eosinophil subtypes in asthmatic patients. I followed their flow cytometric gating strategy to identify if this finding could be applied in the context of COPD. I detected no distinct populations based on the markers they suggested however, I did find that the IL-3 receptor was highly expressed in the blood of eosinophilic COPD patients, possibly corroborating their finding of an inflammatory subtype marker.

Ultimately, eosinophilic inflammation is strongly implicated as a driver of airways disease and eosinophil depleting drugs have already shown their effectiveness in treating this phenotype. ROS production appears to be key mechanism of eosinophil driven acute response and alternative therapies targeting ROS and alternative eosinophil activation pathways may prove beneficial in relieving the acute symptoms in this patient phenotype.

5.4 Discussion

Eosinophilia is present in various unrelated disease states and across multiple tissues (162). A key complication with current attempts to characterise eosinophilic in COPD include the lack of a stringent threshold or cut-off value to define when a patient is considered eosinophilic. This has led to eosinophilic COPD to be present in an estimated region between 19 and 67%, depending on the study (180). This is

clearly a wide and impractical range making any conclusions about the state of inflammation in each study difficult to compare. Other confounding factors may contribute towards assessments of the eosinophil count including the usage of eosinophil depleting drugs including biologics and steroids in the treatment of disease. Moreover, we are also lacking a well-defined description of what a COPD exacerbation is, with no blood measurements, specific observations, or objective scoring system to identify when a patient is undergoing an exacerbation. Therefore, clinicians are reliant on patient self-diagnosis. This is also a subjective measurement and provides little insight into the mechanisms of the underlying pathophysiology (258). The focus of this chapter was to characterise eosinophils in the context of COPD exacerbations in the hopes of unravelling the inflammatory profile of this particular trait. There is increasing evidence that eosinophilic COPD does have certain measurable clinical features and utility in directing therapies to reduce treatment failures (180). Here I sought to identify any measurable biomarkers of exacerbations that could be implicated in the development of eosinophilic airway inflammation.

Eosinophilic inflammation, defined as $>3\%$ of cell differential counts, has been identified in around a third of patients with COPD at stable and exacerbation state (259,260). Increased eosinophil count has also been used as a predictor of corticosteroid response during exacerbations and ICS usage with success to prevent treatment failures (261–264). Although presence of eosinophils in the airways is associated with disease and a paucity of eosinophils with health, patients can often be defined as eosinophilic even though they experience no acute symptoms. I therefore compared the absolute eosinophil count in the blood of patients during stable state to the eosinophil count when I captured a treatment

naïve exacerbation event to establish if their eosinophil count altered between these two time points. Indeed, there was a significant increase in the absolute eosinophil count during an exacerbation suggesting that disease patients have a higher baseline level of eosinophils to begin with and corroborates Fujimoto *et al.*, 2005 findings (265). Although these samples were not matched with the same patients, the percentage eosinophil count was also significantly increased from stable state to exacerbation highlighting the increased influx of eosinophils during an exacerbation. It would be useful to recapitulate these data with samples from more time-points, establishing a baseline level of everyone's eosinophil count over a stable disease period to determine what the 'eosinophilic' threshold could be for that patient. This would aid in discriminating between different types of exacerbation onsets to allocate therapy response correctly and avoid treatment failures.

As neutrophilia is considered a hallmark of inflammation in COPD, I aimed to stratify patients based on their measured IL-8 concentration. A key pro-inflammatory mediator involved in the recruitment and activation of neutrophils (214); it could be expected that IL-8 would be upregulated in neutrophilic COPD when compared to eosinophilic. Interestingly, I found no difference in the levels of IL-8 in the sputum or the serum of either endotype. There are several reasons why this may be the case. IL-8 is reported to also play a role in IgE-mediated lung inflammation, an important pathway that induces other eosinophil related chemoattractants (266). IL-8 can also be secreted by eosinophils; therefore, their activation can result in further IL-8 release (267). I was careful in our choice of patient and stratified each group by their sputum cell differential to give us the best reflection of the inflammation in the airways in the least invasive manner. Nevertheless, this is not a flawless technique and as neutrophils are typically highly

abundant in circulation and in the airways, there is often overlap between patients who are classified as eosinophilic and neutrophilic. I attempted to avoid this overlap, however, as neutrophils can undergo degranulation and release of NETs (221), I cannot rule out the possibility that not wholly intact cells may have been missed. COPD is also a chronic inflammatory disease so it is not unexpected that levels of pro-inflammatory cytokines are present in much higher concentrations than what are ordinarily expected, and IL-8 may not be the optimum marker for neutrophilia in this patient cohort as Fujimoto *et al.*, 2005, showed that IL-8 does increase during an exacerbation without stratifying patients into neutrophilic and eosinophilic subgroups (265).

Further to neutrophil specific cytokines, I designed an extensive detection panel of cytokines critical to eosinophil recruitment, maturation, and survival to determine if a combination of cytokines could be used to predict eosinophilic exacerbations in COPD. Cytokines I found to be undetectable in the platforms utilised included: IL-4, IL-13, IL-17E/IL-25, and IL-33. Vaccination against IL-4 and IL-13 in mice has recently been shown to protect against allergic asthma and eosinophil influx into the airways (268), highlighting the importance of these type 2 cytokines in eosinophil recruitment and response. IL-25 is also strongly implicated in stimulating the release of type 2 cytokines from immune cells in the airways through its release from epithelial cells (269). There is also evidence that IL-25 may promote tissue eosinophilia through local differentiation of eosinophil progenitor cells (270). IL-33 is another cytokine expressed by lung epithelial cells shown to exacerbate eosinophil-mediated airway inflammation in mice in an autocrine manner and can modulate features of eosinophil function related to allergic inflammation (271). It is likely that these cytokines are expressed within the airways

but are unfortunately beyond the limits of detection in this assay. I successfully measured the concentrations of CCL11 (eotaxin-1), CCL24 (eotaxin-2), CCL26 (eotaxin-3), IL-5, TNF, TSLP, IL-1 β in sputum and IL-13 in serum. I found that there was significantly more IL-5 detectable in the sputum of subjects during an eosinophilic exacerbation when compared to stable disease state and following one week after treatment with either corticosteroids or Benralizumab. This contrasts with Stein *et al.*, who reported that paired individual IL-5 changes in 24 subjects before mepolizumab treatment and twenty weeks after showed increased IL-5 levels in plasma (272). However, the short time frame in this current study may reflect treatment effects on biological processes that have inhibited IL-5 release. IL-5 is highly expressed by both cells in the innate and adaptive immune system that are activated to recruit eosinophils to the site of inflammation. Eosinophil progenitor cells, express the high affinity IL-5 receptor, and undergo expansion, proliferation, and terminal differentiation in response to IL-5 (273,274). Therefore, IL-5 is crucial to the development and influx of eosinophils into the airways increases in its production vital to eosinophilia. All other mediators did not show a significant difference in detection in sputum samples. This likely explains the effectiveness of biologics that target IL-5 in the treatment of eosinophilic asthma including mepolizumab as IL-5 is a key mediator in the maturation, recruitment, survival of eosinophils (109,256). Conversely, I found that IL-13 was undetectable in samples following treatment, potentially providing an alternate pathway for the treatment of eosinophilic COPD.

It must also be noted that due to the design of the ABRA trial, patients recruited into the treatment arm were randomised to receive prednisolone, benralizumab or both. This meant that although I had access to larger group of

exacerbating patients and both therapies have been shown to have direct effects on eosinophils, the mechanism of these effects are distinct. As benralizumab is a targeted therapy, it is highly specific in its effect on eosinophils whereas prednisolone is known to affect many aspects of the immune system. This may lead to differences in the measured parameters beyond the selective depletion of the eosinophil.

As a subpopulation of eosinophilic patients experience an exacerbation whilst other eosinophilic patients do not, I investigated whether eosinophil degranulation could be a possible mechanism for driving exacerbations. I found no difference in detectable levels of eosinophil peroxidase, the only eosinophil specific granule protein, in either sputum or serum samples. There was a weak, non-significant correlation of sputum EPX concentration when compared to the sputum eosinophil percentage. I cannot rule out that sputum counts may be skewed by the loss of intact eosinophils following degranulation and changes in their classical morphology following activation. It should also be noted that this EPX ELISA was not validated for use in sputum. Therefore, there is the potential for matrix effect which may have limited detection of the analyte. However, qPCR data from the eosinophilic exacerbation patients and non-eosinophilic stable disease patients support the lack of increased expression between these two groups, making it more unlikely that eosinophil degranulation is a major contributing factor towards the symptoms experienced by patients undergoing an eosinophilic exacerbation. EPX has previously been compared in nasal, pharyngeal, and sputum from an equal number of severe asthma patients and healthy controls ($n = 10$) and found that there was a strong association between EPX levels and eosinophil percentage in sputum suggesting that mucosal surfaces may be a better compartment to measure

eosinophil degranulation (196). Further histological measurements including staining the airway epithelium with antibodies against eosinophil degranulation including free eosinophil granules or Charcot-Leyden crystals (CLCs) may show this conclusively. However, acquiring biopsies from patients experiencing COPD exacerbations may not be recommended or ethically acceptable.

Yang *et al.*, investigated whether eosinophil granule proteins could be a marker of exacerbations by comparing 77 COPD acute exacerbations with 35 healthy controls. They found that there was a significant increase in eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), major basic protein (MBP) and EPX during an acute exacerbation. They also found that there was no correlation between GOLD index severity and eosinophil granule levels. However, they did not stratify their patients by eosinophil blood count or sputum differentials and neither group showed a significant difference in eosinophil blood count (275). Improved biomarkers of eosinophil activation are required to identify eosinophil driven COPD pathogenesis.

A plausible reason as to why some patients present with an eosinophilic exacerbation and others do not may be related to the activation status of eosinophils. Eosinophils exist in various forms of activation and their effector response will depend on signals from the local tissue milieu. This may include the release of noxious particles that contribute towards the experienced symptoms during an eosinophilic exacerbation. As such, I investigated whether eosinophil subsets could be detected in the blood of patients with COPD with the hypothesis that an activated subtype may be driving inflammation. Using the gating strategy outlined by Mesnil *et al.*, 2017, I quantified the percentage of eosinophils that would be classified as 'regulatory' or 'inflammatory' based on their expression of CD62L and the IL-3

receptor (160). I found that although there were cells that could be classified into either category, these populations were low in circulation and lacked a distinct population rather, these cells were present with a spread of expression levels and were not well defined. Unfortunately, the data I gathered was only present in the blood compartment and studies into the airways may yield more convincing results in response to the airway microenvironment. However, Mesnil did report that these cells had matured in the bone marrow with defined functions and therefore their status as a subpopulation should be clear. Classifications of subpopulations require further guidelines on definition, as it is not well defined at what stage an activated cell type responding to its local environment is considered a subtype or is a terminally differentiated cell. In this study I was unable to determine whether stratification by these surface markers would indeed identify distinct functional roles. FACS sorting and a series of functional assays would be required to answer if their functions differed conclusively in a similar fashion to the experiments conducted by Mesnil to validate the functional distinctions reported mice.

To determine whether eosinophil surface marker expression is affected by migration from the blood compartment into the airways, I compared the expression in blood and sputum. I found that in sputum there was a significant increase in the expression of CD63 and the IL-3 receptor once eosinophils had reached the airways. As these samples were acquired from a patient cohort, it could be proposed that the local expression of signalling proteins in the airways cause activation of eosinophils. Further subgroup analysis in a small number of blood samples identified the IL-3 receptor to be upregulated in exacerbation samples, possibly supporting Mesnil's findings that IL-3 receptor expression is associated with an inflammatory type, or at least an altered state of activation. Nelson *et al.*,

recently reported that IL-3 stimulation of isolated peripheral blood eosinophils from healthy donors induced a unique gene expression profile not displayed by the other β -chain cytokines, IL-5, and GM-CSF, suggesting a unique role for IL-3. Genes found in this analysis included those related to eosinophil development, possibly implicating IL-3 as an inducer of an activated eosinophil phenotype (276). Increased expression of the IL-3 receptor may be a marker of an altered cell type by heightening eosinophil sensitivity and response to IL-3. Further validation of these findings on a larger population of samples would be required to confirm this hypothesis and extend beyond the existing gene level evidence and mouse work (160,276).

This chapter aimed to characterise eosinophilic inflammation in COPD, including identifying the effects of a range of cytokines which may influence the recruitment and activation of eosinophils. I have also explored alternate mechanisms of eosinophil activation to identify subsets of cells and surface markers that may identify them. Here, I have shown using a range of eosinophil associated cytokines, that the drivers of exacerbations are not clear-cut. This may be related to individual thresholds that determine responses and further work in a larger cohort is needed to explore the relationship between cytokine production and eosinophil response. Phenotypic markers of eosinophil activation would prove useful in identifying the likelihood eosinophil functional responses and could also be attributed to the cytokine profile of the patient. Next-generation sequencing technologies would be vital in determining characteristics of eosinophil subtypes and activation states and should be employed to advance the findings in this chapter.

Chapter 6

General Discussion

6 General Discussion

COPD is a spectrum of diseases that are responsible for respiratory related chronic disability and mortality worldwide. It is characterised by progressive and irreversible airflow obstruction related to fibrotic structural changes in the small airways (277,278). Smoking is a major cause, although why only a minority of smokers develop COPD is unknown. A combination of destruction to the lung parenchyma, chronic inflammation, and mucus hypersecretion results in loss of lung elasticity and limits airflow to varying extents (279). COPD is complex at the cellular and molecular level, producing a highly heterogeneous set of extrapulmonary effects and symptoms that affect individual patients differently owing to the unique underlying pathophysiology. Even following smoking cessation, disease progression is associated with progressive inflammatory processes in the airways and is related to various cells and mediators (18). Classically associated with Th-1 inflammatory processes including a neutrophilic profile that involves CD8+ T-lymphocytes and alveolar macrophages, it is now clear that eosinophilia also plays an important role in disease progression in some patients (84,90,280). Acute exacerbations punctuate the progression of COPD and contribute heavily to the morbidity and mortality of the disease, measured as decline in lung function, further hastening disease progression (4,281).

COPD affects over 200 million people world-wide, presenting a huge burden on healthcare systems as a substantial proportion of patients experience uncontrolled symptoms, despite optimal pharmacological interventions (6,7,282). There is clearly an unmet need for therapies capable of targeting the specific disease phenotype experienced by the individual patient therefore investigations

into the complex relationship between disease physiology and immune cell interaction are warranted to provide interventions for each treatable trait.

Sputum induction has allowed clinical researchers to study and monitor the inflammatory cell composition within the airways in a non-invasive manner. I have also shown in this thesis that the eosinophil count increases in both sputum and blood during an exacerbation, possibly owing to a higher baseline of eosinophilia in some patients. Eosinophilic COPD has distinct clinical features that separate it from other inflammatory profiles. Although, there is no consensus on the exact definition of phenotypes, with studies often using different measurements and different cut-off thresholds of eosinophil counts to define eosinophilia. Research into eosinophilic COPD is still in its infancy, and although objective and defined parameters would make it easier for clinicians to direct appropriate treatments swiftly, the lack of an agreed definition makes it difficult for research in the field to progress without a unified description. However, it is likely that there may not be a one size fits all approach to treating COPD and a more personalised response may be required to treat patients effectively. This may include understanding what the personal threshold of eosinophilia is for each patient and responding to the patients' individual needs, for example by determining the onset of each exacerbation, before treating them with a blanket response. Furthermore, a high eosinophil count does not necessarily mean a patient is undergoing an exacerbation that is eosinophilic in nature. Bespoke and dynamic treatments for maintenance and exacerbations are demanding but crucial for improving patients' quality of life by reducing treatment failures and improving our understanding of disease.

Eosinophilic inflammation and its relationship with the host response is not well understood in any of the various eosinophilic disorders. This is the result of

conflicting evidence in both animal and human models of disease. Indeed, the specific role of eosinophils is often called into question, having a vast and non-specific arsenal of cytokines and chemokines and the ability to extravasate into multiple organs and tissues makes their position in the immune system difficult to classify. More recent hypotheses have refocussed eosinophils from end-stage effector cells involved in Th-2 responses, to a broadly overlapping immune cell of both the innate and adaptive immune system. The Local Immunity And/or Remodelling/Repair (LIAR) hypothesis, proposed by the late James Lee and colleagues, introduced the eosinophil as a modulator of immune responses and homeostasis in contrast to their seminal role in multi-cellular pathogen defence (120). The non-specific role of eosinophils and the advent of biologics such as Benralizumab that can essentially produce eosinophil 'knock-out' humans with no apparent detrimental effects have brought into question whether they have a role to play in the immune system. Nevertheless, humans and all vertebrate species have evolved with eosinophils, which respond dynamically to given stimuli. Therefore, it is reasonable to assume that they do have a role and it is vital more research is carried out to elucidate it.

The longstanding view that eosinophils are a uniform population of short-lived cells has more recently been overturned with evidence to suggest that they are pleiotropic, with a dynamic set of responses dependent on the inflammatory signals released from the microenvironment to which they extravasate to (156,160). The lifespan of eosinophils has even been found to be extended, with so called resident eosinophils existing in tissues for several weeks, significantly longer than their lifespan when in circulation (283,284). This extended list of functions requires a dynamic transcriptomic response to explain their range of abilities, providing

heterogeneity within a population of cells. This heterogeneity extends to a spectrum of activation states which include phenotypic changes to elicit immunoregulatory roles or cytotoxic effector functions. It is therefore essential that we understand these changes at the gene level so that interpretations of the functional response can be made at the protein level. As such, I carried out transcriptomics on eosinophils from healthy volunteers, stable disease state, and eosinophilic exacerbations to understand how this gene profile might change between each state and allude to genes that are key in defining the activation status during disease.

Due to the presence of multiple RNases in the granule proteins of eosinophils, it has proved difficult to sequence eosinophils successfully. This has even led to eosinophils being excluded from the lung cell atlas (285). To combat this, I needed a method of eosinophil isolation that was suitable for producing eosinophil RNA that was of the optimal quality for RNA-sequencing. Comparing two methods of eosinophil enrichment from whole blood head-to-head showed that an eosinophil activation marker was more highly expressed following Xpress isolation, which likely resulted in lower RNA quality due to eosinophils that would more readily degranulate and degrade RNA. I therefore opted to use the more technically demanding isolation procedure that included density gradient centrifugation, dextran sedimentation, and CD16 negative isolation to produce a single population of cells.

Differential gene set analysis displayed distinct profiles of gene expression between each group, supporting the theory that eosinophils do indeed exist in a spectrum of activation states. The group undergoing an eosinophilic exacerbation displayed the largest number of differentially expressed genes, likely indicating that

these cells are in a heightened state of activation. Further probing of gene ontology enrichment analysis revealed that genes involved in ROS activation were significantly enriched, possibly identifying a pathogenic activation status that could inflict cellular damage and loss of homeostasis as observed in COPD patients undergoing an eosinophilic exacerbation.

In this instance, bulk RNA-seq was clearly fruitful in comparing genes from different groups to produce an overview of the average gene expression. However, these differences are significant between groups rather than at the individual cell level. As previously mentioned, heterogeneity is diverse amongst eosinophils and single cell sequencing would be required to resolve activation subtypes and inform on the transcriptome for each phenotype of cell. It is also apparent that the activation status of the cell should be taken in context of its location within the tissue niche. This is especially important when considering the eosinophils contribution to disease. In the context of eosinophilic COPD, it would be appropriate for next generation sequencing platforms such as scRNA-seq or spatial transcriptomics to unravel these transcriptomic changes as the eosinophil develops, migrates to, and is activated by the cells in the lung tissue niche, such as Th2 cells and ILC2s, where it contributes to the disease state (286). Only then would it be possible to understand the contributions of eosinophil heterogeneity and their dynamic responses to stimuli in COPD. Furthermore, cell heterogeneity may answer why eosinophil directed biologics may be successful at treating some patients and not others even when there are similar levels of eosinophils in circulation. It may be that the eosinophil count taken on its own to direct eosinophil depleting and other type 2 biologic drugs is an oversimplification of a highly diverse cell type interacting with a highly complex disease. Patients with severe COVID-19 have been shown to have eosinopenia,

although the eosinophils that were detectable showed signs of activation (287–289). This reinforces the view that the eosinophil count may not be appropriate at distinguishing when eosinophilic inflammation is present based solely on the number of cells in circulation. Therapies targeted towards certain subtypes of eosinophils or dampening signals that switch on activation statuses may be the more appropriate response. Alternatively, an understanding that a high eosinophil count is ‘normal’ for one individual and not the direct onset of exacerbation may be required to provide the correct treatment.

Following the findings from the differential gene expression analysis, I next sought to identify the specific mechanisms in play that contribute towards ROS release from eosinophils, particularly during an eosinophilic exacerbation. High levels of ROS have also been implicated in the disease progression of COPD. As all cells produce ROS to some degree, it is often difficult to identify the source of detrimental amounts, particularly in a mixed population of cells *in vivo*. I therefore isolated eosinophils and neutrophils from the same donors to compare their production of ROS from healthy volunteers and COPD disease states. I showed that there was rapid and heightened production of ROS during eosinophilic exacerbations when compared to disease state and healthy controls. This was also associated with a higher resting state and an increased production of extracellular ROS when compared to neutrophils. High levels of ROS resulting from the influx of activated eosinophils in the airways would be expected to cause heightened inflammation due to direct aggravation of epithelial cells lining the airways and the release of responding pro-inflammatory signals. This complements other *in vitro* findings that showed disruption in the barrier integrity of epithelial cells resulting in hyperpermeability following co-incubation with activated eosinophils. Of interest,

unstimulated eosinophils also showed an increase in permeability following co-incubation. The cells in this assay were acquired from COPD stable disease patients and as the gene profile differed from the healthy volunteers, it could be that the altered activation state contributes towards other detrimental effects that would not be displayed in healthy volunteers. This lower-level ROS release may contribute to the chronic progression of disease experienced in COPD that is then heightened during an exacerbation.

Heightened ROS production was compounded with a limited expression of the antioxidant system glutathione peroxidase, both at the gene and protein level. This may explain why the production of ROS is so much higher when compared to neutrophils. Eosinophils may have a limited capacity to scavenge any excess ROS and the resulting release is controlled. However, thioredoxin, another antioxidant system, was upregulated and therefore may play a more integral role in this specific type of ROS release.

However, whether the same numbers of eosinophils and neutrophils should be compared in functional assessments is unclear. Neutrophils are the most abundant cells of the immune system whereas eosinophils circulate at low levels in healthy individuals. And although these cells share a similar lineage, they may acquire distinct functional responses upon activation. Nevertheless, these cells carry many of the same abilities and as neutrophils are much more widely studied than eosinophils, inferences about their roles can be made.

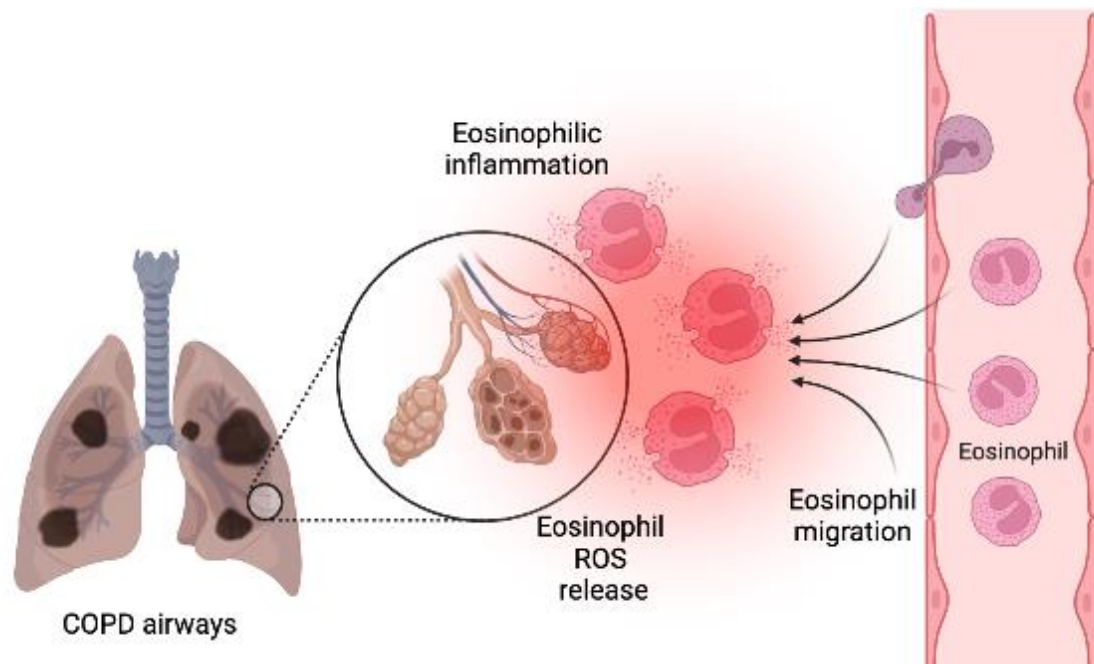


Figure 41. Eosinophil derived ROS causes damage to COPD airways.

During eosinophilic exacerbations, activated eosinophils localise to the airways and release ROS, damaging the epithelium. Created in Biorender.com.

Hydrogen peroxide metabolism was an upregulated gene ontology process in the eosinophilic exacerbation group, with the prominent gene of interest being *Duox2*. Indeed, further validation by qPCR showed that this gene was upregulated in exacerbating subjects when compared to stable disease. Expression of the dual oxidase-2 enzyme has previously been implicated in airway defence owing to its conversion to bactericidal OSCN^- (244). This implicates DUOX2 as the major ROS producer in eosinophils and a contributor towards acute exacerbations of COPD through increases in airway inflammation by acting on the epithelium.

An unexpected finding resulted from the investigation of the eosinophils capacity to carry out phagocytotic functions. Through measurements of Fc mediated phagocytosis of immune complexes and NTHi labelled with pH sensitive dyes, I found that their ability to phagocytose was comparable to that of neutrophils. This was surprising considering eosinophils association with larger multi-cellular parasite defence – organisms too large to engulf clearly required the development of other defence functions. Other more specialised phagocytic cells in much higher

abundance are ordinarily found in the tissues where eosinophils reside bringing their contribution as phagocytic cells into question. A possible explanation may be that eosinophils have retained some redundant phagocytic functions related to similar developmental pathways to neutrophils, as it appeared that eosinophils would not readily respond to in the same manner NTHi unless they were first primed with IL-5 and eotaxin.

To direct an appropriate and effective treatment requires an objective and measurable marker. Through comparisons of serum and sputum samples obtained from patients at stable and during an eosinophilic exacerbation, I measured the levels of a marker for DNA damage, 8-OHdG, to determine its utility in determining ROS damage. I found that the levels of this marker remained the same during these two disease states. However, these fixed timepoints may not be reflective of the underlying or ongoing damage and may require more measurements from the same patients to determine if the levels remain the same or do change over time as DNA repair responses react quickly to prevent possible mutations. Additionally, this 8-OHdG may not be optimal in measuring this type of ROS damage, as damage must occur to the nucleus to produce this marker and consequently other oxidised markers such as lipid or protein oxidation may prove more fruitful.

As eosinophilic inflammation can be present in a diverse range of disorders and organs, it is important to establish the drivers of eosinophilic inflammation specific to COPD. This provides the advantage of providing treatable disease pathways that may prove useful in resolving inflammation without eliciting unwanted side effects and delivering alternate treatments to patients who may be unresponsive to first- or second-line therapies. Therefore, examining the cytokine profile of patients during eosinophilic exacerbations and at stable state to

differentiate what is unique to eosinophilic exacerbations would prove key in blocking migration and activation of eosinophils.

Upon examination of IL-8 levels in patients stratified by eosinophilia or neutrophilia based on their sputum cell differentials, I found that the levels between these two groups was similar. This was surprising considering the integral role IL-8 plays in the activation and recruitment of neutrophils however, IL-8 is released by various cells in the airways to recruit cells into a pro-inflammatory response. It has also been demonstrated that many pro-inflammatory cytokines and chemokines are increased in stable COPD when compared to healthy non-smokers, with patients who are more likely to exacerbate having a higher baseline IL-8 levels (265). Measurements of a range of other cytokines in the sputum (eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, IL-5, TNF, TSLP, IL-1 β) and serum (eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, IL-5, TNF, TSLP, IL-13) that are more specific to eosinophil recruitment revealed that IL-5 is increased in sputum during an eosinophilic exacerbation when compared to stable state and one-week following treatment. As a key mediator involved in the development, recruitment, and survival eosinophils in the airways, IL-5 is clearly integral to the eosinophilic inflammatory response. This is evident in the success of Mepolizumab in lowering the annual rate of moderate or severe exacerbations in patients with eosinophilic COPD compared with placebo (255). IL-13 was also undetectable in the serum following treatment, highlighting the importance of this cytokine in the treatment of eosinophilic exacerbations, and providing supporting evidence for anti-IL-13 therapies - anrukinzumab, lebrikizumab, and tralokinumab - that are currently in development. Further subgroup analysis showed that IL-1 β was increased in eosinophilic exacerbations compared to eosinophilic stable disease.

Further to the findings that eosinophil ROS production is heavily implicated as a source of inflammation during eosinophilic exacerbations, it was appropriate to rule out other potential sources or eosinophil induced damage that could be related to eosinophil activation. Therefore, I measured the levels of the eosinophil specific granule protein EPX in the serum and sputum. There was no significant difference in detectable levels between eosinophilic exacerbation and eosinophilic stable, suggesting that degranulation is unlikely to contribute to the inflammation that is experienced during exacerbations. An alternate effector function that can also be excluded in the involvement of eosinophil induced exacerbations could be eosinophil extracellular traps. Their extrusion from eosinophils would include the granule proteins EPX which would likely be included in the expectorate. This evidence supports the role of eosinophil derived ROS in eosinophil specific exacerbations and an activation state related to this effector function.

Distinct eosinophil gene expression profiles were detected between disease states and healthy volunteers. Recent evidence suggests that eosinophils are not a purely defined population of cells; they exist in heterogeneous states of activation with a diverse range of functions (127,290,291). Therefore, it is logical to conclude that the dynamic transcriptomic profile obtained from the sequencing analysis is indicative of cells being primed to carry out alternative functions. Following the flow cytometric gating strategies proposed by Mesnil *et al.*, 2017, I aimed to identify if it was possible to recapitulate suggested findings of eosinophil subpopulations (160). Based on their data, there should be two definable eosinophil phenotypes, the regulatory and inflammatory eosinophils based on the surface markers CD62L and IL-3 receptor expression. My interpretation of the data I acquired would suggest that these are not a clearly defined homogenous population of cells with most cells

falling outside these two groups. It seems more likely that these two markers are not entirely adequate to categorise a heterogeneous population of cells. It could be proposed that eosinophils exist in a spectrum of activation states with varying levels of surface marker expression and the grouping of subpopulations may not be as clear cut. There may also be a combination of gene expression profiles, intracellular and extracellular surface markers, metabolic profiles, and production of intracellular contents related to subtype function. Unfortunately, it was not possible to acquire the planned sputum samples from exacerbating patients to investigate if the local tissue milieu could also influence the surface marker expressed that reflects the activation status of these cells. Additional surface marker measurements indicated that receptors are generally upregulated during an exacerbation however, only the IL-3 receptor was significantly overexpressed. Further measurements are required to validate these findings as sample size was small. This is the first study of its type to investigate the surface marker expression of eosinophils between disease states and further research is warranted to explore other expression markers and in different body compartments including the blood and airways.

The advent of eosinophil depleting drugs e.g., Benralizumab have brought into question the safety of the complete removal of a cell type from the immune system (292,293). It is evident from the research in this thesis that eosinophils do indeed exist in various states of activation and are capable of a diverse range of functions. It is likely that not all eosinophils are pathogenic, and they do have the capacity to carry out homeostatic roles. Furthermore, although an influx of eosinophils into the airways is strongly associated with detrimental effects in airways disease, Benralizumab was unsuccessful at achieving statistically significant improvements in reducing exacerbation rates in COPD. Eosinophil

depletion has thus far not been associated with detrimental effects and there has even been a child born to a patient whilst being prescribed Benralizumab throughout their pregnancy, recording no ill effects. Ultimately, eosinophils are complex cells with the capacity to carry out various functions interacting with a highly complex heterogenous disease. It is likely that eosinophils can be advantageous in some contexts and their role requires further research before considering absolute removal from the immune system.

6.1 Future work

This thesis aimed to provide a comprehensive overview of eosinophils features relevant to the pathophysiology of COPD using RNA-seq profiles to guide exploration of eosinophil functions in disease. However, this body of work is intended to provide a foundation from which further studies can elucidate mechanisms in greater detail. Next generation sequencing platforms are advancing at a remarkable rate and novel methods provide greater resolution of the genome with astonishing detail and should be utilised appropriately to aid our understanding of eosinophils in disease.

1. Single-cell RNA sequencing would advance the group gene expression profiles in this study. It is likely heterogeneity is present at the individual cell level and this resolution is lost during bulk-RNA sequencing.
2. Spatial transcriptomics would contextualise the cellular landscape in its journey to the destined tissue microenvironment, providing gene expression data specific to the *in vivo* location and the environment that influences these

changes. This would provide information on what signals may influence the activation status of the eosinophil.

3. Single-cell functional phenotyping to identify individual cell cytokine secretions and how they correlate with specific responses / disease traits to identify eosinophil subsets and changes in activation status.
4. Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) to phenotype individual cell's transcriptome and the surface proteins to provide insights into the potential surface marker expression of subtypes of eosinophils that could be utilised to detect pathogenic cells during disease.
5. This study explored a range of eosinophil specific cytokines however this was not exhaustive. Other chemokines and cytokines may be relevant to the activation status of eosinophils during disease.
6. Inhibition of ROS production to detect any differences in epithelial barrier hyperpermeability.
7. Inhibition of thioredoxin to confirm its role in ROS scavenging during stimulation. Eosinophil ROS production could also be measured along with the effects on cellular metabolism/apoptosis.
8. Repeat the epithelial permeability assay in primary lung epithelial cells to determine how a mixed population of cells are affected by stimulated eosinophils.
9. Measurements of lung surfactant secretions to determine the effects of ROS production on epithelial surface secretions.
10. Longitudinal sampling of eosinophil counts / sputum cell differentials to establish individual baselines and indicators of eosinophilic inflammation.

11. Identification of biomarkers of oxidative stress during eosinophilic exacerbations including malondialdehyde, 8-isoprostane, H₂O₂ and activation of antioxidant enzymes.
12. Functional experiments comparing younger donor eosinophils to eosinophils isolated from COPD age-matched controls to determine the effects of aging on eosinophil responses.

6.2 Concluding remarks

In conclusion, this thesis has elucidated the role of the eosinophil in chronic obstructive pulmonary disease and, more specifically, understanding how the eosinophil may contribute towards acute periods of disease severity known as eosinophilic exacerbations. Data in this thesis addressed three main areas of focus. The first focus concerned the feasibility of acquiring sufficient and stable RNA from eosinophils for the purposes of comparing the transcriptome between disease states and healthy volunteers. Owing to the success of an optimised isolation and RNA extraction pipeline, gene expression analysis was conducted to explore the expression profile of eosinophils in health and disease. The second focus relied on the findings of the differential expression gene set, namely that there appeared to be changes in the expression profile during an eosinophilic exacerbation relating to mitochondrial function, production of reactive oxygen species, and antioxidant responses. This chapter centred around unravelling the mechanisms involved in ROS activity and linking the implications from the gene set analysis to understanding the mechanistic functions of eosinophils during an eosinophilic exacerbation. The third and final focus was to appropriately phenotype eosinophilic

inflammation in the context of COPD to further understand the drivers of exacerbations and identify any possible markers of eosinophil activation states. These studies provided evidence of unique eosinophil gene expression profiles associated with disease and a possible mechanism by which eosinophils contribute towards eosinophilic exacerbations of COPD.

Chapter 7

Covid-19 Impact

7 Covid-19 Impact

7.1 Covid-19 Impact Statement

The University of Oxford has released guidance to graduate students regarding the effects of the coronavirus pandemic on our studies. We were advised to either apply for an extension, or to submit a thesis on time containing details of how our work was impacted. I was advised by my supervisor to choose the latter of these two options.

As the planned experiments in this thesis were reliant on access to clinical samples, my research was heavily disrupted by the pandemic. Along with general University closures, clinical trials not related to Covid-19 were halted. Our laboratory was shut between March 13th and August 7th, 2020. I used this time to begin writing my thesis and analyse data that I had collected up to that point. Access to the laboratories was also restricted due to proximity within the John Radcliffe Hospital. In accordance with the University and national guidance, we had limited access once laboratories did open to maintain social distancing measures. The availability of sputum has also been limited since then as the University designated these samples as high risk, preventing sputum induction procedures.

As both the ABRA and CORAL trials were paused and due to safety concerns, I could not obtain clinical samples. In the meantime, I began working with the eosinophil-like cell line HL-60. Details of these experiments and a description of work that could not be completed are included here.

7.2 Discussion

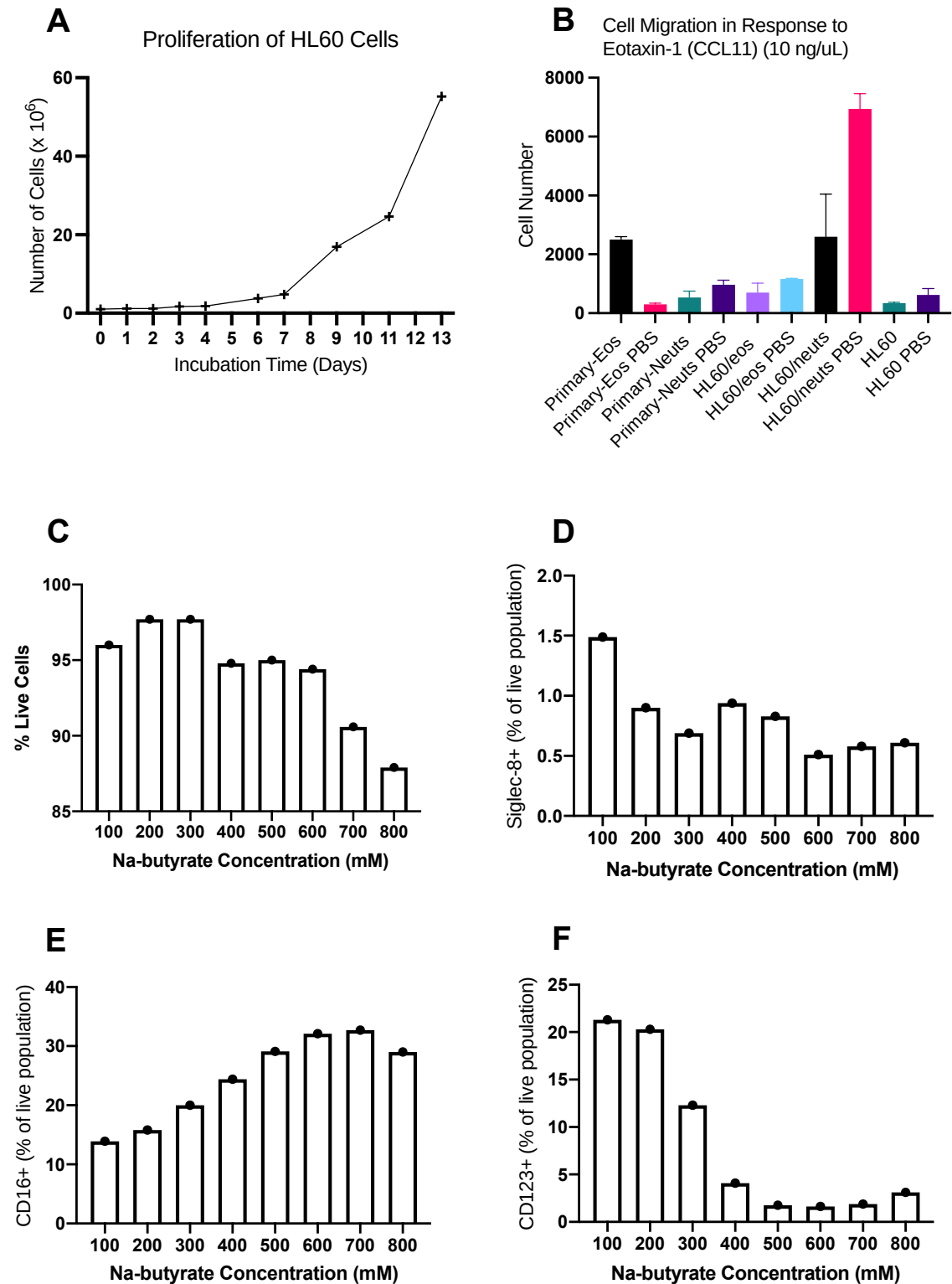


Figure 42. Eosinophil-like cell line HL-60 does not recapitulate human eosinophils.

(A) Proliferation of cells in culture over 13 days. (B) cell migration to 10 ng/ μ L of eotaxin-1 in a Boyden chamber. Culture of HL-60 cells in 100 – 800 mM sodium butyrate showing (C) percentage of live cells; (D) siglec-8 positive cells; (E) CD16 positive cells; (F) CD123 positive cells.

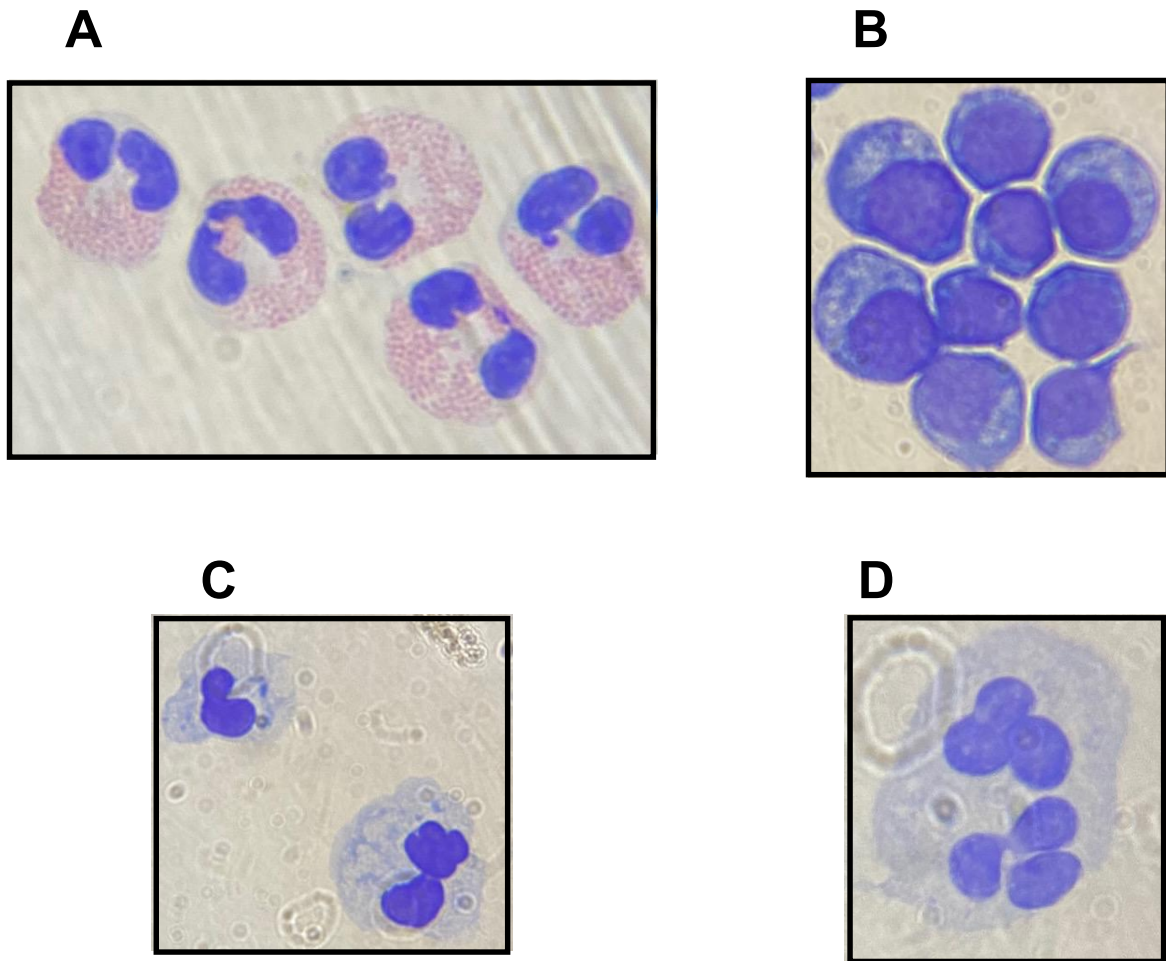


Figure 43. Morphology assessment.

(A) Isolated human eosinophils. (B) HL-60 undifferentiated cells. (C) HL-60 eosinophil-like cells. (D) HL-60 neutrophil-like cells. X100 magnification under oil immersion.

To begin working with the HL-60 cell line, I first wanted to be confident that these cells would recapitulate primary eosinophils I had used in my previous experiments. Although I followed the European Collection of Authenticated Cell Cultures (ECACC) culture protocol for eosinophil and neutrophil differentiation (see methods section 2.12.3), I was not convinced that these cells had many of the characteristic features of the primary cells they were meant to behave like.

Following thawing and initial culture, HL-60 cells were slow to proliferate up to their first week before expanding rapidly (fig. 42A). Once differentiation was complete, I measured their migration to the eosinophil chemoattractant CCL11. 1×10^4 cells were seeded in the top well of a Boyden chamber and the number of

cells that migrated towards the bottom chamber quantified. From previous human experiments, I found that around 2×10^3 cells would migrate at this concentration. With the eosinophil-like HL-60 cells, I found that there was limited migration and when compared to the PBS negative control, this migration was similar indicating that eotaxin-1 did not exhibit its known chemoattractant effects on these cells. This was in contrast with the neutrophil-like cells which displayed a greater affinity for the eotaxin-1. However, upon comparison to the PBS control, the largest number of migratory cells was seen, the likelihood that these cells were responding to eotaxin-1 was diminished (fig. 42B).

Following these initial experiments, I decided to explore the effects of various concentrations of sodium butyrate, the eosinophil differentiating factor, on surface marker expression. I found that although higher concentrations did decrease the proportion on live cells (fig. 42C), the canonical eosinophil marker siglec-8 (fig. 42D), remained at a very low percentage. I also found that CD16 expression (fig. 42E), expressed highly on neutrophils, was expressed much higher on these cells. The percentage of CD123 positive cells, another marker of eosinophils, dropped to its lowest level at 500 nm, which was also the recommended concentration for eosinophil-like culture (fig. 42F).

Morphology assessment by eosin and methylene blue staining highlighted the absence of granule proteins in the cytoplasm of HL-60 cells. Human primary eosinophils characteristically stain with pink granules and with a blue bi-lobed nucleus under these conditions (fig. 43A). Undifferentiated HL-60 cells had a large nucleus to cytoplasm ratio (fig. 43B), whereas eosinophil-like HL-60s displayed a bilobed nucleus (fig. 43C) and neutrophil-like cells a hyper segmented nucleus (fig. 43D).

In summary, HL-60 cells were absent in many of the feature's characteristic of the primary human eosinophils I had previously conducted experiments with. This prohibited their use as a model system to replace clinical samples and I did not conduct further experiments with this cell line.

An additional study which I could not complete was exploration of the lung microbiome following eosinophil depletion. As eosinophilic exacerbations have shown an inverse correlation with pathogenic bacterial infection, I hypothesised that eosinophil depletion may result in changes in the lung microbiome. Microbiome analysis of sputum samples is an increasingly common tool utilised for the study of chronic respiratory diseases and the investigation of bacterial population diversity. Due to the non-invasive nature of sputum collection and recent advances in next-generation sequencing techniques, the study of the respiratory microbiome has become more accessible in scientific research. However, there is currently no gold standard sputum bacterial DNA extraction method whilst the availability of numerous commercial kits may lead to inconsistencies between studies.

I conducted preliminary experiments to determine which was the optimal DNA extraction method from three commercially available kits sample of five patients. 1) ZymoBIOMICS DNA miniprep kit (Zymo. Irvine. CA. USA); 2) Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany); 3) MP Bio FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA). The Qiagen kit uses an enzyme-based lysis, whereas MP Bio utilises bead beating for physical disruption and Zymo a combination of the two.

Each sample was then divided equally into thirds for DNA extraction. DNA yield and A260/A280 ratio was quantified using a NanoDrop 2000 spectrophotometer and agarose gel electrophoresis. 16S amplicon metagenomic

sequencing was carried out on the Illumina NovaSeq 6000 platform.

As can be seen in figure 44, most bacteria found in sputum are made up of firmicutes or proteobacteria to varying degrees. However, inconsistencies arise when comparing the relative abundance of bacterial phylum depending on the DNA extraction kit used. For example, in specimen 2 the relative abundance of proteobacteria using either the Qiagen or MP bio extraction kit was more than double that of what was found using the Zymo extraction kit. From this small sample size, it is apparent that each kit offers various inconsistencies between bacterial phyla making it difficult to establish which method is most representative of the bacterial community and therefore sufficient for the extraction of sputum bacterial DNA. Further investigations were not conducted due to the lack of access to sputum samples.

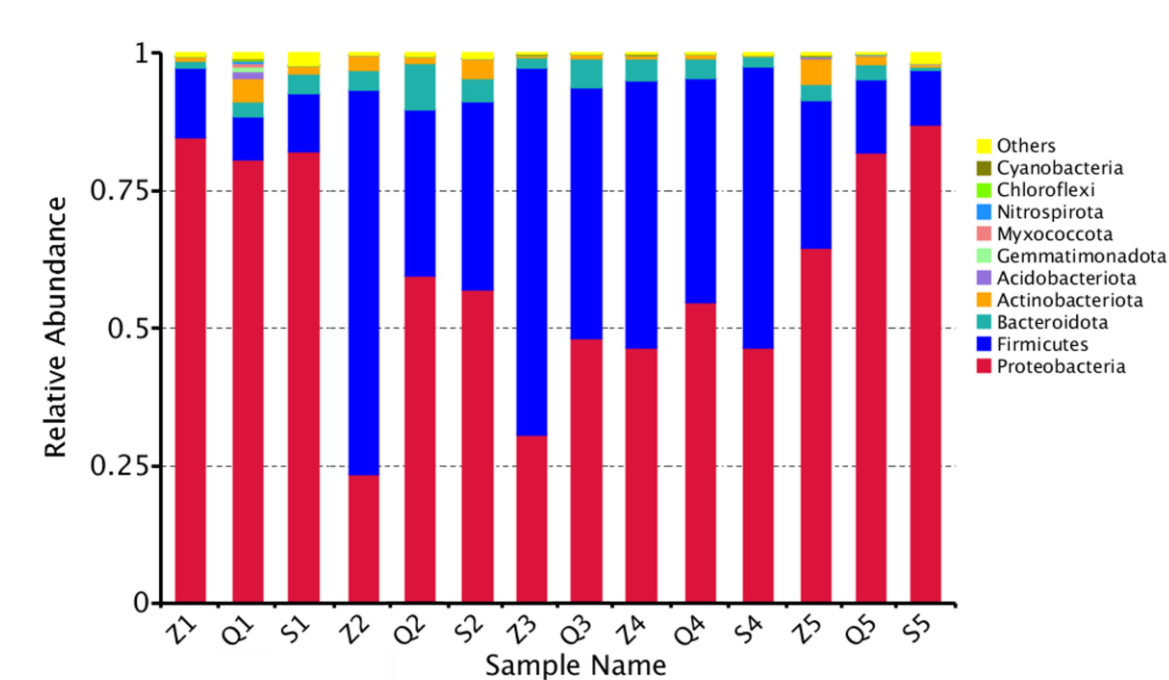


Figure 44. Relative abundance of bacteria in each sputum specimen.

Z: Zymo; Q: Qiagen; S: MP Bio. Each number corresponds to the same sputum specimen. Sample S3 was not of sufficient quality following extraction and therefore was not included for amplification.

Chapter 8

Appendix

8 Appendix

Supplementary Table 1. Reads, Q20/Q30, and GC contents of samples

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Error Rate	Q20	Q30	GC (%)
HC_1	102,851,610	15.43G	101917296	15.29G	0.03	97.41	93.26	51.71
HC_2	99,783,556	14.97G	98804660	14.82G	0.03	97.57	93.62	51.43
HC_3	100,211,212	15.03G	99189634	14.88G	0.03	97.58	93.58	50.96
HC_4	108,893,828	16.33G	107938966	16.19G	0.03	97.63	93.68	51.15
AB_1002	99,710,268	14.96G	98726234	14.81G	0.03	97.7	93.89	52.64
AB_1010	121,714,774	18.26G	120699046	18.1G	0.03	97.69	93.86	52.14
AB_1012	141,555,242	21.23G	140029540	21.0G	0.03	97.6	93.67	51.79
C_1026	128,760,876	19.31G	127557238	19.13G	0.03	97.77	94.06	52.3
AB_1005	109,733,684	16.46G	108713068	16.31G	0.03	97.61	93.68	52.47
C_1007	107,169,294	16.08G	106126276	15.92G	0.03	97.59	93.59	51.91
C_1017	104,797,156	15.72G	103706784	15.56G	0.03	97.64	93.74	51.66
AB_1021	103,453,694	15.52G	102394852	15.36G	0.03	97.58	93.65	52.29

Q20: The percentage of the total bases have Phred greater than 20. Phred= $-10\log_{10}(e)$

Q30: The percentage of the total bases have Phred greater than 30. Phred= $-10\log_{10}(e)$

Supplementary Table 2. Number of various read types per sample comparing sample and reference genome for healthy donors

Sample	HC_1	HC_2	HC_3	HC_4
Total Reads	101917296	98804660	99189634	107938966
Total Mapped	97778144(95.94%)	94748042(95.89%)	95301849(96.08%)	102473602(94.94%)
Uniquely Mapped	95836476(94.03%)	92379081(93.5%)	93141193(93.9%)	99927749(92.58%)
Multimap	1941668(1.91%)	2368961(2.4%)	2160656(2.18%)	2545853(2.36%)
Read 1	48195070(47.29%)	46336296(46.9%)	46711425(47.09%)	50144346(46.46%)
Read 2	47641406(46.75%)	46042785(46.6%)	46429768(46.81%)	49783403(46.12%)
Forward Strand	47845006(46.94%)	46144475(46.7%)	46527566(46.91%)	49950086(46.28%)
Reverse Strand	47991470(47.09%)	46234606(46.79%)	46613627(46.99%)	49977663(46.3%)
Spliced	24349347(23.89%)	29208037(29.56%)	27630657(27.86%)	36086946(33.43%)
Un-spliced	71487129(70.14%)	63171044(63.94%)	65510536(66.05%)	63840803(59.15%)
Proper Mapped	92923760(91.18%)	89819068(90.91%)	90547388(91.29%)	97179180(90.03%)

Supplementary Table 3. Number of various read types per sample comparing sample and reference genome for stable disease state donors

Sample	AB_1002	AB_1010	AB_1012	C_1026
Total Reads	98726234	120699046	140029540	127557238
Total Mapped	92934371(94.13%)	115336492(95.56%)	134624745(96.14%)	122121798(95.74%)
Uniquely Mapped	90041879(91.2%)	112473294(93.18%)	131503327(93.91%)	118985413(93.28%)
Multimap	2892492(2.93%)	2863198(2.37%)	3121418(2.23%)	3136385(2.46%)
Read 1	45165556(45.75%)	56407968(46.73%)	65983192(47.12%)	59626329(46.74%)
Read 2	44876323(45.46%)	56065326(46.45%)	65520135(46.79%)	59359084(46.54%)
Forward Strand	44973092(45.55%)	56188533(46.55%)	65670842(46.9%)	59432689(46.59%)

Reverse Strand	45068787(45.65%)	56284761(46.63%)	65832485(47.01%)	59552724(46.69%)
Spliced	29866261(30.25%)	37930601(31.43%)	41542432(29.67%)	41049453(32.18%)
Un-spliced	60175618(60.95%)	74542693(61.76%)	89960895(64.24%)	77935960(61.1%)
Proper Mapped	87307660(88.43%)	109499562(90.72%)	127894004(91.33%)	115779366(90.77%)

Supplementary Table 4. Number of various read types per sample comparing sample and reference genome for eosinophilic crisis donors

Sample	AB_1005	C_1007	C_1017	AB_1021
Total Reads	108713068	106126276	103706784	102394852
Total Mapped	103186185(94.92%)	101658560(95.79%)	98731363(95.2%)	97814698(95.53%)
Uniquely Mapped	100254640(92.22%)	99026815(93.31%)	96373859(92.93%)	95235036(93.01%)
Multimap	2931545(2.7%)	2631745(2.48%)	2357504(2.27%)	2579662(2.52%)
Read 1	50328715(46.3%)	49755543(46.88%)	48358905(46.63%)	47803648(46.69%)
Read 2	49925925(45.92%)	49271272(46.43%)	48014954(46.3%)	47431388(46.32%)
Positive map	50103276(46.09%)	49491597(46.63%)	48151173(46.43%)	47596761(46.48%)
Negative map	50151364(46.13%)	49535218(46.68%)	48222686(46.5%)	47638275(46.52%)
Spliced	35465643(32.62%)	38537748(36.31%)	30429422(29.34%)	35750850(34.91%)
Un-spliced	64788997(59.6%)	60489067(57.0%)	65944437(63.59%)	59484186(58.09%)
Proper Mapped	97328516(89.53%)	96218832(90.66%)	93580598(90.24%)	92772550(90.6%)

- **Sample:** SampleID.
- **Total reads:** Total clean reads used for analysis.
- **Total mapped:** Number and percentage of reads aligned to the genome, the ratio should higher than 70%, total mapping rate: (mapped reads)/(total reads)*100.
- **Uniquely mapped:** Number and percentage of reads aligned to the unique position of the reference genome (for subsequent quantitative data analysis), unique mapping rate: (uniquely mapped reads)/(total reads)*100.
- **Multimap:** number and percentage of reads aligned to multiple locations in the reference genome, multiple mapping rate: (multiple mapped reads)/(total reads)*100.
- **Read 1:** Number and percentage of read1 aligned to the reference genome.
- **Read 2:** Number and percentage of read2 aligned to the reference genome.
- **Positive map:** Number and percentage of reads aligned to the positive chain of the reference genome.
- **Negative map:** Number and percentage of reads aligned to the negative chain of the reference genome.
- **Spliced:** Number of spliced reads on the genome and its percentage.
- **Un-spliced:** Number of complete reads aligned to genome and its percentage.
- **Proper mapped:** Number of paired read1 and read2 aligned to the genome and its percentage.

Supplementary Table 5. Mapped bases according to the hg38 reference genome

sample	exon	intron	intergenic
HC_1	11391465711(77.8538%)	2851638225(19.4892%)	388772024(2.6570%)
HC_2	11367122897(80.1711%)	2508995386(17.6957%)	302454224(2.1332%)
HC_3	11206343944(78.5656%)	2740479277(19.2130%)	316866488(2.2215%)
HC_4	12551533755(81.8286%)	2465391791(16.0729%)	321882064(2.0985%)
AB_1002	12591428577(90.5185%)	1010188770(7.2621%)	308719585(2.2194%)
AB_1010	14528483725(84.1467%)	2348182220(13.6003%)	388976367(2.2529%)
AB_1012	17048832564(84.6061%)	2647680050(13.1393%)	454320440(2.2546%)
C_1026	15979370034(87.4139%)	1927826421(10.5460%)	372918141(2.0400%)
AB_1005	13871389090(89.8053%)	1277202624(8.2688%)	297483910(1.9260%)
C_1007	13617250308(89.4758%)	1348991770(8.8639%)	252678012(1.6603%)
C_1017	12299297585(83.2279%)	2167319903(14.6660%)	311233080(2.1061%)
AB_1021	13502572089(92.2073%)	915598534(6.2525%)	225550129(1.5403%)

The calculation of FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) considers the influence of sequencing depth and gene length on the count of fragments, and it is a commonly used method for estimating gene expression levels.

Supplementary Table 6. FPKM intervals for healthy donors

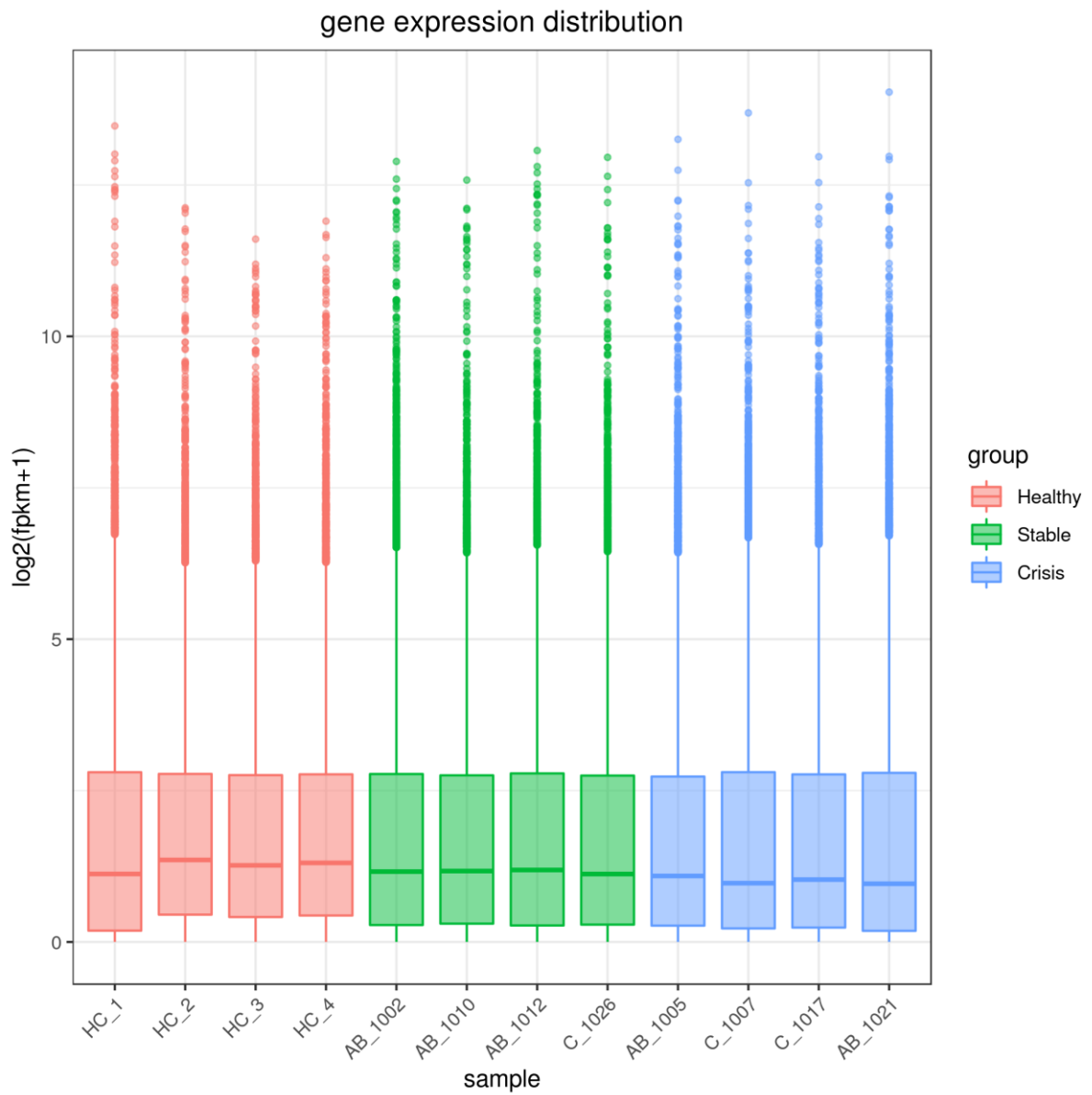
FPKM Interval	HC_1	HC_2	HC_3	HC_4
0~1	46952(79.94%)	45733(77.86%)	46166(78.6%)	45881(78.12%)
1~3	3774(6.43%)	4474(7.62%)	4337(7.38%)	4521(7.7%)
3~15	5181(8.82%)	6188(10.54%)	5716(9.73%)	5829(9.92%)
15~60	2129(3.62%)	1851(3.15%)	1973(3.36%)	1912(3.26%)
>60	699(1.19%)	489(0.83%)	543(0.92%)	592(1.01%)

Supplementary Table 7. FPKM intervals for stable disease state donors

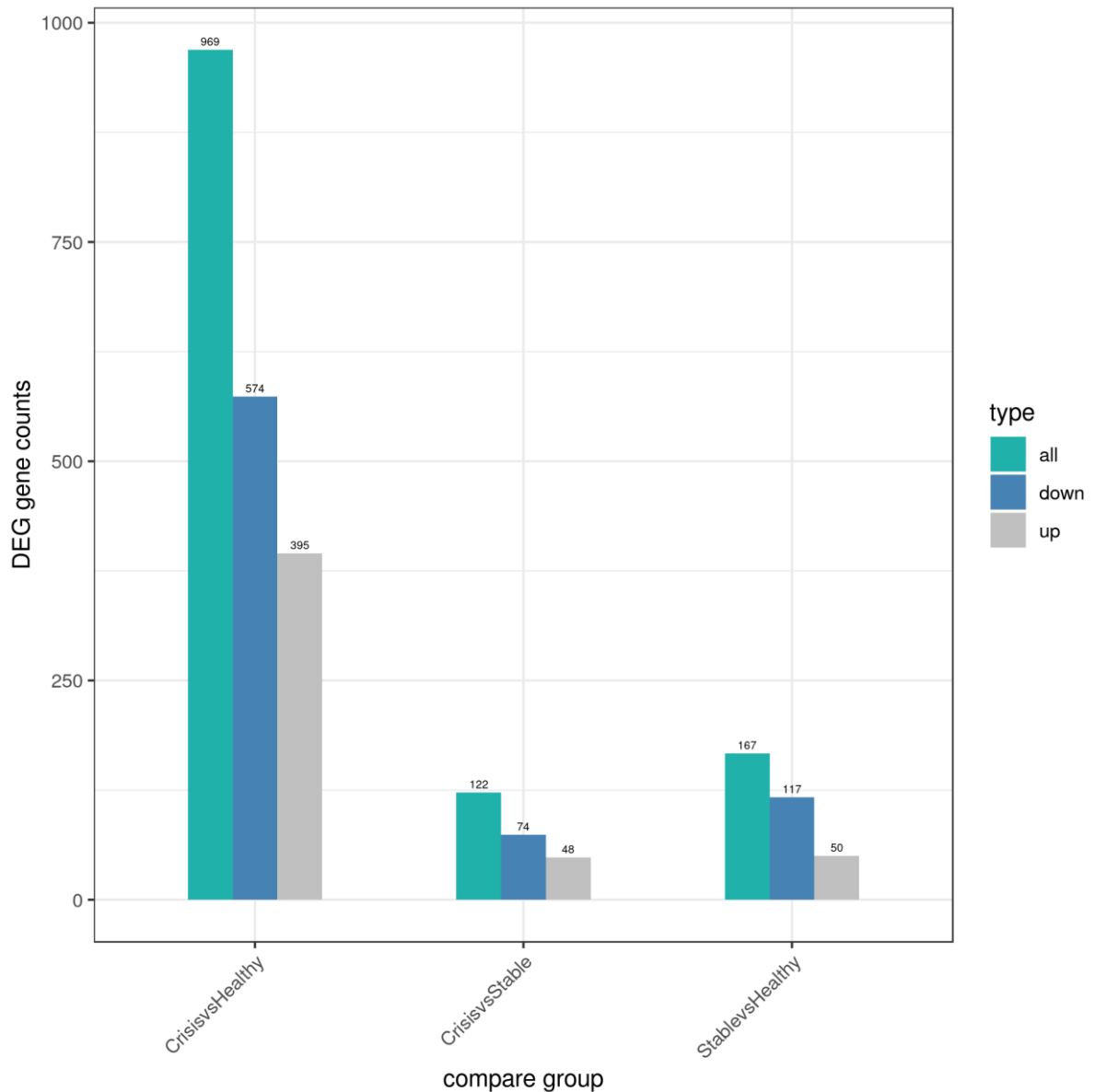
FPKM Interval	AB_1002	AB_1010	AB_1012	C_1026
0~1	46774(79.64%)	46733(79.57%)	46644(79.41%)	46946(79.93%)
1~3	3936(6.7%)	3963(6.75%)	3983(6.78%)	3821(6.51%)
3~15	5181(8.82%)	5364(9.13%)	5338(9.09%)	5222(8.89%)
15~60	2036(3.47%)	2066(3.52%)	2101(3.58%)	2091(3.56%)
>60	808(1.38%)	609(1.04%)	669(1.14%)	655(1.12%)

Supplementary Table 8. FPKM intervals for eosinophilic crisis donors

FPKM Interval	AB_1005	C_1007	C_1017	AB_1021
0~1	47117(80.22%)	47628(81.09%)	47369(80.65%)	47661(81.15%)
1~3	3799(6.47%)	3204(5.46%)	3520(5.99%)	3277(5.58%)
3~15	5002(8.52%)	4962(8.45%)	4973(8.47%)	4751(8.09%)
15~60	2090(3.56%)	2205(3.75%)	2155(3.67%)	2184(3.72%)
>60	727(1.24%)	736(1.25%)	718(1.22%)	862(1.47%)



Supplementary Figure 1. Gene expression distribution between samples.
X-axis represents the name of the sample. Y-axis indicates the $\log_2(\text{FPKM}+1)$.



Supplementary Figure 2. Significantly differentially expressed genes between groups. X-axis shows the differentially expressed gene counts based on the screening threshold, y-axis shows the group names. Grey represents the number of up regulated genes, blue downregulated and green the total of differentially expressed genes.

Supplementary Table 9. Crisis vs. healthy: significantly upregulated genes

#	Gene ID	Gene Name	log2FoldChange	padj
1	ENSG00000283579	AL160035.1	10.15777699	1.63E-15
2	ENSG00000274736	CCL23	3.680971801	3.03E-09
3	ENSG00000144681	STAC	5.346093445	3.52E-09
4	ENSG00000163993	S100P	2.889355478	4.13E-08
5	ENSG00000218198	RPS20P32	4.747759602	9.43E-08
6	ENSG00000188933	USP32P1	8.723809732	1.13E-07
7	ENSG00000120262	CCDC170	2.291515856	3.76E-06
8	ENSG00000267452	LINC02073	4.864831921	9.70E-06
9	ENSG00000188536	HBA2	7.261774092	1.12E-05
10	ENSG00000147872	PLIN2	1.899863707	1.37E-05
11	ENSG00000131203	IDO1	2.727735774	1.71E-05
12	ENSG00000257108	NHLRC4	2.200325688	3.30E-05
13	ENSG00000167779	IGFBP6	2.126027355	3.30E-05
14	ENSG00000142606	MMEL1	2.833982174	4.15E-05
15	ENSG00000145920	CPLX2	2.263530159	4.24E-05
16	ENSG00000185885	IFITM1	1.87669411	4.83E-05
17	ENSG00000140279	DUOX2	2.898572559	5.01E-05
18	ENSG00000279024	AC112255.1	2.022555494	7.44E-05
19	ENSG00000267416	AC025048.4	2.912316198	0.00010332
20	ENSG00000109099	PMP22	2.488133535	0.00010858
21	ENSG00000215915	ATAD3C	3.364454601	0.00012036
22	ENSG00000189159	JPT1	1.733241747	0.00012036
23	ENSG00000261040	WFDC21P	2.837577403	0.00012036
24	ENSG00000169442	CD52	1.813319606	0.00012036
25	ENSG00000074416	MGLL	1.457629032	0.00017556
26	ENSG00000206172	HBA1	9.189844416	0.00019065
27	ENSG00000251533	LINC00605	4.021777761	0.00023043
28	ENSG00000284999	AL591518.1	2.340028902	0.0003232
29	ENSG00000129559	NEDD8	1.389360736	0.00033548
30	ENSG00000170160	CCDC144A	4.83824032	0.00043146
31	ENSG00000244734	HBB	6.837317954	0.00043146
32	ENSG00000161055	SCGB3A1	3.385928049	0.00043146
33	ENSG00000180573	HIST1H2AC	1.603351755	0.00047023
34	ENSG00000246089	AC016065.1	1.456651908	0.00049103
35	ENSG00000228594	FNDC10	1.766167925	0.00049321
36	ENSG00000272398	CD24	2.067171719	0.00072999
37	ENSG00000121766	ZCCHC17	1.385106033	0.00081831
38	ENSG00000103363	ELOB	1.463562629	0.00084413
39	ENSG00000176834	VSIG10	2.581079555	0.00084837
40	ENSG00000134460	IL2RA	2.414113085	0.00084837
41	ENSG00000270030	AC136475.7	2.184114824	0.00098555

42	ENSG00000157303	SUSD3	1.481475802	0.00108723
43	ENSG00000168350	DEGS2	2.272337934	0.00109481
44	ENSG00000160180	TFF3	2.269210587	0.00109481
45	ENSG00000179933	C14orf119	2.10565111	0.00110673
46	ENSG00000144485	HES6	1.720394053	0.00139106
47	ENSG00000187608	ISG15	2.042438253	0.00143674
48	ENSG00000259704	AC124248.1	2.153181841	0.0018185
49	ENSG00000153485	TMEM251	1.534131152	0.0018274
50	ENSG00000111328	CDK2AP1	1.887206527	0.00188017
51	ENSG00000123975	CKS2	1.54344811	0.00188017
52	ENSG00000152082	MZT2B	1.344559772	0.00188017
53	ENSG00000147852	VLDLR	1.612422413	0.00205374
54	ENSG00000010278	CD9	1.286166307	0.00221796
55	ENSG00000107438	PDLIM1	1.966714841	0.00227689
56	ENSG00000136810	TXN	1.603676005	0.00253594
57	ENSG00000258114	AC005871.2	3.136704308	0.00255022
58	ENSG00000092841	MYL6	1.454491569	0.00268937
59	ENSG00000134594	RAB33A	1.861136572	0.00272509
60	ENSG00000205352	PRR13	1.398819794	0.00287321
61	ENSG00000103187	COTL1	1.303460272	0.00304524
62	ENSG00000158710	TAGLN2	1.539893821	0.00310093
63	ENSG00000163453	IGFBP7	1.340085557	0.00314548
64	ENSG00000255328	AC136475.5	2.015375468	0.00315118
65	ENSG00000205106	DKFZp779M0652	2.037529354	0.00320864
66	ENSG00000143158	MPC2	1.208922877	0.00363535
67	ENSG00000187837	HIST1H1C	1.773046945	0.00363535
68	ENSG00000225873	C3orf86	1.624401182	0.00363535
69	ENSG00000198892	SHISA4	2.046739223	0.00363535
70	ENSG00000125868	DSTN	1.424622908	0.00363535
71	ENSG00000204308	RNF5	1.463247833	0.00368878
72	ENSG00000247095	MIR210HG	2.071442261	0.0038996
73	ENSG00000130772	MED18	1.537345661	0.0038996
74	ENSG00000163517	HDAC11	1.126698688	0.00407745
75	ENSG00000131773	KHDRBS3	1.674015123	0.00411952
76	ENSG00000169291	SHE	1.976517868	0.00411952
77	ENSG00000229809	ZNF688	1.302784775	0.00420124
78	ENSG00000234506	LINC01506	1.749443897	0.0043574
79	ENSG00000125534	PPDPF	1.439113995	0.0046542
80	ENSG00000087086	FTL	1.41076315	0.00470193
81	ENSG00000125266	EFNB2	1.622665644	0.00473089
82	ENSG00000232671	AL391069.2	1.886243494	0.00473089
83	ENSG00000188290	HES4	2.149356869	0.00479367
84	ENSG00000118680	MYL12B	1.425895325	0.00489889

85	ENSG00000135116	HRK	2.763397502	0.00493959
86	ENSG00000140274	DUOXA2	4.263281677	0.00516227
87	ENSG00000270972	AC136475.9	2.743340192	0.00520029
88	ENSG00000146540	C7orf50	1.581082139	0.00551128
89	ENSG00000196544	BORCS6	1.413262476	0.00559988
90	ENSG00000188428	BLOC1S5	1.257562824	0.00582168
91	ENSG00000176386	CDC26	1.159505108	0.00590976
92	ENSG00000114956	DGUOK	1.120959803	0.00597013
93	ENSG00000181061	HIGD1A	1.446142976	0.00598136
94	ENSG00000235863	B3GALT4	1.498517765	0.00610187
95	ENSG00000177700	POLR2L	1.564359243	0.00637614
96	ENSG00000238072	AC009244.1	1.383110189	0.00650637
97	ENSG00000215845	TSTD1	1.327349572	0.0066931
98	ENSG00000140264	SERF2	1.107574958	0.00694273
99	ENSG00000106245	BUD31	1.034093516	0.00700949
100	ENSG00000034713	GABARAPL2	1.199835313	0.00706773
101	ENSG00000132581	SDF2	1.18681315	0.00713699
102	ENSG00000173915	ATP5MD	1.37223402	0.00724123
103	ENSG00000167985	SDHAF2	1.324677135	0.00734859
104	ENSG00000160446	ZDHHC12	1.407436275	0.00734859
105	ENSG00000187049	TMEM216	1.550893881	0.00734859
106	ENSG00000176973	FAM89B	1.386293498	0.00736742
107	ENSG00000175130	MARCKSL1	1.5375742	0.00751358
108	ENSG00000204261	PSMB8-AS1	1.335383933	0.00817669
109	ENSG00000100075	SLC25A1	1.283432811	0.00817669
110	ENSG00000241553	ARPC4	1.30942334	0.00817669
111	ENSG00000107960	STN1	1.288533073	0.00822469
112	ENSG00000152904	GGPS1	1.504115066	0.00822469
113	ENSG00000150779	TIMM8B	1.329225843	0.00836223
114	ENSG00000185201	IFITM2	1.620257274	0.00846158
115	ENSG00000144118	RALB	1.356575968	0.00853784
116	ENSG00000254635	WAC-AS1	1.166515765	0.00862793
117	ENSG00000119514	GALNT12	1.131032486	0.00869175
118	ENSG00000149418	ST14	1.675728129	0.00890718
119	ENSG00000198830	HMGN2	1.155675295	0.00899225
120	ENSG00000068784	SRBD1	1.248543186	0.009476
121	ENSG00000158769	F11R	1.464304996	0.00948262
122	ENSG00000111229	ARPC3	1.230842047	0.00952839
123	ENSG00000165264	NDUFB6	1.195041716	0.00960823
124	ENSG00000187653	TMSB4XP8	1.472224688	0.00972272
125	ENSG00000167642	SPINT2	1.272113887	0.00972272
126	ENSG00000101608	MYL12A	1.168656167	0.00972272
127	ENSG00000089486	CDIP1	1.154500994	0.00972272

128	ENSG00000057294	PKP2	1.74941548	0.00972272
129	ENSG00000284882	AL359762.1	1.391563082	0.00972272
130	ENSG00000169193	CCDC126	1.207354894	0.00972272
131	ENSG00000254910	AC136475.2	1.259161004	0.00972272
132	ENSG00000166165	CKB	1.534754915	0.01008455
133	ENSG00000197747	S100A10	1.693358133	0.01012681
134	ENSG00000105205	CLC	1.760599971	0.010288
135	ENSG00000178458	H3F3AP6	1.20700962	0.010288
136	ENSG00000143110	C1orf162	1.207065365	0.010288
137	ENSG00000104904	OAZ1	1.166224906	0.01029188
138	ENSG00000132965	ALOX5AP	1.363750263	0.01029188
139	ENSG00000197903	HIST1H2BK	1.474603408	0.01061244
140	ENSG00000072954	TMEM38A	1.576947396	0.01094431
141	ENSG00000178988	MRFAP1L1	1.179886471	0.01094431
142	ENSG00000111348	ARHGDIB	1.215654616	0.01108038
143	ENSG00000100744	GSKIP	1.182747374	0.01119559
144	ENSG00000185518	SV2B	5.391894588	0.01130207
145	ENSG00000156973	PDE6D	1.248944286	0.01130207
146	ENSG00000140263	SORD	1.605704382	0.0114586
147	ENSG00000176871	WSB2	1.495742269	0.0114586
148	ENSG00000184319	RPL23AP82	1.278742694	0.01146693
149	ENSG00000184831	APOO	1.662771805	0.01146693
150	ENSG00000166428	PLD4	1.415507577	0.01178397
151	ENSG00000179840	PIK3CD-AS1	1.470015839	0.01178397
152	ENSG00000159176	CSRP1	1.174965873	0.01194276
153	ENSG00000135046	ANXA1	1.187610375	0.01210823
154	ENSG00000272799	AC006238.1	1.756281674	0.01234328
155	ENSG00000204020	LIPN	1.801697002	0.01268274
156	ENSG00000276180	HIST1H4I	1.656830175	0.01268274
157	ENSG00000126432	PRDX5	1.180951277	0.01268274
158	ENSG00000090097	PCBP4	1.313633871	0.01277808
159	ENSG00000125356	NDUFA1	1.170073652	0.01290521
160	ENSG00000230257	NFE4	1.836288148	0.01318444
161	ENSG00000189068	VSTM1	1.626548563	0.01320973
162	ENSG00000266302	AC098850.3	4.776604225	0.01353771
163	ENSG00000226976	COX6A1P2	1.936893397	0.01398266
164	ENSG00000238142	BX284668.5	1.204329189	0.01398266
165	ENSG00000169385	RNASE2	3.051431941	0.01406122
166	ENSG00000172172	MRPL13	1.178903767	0.01418178
167	ENSG00000130755	GMFG	1.206189718	0.01436275
168	ENSG00000122694	GLIPR2	1.341633505	0.01446078
169	ENSG00000256745	AP002784.2	1.374696637	0.01446078
170	ENSG00000171428	NAT1	1.371546765	0.01446078

171	ENSG00000143933	CALM2	1.380458843	0.01448799
172	ENSG00000178537	SLC25A20	1.393972982	0.01458915
173	ENSG00000253652	AC136628.4	5.471145564	0.01492976
174	ENSG00000284648	AC097493.4	1.485572657	0.01530284
175	ENSG00000110876	SELPLG	1.525690551	0.01536307
176	ENSG00000268001	CARD8-AS1	1.520070521	0.01536307
177	ENSG00000171159	C9orf16	1.112317274	0.01562585
178	ENSG00000223891	OSER1-DT	1.557631633	0.01565115
179	ENSG00000146476	ARMT1	1.893142364	0.01565195
180	ENSG00000184076	UQCR10	1.316552427	0.01565937
181	ENSG00000106330	MOSPD3	1.093077766	0.01598002
182	ENSG00000099804	CDC34	1.026599145	0.01598002
183	ENSG00000135940	COX5B	1.118601207	0.01631613
184	ENSG00000116663	FBXO6	1.345184213	0.01723526
185	ENSG00000125910	S1PR4	1.507791519	0.01725347
186	ENSG00000155876	RRAGA	1.216410854	0.01729361
187	ENSG00000283633	AP000547.3	1.716639491	0.01729361
188	ENSG00000128606	LRRC17	2.318941066	0.01729361
189	ENSG00000261485	PAN3-AS1	1.460084156	0.01734262
190	ENSG00000244459	AC147067.1	1.600376679	0.01745962
191	ENSG00000139719	VPS33A	1.11530699	0.01745962
192	ENSG00000176340	COX8A	1.130712096	0.01756753
193	ENSG00000147400	CETN2	1.238039856	0.01760976
194	ENSG00000198168	SVIP	1.226863502	0.01760976
195	ENSG00000168894	RNF181	1.095796116	0.01760976
196	ENSG00000104687	GSR	1.270548331	0.01766943
197	ENSG00000121053	EPX	1.488955455	0.01776971
198	ENSG00000185527	PDE6G	1.587045162	0.01780209
199	ENSG00000105088	OLFM2	1.872098638	0.01797458
200	ENSG00000274012	RN7SL2	2.184023578	0.01797458
201	ENSG00000116586	LAMTOR2	1.290587158	0.01819251
202	ENSG00000166557	TMED3	1.109435075	0.01820462
203	ENSG00000172757	CFL1	1.07111643	0.0183385
204	ENSG00000197766	CFD	1.665917076	0.01865978
205	ENSG00000124172	ATP5F1E	1.270929504	0.01873457
206	ENSG00000198468	FLVCR1-DT	1.675146933	0.01898837
207	ENSG00000123080	CDKN2C	1.431977649	0.01901256
208	ENSG00000220586	TUBBP9	4.036552246	0.01901256
209	ENSG00000168556	ING2	1.241132755	0.01912975
210	ENSG00000166710	B2M	1.106444735	0.01912975
211	ENSG00000187134	AKR1C1	1.820126618	0.01925061
212	ENSG00000109390	NDUFC1	1.223127765	0.01929942
213	ENSG00000170017	ALCAM	1.308412143	0.02002546

214	ENSG00000241468	ATP5MF	1.066543789	0.02006158
215	ENSG00000110651	CD81	1.05595798	0.02006158
216	ENSG00000235169	SMIM1	1.731117082	0.02056435
217	ENSG00000243449	C4orf48	1.265483188	0.02068922
218	ENSG00000265735	RN7SL5P	3.444278144	0.02076571
219	ENSG00000155368	DBI	1.102607488	0.02103006
220	ENSG00000182551	ADI1	1.22451871	0.02121373
221	ENSG00000085741	WNT11	1.731899874	0.02130528
222	ENSG00000110203	FOLR3	2.568532345	0.02239917
223	ENSG00000177692	DNAJC28	1.811136864	0.02239917
224	ENSG00000175857	GAPT	1.690063347	0.02239917
225	ENSG00000237311	AL034397.2	2.234057749	0.02239917
226	ENSG00000104723	TUSC3	3.506570026	0.02239917
227	ENSG00000134072	CAMK1	1.394879531	0.02249667
228	ENSG00000178980	SELENOW	1.054363624	0.02308439
229	ENSG00000102996	MMP15	1.669188736	0.02315718
230	ENSG00000205542	TMSB4X	1.161657378	0.02346952
231	ENSG00000135211	TMEM60	1.503870902	0.02358995
232	ENSG00000156831	NSMCE2	1.104842004	0.02365629
233	ENSG00000213080	AL354714.2	1.545867549	0.02365629
234	ENSG00000099385	BCL7C	1.035345364	0.02400141
235	ENSG00000228305	AC016734.1	1.454083756	0.02409963
236	ENSG00000130561	SAG	1.876128029	0.02418899
237	ENSG00000163191	S100A11	1.423425787	0.02429105
238	ENSG00000007350	TKTL1	1.505220499	0.02429105
239	ENSG00000198258	UBL5	1.130230714	0.02487117
240	ENSG00000143420	ENSA	1.078165442	0.02487117
241	ENSG00000116918	TSNAX	1.138055147	0.02487117
242	ENSG00000179029	TMEM107	1.190224382	0.02503246
243	ENSG00000151773	CCDC122	1.482136091	0.02503246
244	ENSG00000198178	CLEC4C	2.74561153	0.02515443
245	ENSG00000137500	CCDC90B	1.03192001	0.02515919
246	ENSG00000277791	PSMB3	1.045108838	0.02550779
247	ENSG00000158292	GPR153	1.502360139	0.02550779
248	ENSG00000221510	MIR548O	1.567479709	0.02550779
249	ENSG00000197894	ADH5	1.012383798	0.02573036
250	ENSG00000168060	NAALADL1	1.138332802	0.02651029
251	ENSG00000182165	TP53TG1	1.383907415	0.02670081
252	ENSG00000119943	PYROXD2	1.360359887	0.02694326
253	ENSG00000066379	ZNRD1	1.002475251	0.02699925
254	ENSG00000140030	GPR65	1.367025553	0.02725246
255	ENSG00000105701	FKBP8	1.028323458	0.02735693
256	ENSG00000131495	NDUFA2	1.073867565	0.02735693

257	ENSG00000204315	FKBPL	1.627150739	0.02763833
258	ENSG00000121851	POLR3GL	1.209906342	0.02802524
259	ENSG00000138738	PRDM5	1.179002843	0.02802524
260	ENSG00000272839	AC011899.3	1.401330717	0.02823318
261	ENSG00000241764	AC002467.1	1.553595057	0.02839238
262	ENSG00000120656	TAF12	1.131858002	0.02839826
263	ENSG00000197956	S100A6	1.091012343	0.02843127
264	ENSG00000165118	C9orf64	1.662380555	0.02865426
265	ENSG00000184106	TREML3P	1.68488407	0.02865426
266	ENSG00000116171	SCP2	1.010195909	0.02889363
267	ENSG00000161677	JOSD2	1.044180065	0.02905424
268	ENSG00000229847	EMX2OS	1.880765215	0.02909678
269	ENSG00000198133	TMEM229B	1.114972623	0.02969112
270	ENSG00000224650	IGHV3-74	1.688512092	0.03001036
271	ENSG00000150281	CTF1	1.374710059	0.03001036
272	ENSG00000169397	RNASE3	3.957037122	0.03001036
273	ENSG00000239697	TNFSF12	1.214732913	0.03001036
274	ENSG00000091181	IL5RA	1.348658132	0.03001036
275	ENSG00000106003	LFNG	1.644260836	0.03001036
276	ENSG00000171552	BCL2L1	1.208322057	0.03012104
277	ENSG00000197299	BLM	1.734216799	0.03056308
278	ENSG00000163956	LRPAP1	1.179342531	0.03093102
279	ENSG00000276644	DACH1	1.214812587	0.03093102
280	ENSG00000224051	CPTP	1.19777411	0.03117939
281	ENSG00000187569	DPPA3	4.202976656	0.03143003
282	ENSG00000127540	UQCR11	1.018406359	0.03145137
283	ENSG00000214114	MYCBP	1.278041537	0.03152835
284	ENSG00000183386	FHL3	1.22955958	0.03152835
285	ENSG00000137312	FLOT1	1.039275913	0.03179321
286	ENSG00000120885	CLU	2.153635411	0.03179321
287	ENSG00000176108	CHMP6	1.084608755	0.03197657
288	ENSG00000125870	SNRPB2	1.019418395	0.03197657
289	ENSG00000137806	NDUFAF1	1.377293978	0.03218009
290	ENSG00000143549	TPM3	1.094946583	0.03218795
291	ENSG00000159496	RGL4	1.085680459	0.03220881
292	ENSG00000274976	AC087588.2	4.010916847	0.03239364
293	ENSG00000054983	GALC	1.150197791	0.03256619
294	ENSG00000134321	RSAD2	2.090599652	0.03282104
295	ENSG00000233554	B4GALT1-AS1	1.963608954	0.03327656
296	ENSG00000243317	STMP1	1.114188628	0.03335186
297	ENSG00000135114	OASL	1.436003773	0.03335186
298	ENSG00000099785	MAR2	1.064958963	0.03345155
299	ENSG00000113732	ATP6V0E1	1.151179556	0.03354939

300	ENSG00000175324	LSM1	1.019567241	0.03377613
301	ENSG00000174977	AC026271.1	1.056815451	0.03377613
302	ENSG00000171148	TADA3	1.238156941	0.03377613
303	ENSG00000175416	CLTB	1.039828268	0.03377613
304	ENSG00000165030	NFIL3	1.210744861	0.03398618
305	ENSG00000105223	PLD3	1.150037432	0.03398618
306	ENSG00000112576	CCND3	1.081104531	0.03398812
307	ENSG00000125352	RNF113A	1.008549397	0.03455623
308	ENSG00000203705	TATDN3	1.163047311	0.03465843
309	ENSG00000279673	AC092919.2	1.552444052	0.03465843
310	ENSG00000131100	ATP6V1E1	1.121172738	0.03468306
311	ENSG00000125871	MGME1	1.245169435	0.03478733
312	ENSG00000022277	RTF2	1.039231947	0.03497147
313	ENSG00000187446	CHP1	1.104673458	0.03500091
314	ENSG00000162746	FCRLB	1.222413809	0.03515702
315	ENSG00000165140	FBP1	1.237397304	0.03515702
316	ENSG00000282608	ADORA3	1.462098912	0.035551
317	ENSG00000103769	RAB11A	1.191542036	0.035551
318	ENSG00000233461	AL445524.1	1.086668271	0.03603478
319	ENSG00000101335	MYL9	2.032171175	0.03603478
320	ENSG00000179918	SEPHS2	1.130645436	0.03639853
321	ENSG00000166664	CHRFAM7A	1.564181401	0.03651292
322	ENSG00000253838	AC007991.2	1.721203833	0.03677448
323	ENSG00000168118	RAB4A	1.030530155	0.0368016
324	ENSG00000162961	DPY30	1.140891985	0.03690978
325	ENSG00000119048	UBE2B	1.073599219	0.03709032
326	ENSG00000077549	CAPZB	1.06114488	0.03741546
327	ENSG00000240065	PSMB9	1.154631209	0.03744853
328	ENSG00000092067	CEBPE	1.835587298	0.0375575
329	ENSG00000158825	CDA	1.297223555	0.0375575
330	ENSG00000278771	RN7SL3	2.51920743	0.03758188
331	ENSG00000111669	TPI1	1.056688853	0.03758188
332	ENSG00000265369	PCAT18	4.723603407	0.03812186
333	ENSG00000062282	DGAT2	1.717486516	0.03834096
334	ENSG00000169122	FAM110B	1.363988256	0.0389342
335	ENSG00000119917	IFIT3	1.882527409	0.0389342
336	ENSG00000172086	KRCC1	1.364102175	0.03901494
337	ENSG00000182952	HMGN4	1.045995086	0.03920908
338	ENSG00000177156	TALDO1	1.354025922	0.03923261
339	ENSG00000275160	AL354718.1	2.322735662	0.03923261
340	ENSG00000171700	RGS19	1.174108814	0.03926727
341	ENSG00000126267	COX6B1	1.136873863	0.03939195
342	ENSG00000277203	F8A1	1.164619674	0.03940207

343	ENSG00000160325	CACFD1	1.254728869	0.04054863
344	ENSG00000140379	BCL2A1	1.432476982	0.04054863
345	ENSG00000272456	AC087045.2	3.809438785	0.04054863
346	ENSG00000108518	PFN1	1.026417001	0.04054863
347	ENSG00000167315	ACAA2	1.047525246	0.04063799
348	ENSG00000169714	CNBP	1.090792498	0.04091109
349	ENSG00000237773	AC073332.1	1.790468453	0.04125661
350	ENSG00000272288	AL451165.2	1.342416733	0.04134717
351	ENSG00000125148	MT2A	1.500521055	0.0413784
352	ENSG00000197933	ZNF823	1.468407512	0.0413784
353	ENSG00000165886	UBTD1	1.188052781	0.04138857
354	ENSG00000059377	TBXAS1	1.031305784	0.04138857
355	ENSG00000233859	ADH5P4	2.721539081	0.04138857
356	ENSG00000182117	NOP10	1.256554902	0.04138857
357	ENSG00000111666	CHPT1	1.093475935	0.04138857
358	ENSG00000204439	C6orf47	1.019414852	0.04138857
359	ENSG00000007520	TSR3	1.002975357	0.04139749
360	ENSG00000140105	WARS	1.037389482	0.04141512
361	ENSG00000213719	CLIC1	1.162089043	0.04144063
362	ENSG00000087586	AURKA	1.463421505	0.041646
363	ENSG00000134755	DSC2	1.466413961	0.04250412
364	ENSG00000149600	COMMD7	1.01944173	0.04250412
365	ENSG00000238121	LINC00426	1.007419974	0.04286029
366	ENSG00000138381	ASNSD1	1.037654728	0.04337546
367	ENSG00000253683	AC027309.2	1.103003355	0.04347117
368	ENSG00000135519	KCNH3	1.285754871	0.04398472
369	ENSG00000244617	ASPRV1	1.430301263	0.04416459
370	ENSG00000125753	VASP	1.266000692	0.04421056
371	ENSG00000135441	BLOC1S1	1.095489061	0.04421056
372	ENSG00000278970	HEIH	1.100707004	0.04421056
373	ENSG00000126262	FFAR2	1.3577689	0.04430624
374	ENSG00000103355	PRSS33	1.38653475	0.04430624
375	ENSG00000143740	SNAP47	1.026717293	0.04458831
376	ENSG00000168899	VAMP5	1.234221097	0.04471911
377	ENSG00000176845	METRNL	1.212903568	0.04483044
378	ENSG00000174307	PHLDA3	2.704568679	0.04506535
379	ENSG00000267174	AC011472.2	1.90438794	0.0451311
380	ENSG00000144746	ARL6IP5	1.207064986	0.04555204
381	ENSG00000163319	MRPS18C	1.083288762	0.04585948
382	ENSG00000142657	PGD	1.254617378	0.04624816
383	ENSG00000154822	PLCL2	1.139281096	0.04624816
384	ENSG00000115685	PPP1R7	1.024358849	0.04624816
385	ENSG00000102172	SMS	1.017376574	0.04663667

386	ENSG00000269293	ZSCAN16-AS1	1.160928066	0.04663667
387	ENSG00000175854	SWI5	1.168470158	0.04737275
388	ENSG00000143977	SNRPG	1.110039751	0.04759918
389	ENSG00000141429	GALNT1	1.132370904	0.0481811
390	ENSG00000128340	RAC2	1.055170146	0.04846898
391	ENSG00000066455	GOLGA5	1.02312989	0.04847356
392	ENSG00000178449	COX14	1.05108259	0.04847356
393	ENSG00000225217	HSPA7	1.577576773	0.04871877
394	ENSG00000226210	WASH8P	2.012411145	0.04883444
395	ENSG00000198736	MSRB1	1.539448286	0.04998957

Supplementary Table 10. Crisis vs. healthy: significantly downregulated genes

#	Gene ID	Gene Name	log2FoldChange	padj
1	ENSG00000169429	CXCL8	-4.335598217	6.58E-15
2	ENSG00000250138	AC139495.3	-3.966122409	1.08E-07
3	ENSG00000081041	CXCL2	-5.194013954	1.96E-06
4	ENSG00000233578	EIF4EP1	-3.00437958	1.17E-05
5	ENSG00000112245	PTP4A1	-4.476701432	1.31E-05
6	ENSG00000255398	HCAR3	-2.45049772	4.07E-05
7	ENSG00000162510	MATN1	-4.713450426	4.53E-05
8	ENSG00000267858	MZF1-AS1	-2.090005581	8.22E-05
9	ENSG00000172243	CLEC7A	-2.922972157	9.47E-05
10	ENSG00000274021	AC024909.2	-1.996553022	0.000103229
11	ENSG00000175556	LONRF3	-3.669134903	0.000108582
12	ENSG00000157734	SNX22	-2.088075167	0.000108582
13	ENSG00000167034	NKX3-1	-4.634729214	0.000108582
14	ENSG00000196247	ZNF107	-2.218928437	0.000120357
15	ENSG00000125538	IL1B	-4.296358079	0.000126482
16	ENSG00000162804	SNED1	-2.299091024	0.000126482
17	ENSG00000187862	TTC24	-3.609428445	0.000135813
18	ENSG00000197558	SSPO	-2.396619126	0.000152072
19	ENSG00000136826	KLF4	-4.031806169	0.000161464
20	ENSG00000258302	AC025034.1	-3.301978087	0.000185329
21	ENSG00000142599	RERE	-1.576645535	0.000187397
22	ENSG00000146592	CREB5	-3.639946873	0.00019065
23	ENSG00000119139	TJP2	-4.632551497	0.000209686
24	ENSG00000188383	GPAT2P2	-6.714833693	0.000233101
25	ENSG00000238018	AC093110.1	-2.125666217	0.000233101
26	ENSG00000269954	AC022239.1	-3.889805108	0.000271393
27	ENSG00000185650	ZFP36L1	-2.702109386	0.000323198
28	ENSG00000279192	PWAR5	-4.176952567	0.000323198
29	ENSG00000224796	RPL32P1	-3.37127358	0.000375292
30	ENSG00000277117	FP565260.3	-5.74406607	0.000409387

31	ENSG00000211821	TRDV2	-2.997031587	0.000429328
32	ENSG00000232810	TNF	-3.300470612	0.000431464
33	ENSG00000260075	NSFP1	-4.7554122	0.000781304
34	ENSG00000176490	DIRAS1	-2.38341372	0.000801054
35	ENSG00000134909	ARHGAP32	-3.217456601	0.000801054
36	ENSG00000124731	TREM1	-2.044694605	0.000818305
37	ENSG00000114923	SLC4A3	-2.020631583	0.000848366
38	ENSG00000128578	STRIP2	-2.637759468	0.000848366
39	ENSG00000117400	MPL	-3.601321096	0.000848366
40	ENSG00000161681	SHANK1	-5.379196111	0.000848366
41	ENSG00000165507	DEPP1	-3.798556219	0.000932488
42	ENSG00000138670	RASGEF1B	-3.373743146	0.00104124
43	ENSG00000279825	AC003957.1	-3.720998266	0.001098204
44	ENSG00000188158	NHS	-3.393418846	0.001457155
45	ENSG00000163635	ATXN7	-2.071637955	0.001749895
46	ENSG00000271550	BNIP3P11	-2.716771253	0.001806506
47	ENSG00000137502	RAB30	-2.730048064	0.0018185
48	ENSG00000042062	RIPOR3	-3.511774657	0.0018185
49	ENSG00000182389	CACNB4	-2.318215132	0.00188017
50	ENSG00000049249	TNFRSF9	-2.030183252	0.002053744
51	ENSG00000214787	MS4A4E	-2.633332138	0.002053744
52	ENSG00000272498	AC090948.2	-4.106241276	0.002269227
53	ENSG00000171444	MCC	-1.892610986	0.002269227
54	ENSG00000182782	HCAR2	-1.76610267	0.002276893
55	ENSG00000170873	MTSS1	-2.318139187	0.002276893
56	ENSG00000102755	FLT1	-4.702185181	0.002350493
57	ENSG00000251194	AL133330.1	-3.032076761	0.002397971
58	ENSG00000111300	NAA25	-2.140309101	0.002535942
59	ENSG00000100154	TTC28	-2.369808245	0.002733569
60	ENSG00000183960	KCNH8	-4.299455225	0.002822049
61	ENSG00000155846	PPARGC1B	-3.123077058	0.002822049
62	ENSG00000278743	AC087239.1	-2.15193331	0.00287321
63	ENSG00000050628	PTGER3	-2.960061295	0.003027776
64	ENSG00000172915	NBEA	-3.372466104	0.003045236
65	ENSG00000247982	LINC00926	-2.936292687	0.003084576
66	ENSG00000200169	RNU5D-1	-7.094348122	0.003151181
67	ENSG00000279433	AC018529.3	-3.184525104	0.003200878
68	ENSG00000167483	FAM129C	-1.888229345	0.003342261
69	ENSG00000152894	PTPRK	-5.064874954	0.003342261
70	ENSG00000090924	PLEKHG2	-3.176302155	0.003422248
71	ENSG00000144645	OSBPL10	-3.15838944	0.003512486
72	ENSG00000082293	COL19A1	-4.947408124	0.003518888
73	ENSG00000125740	FOSB	-3.376348285	0.003635354

74	ENSG00000261573	AL157402.2	-3.846869292	0.003635354
75	ENSG00000142798	HSPG2	-3.288453584	0.00368341
76	ENSG00000173898	SPTBN2	-3.056370805	0.003744296
77	ENSG00000107611	CUBN	-2.535938942	0.003777221
78	ENSG00000009694	TENM1	-3.448928135	0.003777221
79	ENSG00000164330	EBF1	-4.643891271	0.003924517
80	ENSG00000170113	NIPA1	-1.286652915	0.00410727
81	ENSG00000157554	ERG	-3.847714448	0.004360642
82	ENSG00000213782	DDX47	-2.709301939	0.004551672
83	ENSG00000177272	KCNA3	-3.282059988	0.004906142
84	ENSG00000251322	SHANK3	-3.554397987	0.005054896
85	ENSG00000108179	PPIF	-1.233651645	0.005106007
86	ENSG00000240487	AC093010.1	-6.047096064	0.00516227
87	ENSG00000251158	AC131392.2	-3.974300751	0.005368805
88	ENSG00000237111	IGHJ3P	-5.487509222	0.00547631
89	ENSG00000272533	SNORA28	-2.875997994	0.00551128
90	ENSG00000170345	FOS	-2.393784007	0.00551128
91	ENSG00000133048	CHI3L1	-3.200094411	0.005826444
92	ENSG00000177606	JUN	-3.66517061	0.005981365
93	ENSG00000214595	EML6	-2.320429418	0.006247867
94	ENSG00000174885	NLRP6	-2.689345607	0.006247867
95	ENSG00000280195	AC245140.2	-2.090899679	0.006361777
96	ENSG00000155980	KIF5A	-3.675766468	0.006520336
97	ENSG00000148773	MKI67	-2.068098938	0.006562563
98	ENSG00000203757	OR6K3	-3.628780034	0.006794216
99	ENSG00000146555	SDK1	-6.315981476	0.006826969
100	ENSG00000271913	AL035530.2	-1.950586653	0.00689023
101	ENSG00000177494	ZBED2	-3.016693491	0.007009492
102	ENSG00000154252	GAL3ST2	-6.82727578	0.007161793
103	ENSG00000179869	ABCA13	-3.550288513	0.007161793
104	ENSG00000076067	RBMS2	-2.82059605	0.007166294
105	ENSG00000198363	ASPH	-1.544287825	0.007348594
106	ENSG00000048052	HDAC9	-2.951034335	0.007348594
107	ENSG00000257599	OVCH1-AS1	-3.393038954	0.007348594
108	ENSG00000211937	IGHV2-5	-6.260244748	0.007348594
109	ENSG00000168026	TTC21A	-2.107507442	0.007348594
110	ENSG00000279530	AC092881.1	-2.887667591	0.007896228
111	ENSG00000176438	SYNE3	-1.653255526	0.008176692
112	ENSG00000132704	FCRL2	-3.468576407	0.008176692
113	ENSG00000141934	PLPP2	-5.396092559	0.00822469
114	ENSG00000142513	ACP4	-3.735711401	0.008442464
115	ENSG00000206633	SNORA80B	-5.172666419	0.008537844
116	ENSG00000214357	NEURL1B	-5.672970766	0.008627926

117	ENSG00000180139	ACTA2-AS1	-2.695683071	0.008627926
118	ENSG00000145428	RNF175	-2.300852388	0.008627926
119	ENSG00000231508	RPL34P20	-4.387656406	0.008627926
120	ENSG00000154258	ABCA9	-3.193610298	0.008628131
121	ENSG00000086548	CEACAM6	-2.580552717	0.008708117
122	ENSG00000278330	AC018529.2	-2.124638292	0.008844532
123	ENSG00000130052	STARD8	-2.943448047	0.00890718
124	ENSG00000069188	SDK2	-4.008670911	0.009035091
125	ENSG00000091262	ABCC6	-1.583224215	0.009035091
126	ENSG00000272476	AL024507.2	-5.273414922	0.009035091
127	ENSG00000173894	CBX2	-3.731829331	0.009109463
128	ENSG00000259598	AC116158.2	-5.482858634	0.009403918
129	ENSG00000028277	POU2F2	-1.877954064	0.009403918
130	ENSG00000284669	AC092053.3	-2.181979071	0.009403918
131	ENSG00000137094	DNAJB5	-2.379172615	0.009556927
132	ENSG00000183023	SLC8A1	-2.95807007	0.009588195
133	ENSG00000169031	COL4A3	-4.318876905	0.009722721
134	ENSG00000263826	AC112907.3	-1.809466715	0.009722721
135	ENSG00000166833	NAV2	-4.093569154	0.009722721
136	ENSG00000123384	LRP1	-3.280526089	0.009722721
137	ENSG00000253302	STAU2-AS1	-5.974004466	0.009803392
138	ENSG00000223960	AC009948.1	-2.174001837	0.009864246
139	ENSG00000119535	CSF3R	-2.377699219	0.010018207
140	ENSG00000174807	CD248	-4.431440694	0.010241372
141	ENSG00000241106	HLA-DOB	-2.036675118	0.010287999
142	ENSG00000029534	ANK1	-2.397037765	0.010287999
143	ENSG00000186049	KRT73	-3.885409758	0.010406744
144	ENSG00000121454	LHX4	-2.629815754	0.010406744
145	ENSG00000267702	AP005131.6	-2.533161562	0.01062099
146	ENSG00000153914	SREK1	-1.366754113	0.010877934
147	ENSG00000157927	RADIL	-3.410014575	0.010944308
148	ENSG00000175899	A2M	-4.554978162	0.011375002
149	ENSG00000151208	DLG5	-1.997549904	0.011458597
150	ENSG00000183723	CMTM4	-1.259327232	0.011458597
151	ENSG00000169855	ROBO1	-5.324228824	0.011514967
152	ENSG00000197959	DNM3	-2.628981528	0.01159846
153	ENSG00000124920	MYRF	-1.553023087	0.011648318
154	ENSG00000232104	RFX3-AS1	-2.122989629	0.01173705
155	ENSG00000237232	ZNF295-AS1	-3.699970771	0.011783971
156	ENSG00000111696	NT5DC3	-2.666166637	0.011783971
157	ENSG00000069493	CLEC2D	-1.66506844	0.011783971
158	ENSG00000230606	AC092683.1	-4.469283897	0.011783971
159	ENSG00000248734	AC008906.1	-2.073602245	0.012343279

160	ENSG00000214279	SCART1	-3.2990935	0.012366527
161	ENSG00000124107	SLPI	-2.588255607	0.012405919
162	ENSG00000012124	CD22	-3.10711106	0.012672054
163	ENSG00000259248	USP3-AS1	-2.119384316	0.012682742
164	ENSG00000276517	AL133243.3	-2.150694789	0.012682742
165	ENSG00000099194	SCD	-2.18587552	0.012843125
166	ENSG00000120278	PLEKHG1	-3.570038899	0.013030224
167	ENSG00000204186	ZDBF2	-2.550526534	0.013184441
168	ENSG00000237706	TRIM51EP	-2.252493131	0.013217153
169	ENSG00000279602	AC109326.1	-2.301242566	0.013431076
170	ENSG00000152217	SETBP1	-2.553404319	0.013537705
171	ENSG00000269951	AC090181.2	-2.490252692	0.01364885
172	ENSG00000222043	AC079305.1	-1.935921851	0.013982659
173	ENSG00000173542	MOB1B	-1.536732894	0.013982659
174	ENSG00000148737	TCF7L2	-2.595675504	0.013982659
175	ENSG00000245648	AC022075.1	-3.848900404	0.013982659
176	ENSG00000245105	A2M-AS1	-3.997806709	0.014061217
177	ENSG00000283674	AC068587.4	-2.415973647	0.014061217
178	ENSG00000060749	QSER1	-1.718126709	0.014061217
179	ENSG00000226383	LINC01876	-5.626963204	0.014204243
180	ENSG00000158402	CDC25C	-2.862373997	0.014362754
181	ENSG00000074590	NUAK1	-2.569324219	0.014407834
182	ENSG00000172037	LAMB2	-2.571181515	0.014589153
183	ENSG00000279897	BIRC6-AS2	-5.612434024	0.014589153
184	ENSG00000214018	RRM2P3	-2.747694711	0.014589153
185	ENSG00000262312	AC004494.1	-2.634752563	0.01530284
186	ENSG00000285103	AL451123.1	-2.218338483	0.015363071
187	ENSG00000240505	TNFRSF13B	-4.059970343	0.015406471
188	ENSG00000151612	ZNF827	-2.813171853	0.015562788
189	ENSG00000104804	TULP2	-4.282987176	0.015616185
190	ENSG00000072182	ASIC4	-4.837916083	0.015651949
191	ENSG00000110777	POU2AF1	-3.150616571	0.015659366
192	ENSG00000069667	RORA	-3.047619978	0.015758531
193	ENSG00000163694	RBM47	-2.411279979	0.015915067
194	ENSG00000255397	AC022182.3	-4.929520463	0.015915067
195	ENSG00000138640	FAM13A	-1.408285051	0.016263075
196	ENSG00000186642	PDE2A	-2.282403996	0.016286174
197	ENSG00000104177	MYEF2	-2.47435379	0.016545532
198	ENSG00000111788	AC009533.1	-2.107025069	0.016973093
199	ENSG00000123411	IKZF4	-1.246687241	0.017250995
200	ENSG00000151117	TMEM86A	-2.243719956	0.017293605
201	ENSG00000113389	NPR3	-2.81551972	0.017293605
202	ENSG00000232931	LINC00342	-1.642097662	0.017342622

203	ENSG00000280347	AC000123.3	-1.546516462	0.017396014
204	ENSG00000119866	BCL11A	-2.010362222	0.01745962
205	ENSG00000259709	AC023906.4	-6.297305635	0.01745962
206	ENSG00000204620	AC115618.1	-2.111501879	0.01745962
207	ENSG00000144802	NFKBIZ	-1.42303065	0.01745962
208	ENSG00000197183	NOL4L	-1.955214675	0.017609757
209	ENSG00000011028	MRC2	-2.730236675	0.017662779
210	ENSG00000249661	TNRC18P1	-5.64518794	0.017669434
211	ENSG00000129204	USP6	-4.416110616	0.017669434
212	ENSG00000103647	CORO2B	-5.146448603	0.017669434
213	ENSG00000252355	RN7SKP287	-3.855948782	0.017669434
214	ENSG00000246130	AC107959.2	-1.95204188	0.017761886
215	ENSG00000173083	HPSE	-2.436954124	0.017802088
216	ENSG00000211829	TRDC	-2.656135397	0.017884623
217	ENSG00000269069	AC007842.1	-2.602486756	0.017906436
218	ENSG00000159433	STARD9	-2.027238563	0.017906436
219	ENSG00000261596	AC005632.2	-1.627671088	0.017974577
220	ENSG00000107614	TRDMT1	-1.261254159	0.018071444
221	ENSG00000235703	LINC00894	-1.490333627	0.018156871
222	ENSG00000163251	FZD5	-2.278396776	0.018192513
223	ENSG00000254017	IGHEP2	-1.950855755	0.018202424
224	ENSG00000240563	L1TD1	-6.129301527	0.018204621
225	ENSG00000252759	RF00019	-1.894517588	0.018509402
226	ENSG00000166736	HTR3A	-4.020438245	0.018602843
227	ENSG00000196628	TCF4	-2.934446806	0.018659779
228	ENSG00000188211	NCR3LG1	-2.88080784	0.018906916
229	ENSG00000040199	PHLPP2	-1.465140812	0.018988365
230	ENSG00000239653	PSMD6-AS2	-1.681505478	0.018988365
231	ENSG00000224078	SNHG14	-2.133806715	0.018988365
232	ENSG00000065923	SLC9A7	-2.652328843	0.019012563
233	ENSG00000181619	GPR135	-2.365042343	0.019012563
234	ENSG00000208028	MIR616	-2.185224495	0.019012563
235	ENSG00000140022	STON2	-2.652464367	0.019085719
236	ENSG00000115977	AAK1	-1.322088662	0.019085719
237	ENSG00000010704	HFE	-2.227402456	0.019085719
238	ENSG00000151914	DST	-2.353365841	0.019104602
239	ENSG00000177455	CD19	-2.820177903	0.019247677
240	ENSG00000162576	MXRA8	-2.160732404	0.019299416
241	ENSG00000181666	HKR1	-1.187046435	0.019399211
242	ENSG00000103067	ESRP2	-2.62273683	0.01973592
243	ENSG00000268366	AC010271.1	-1.845314794	0.019755051
244	ENSG00000197121	PGAP1	-2.253692644	0.019901808
245	ENSG00000091831	ESR1	-2.283901865	0.020036427

246	ENSG00000264456	AC138207.4	-1.604939935	0.020102982
247	ENSG00000150048	CLEC1A	-2.939272403	0.020102982
248	ENSG00000163629	PTPN13	-3.428905306	0.020303444
249	ENSG00000275538	RNVU1-19	-4.749859227	0.020417835
250	ENSG00000186088	GSAP	-1.181893351	0.020442733
251	ENSG00000237513	AC007384.1	-2.27009265	0.020557777
252	ENSG00000284828	AC012020.2	-1.84660806	0.020564351
253	ENSG00000265018	AGAP12P	-3.988681115	0.020918777
254	ENSG00000120889	TNFRSF10B	-1.367277266	0.020965356
255	ENSG00000184384	MAML2	-2.731556006	0.021061082
256	ENSG00000124766	SOX4	-2.321241143	0.021061082
257	ENSG00000186115	CYP4F2	-4.902171433	0.021061082
258	ENSG00000272625	AP000919.4	-1.813027891	0.021061082
259	ENSG00000278985	AC092718.7	-3.028713181	0.021213733
260	ENSG00000135052	GOLM1	-1.696094068	0.021213733
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262	ENSG00000102445	RUBCNL	-2.452895208	0.021866251
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542	ENSG00000279198	AC008894.3	-2.655132898	0.046701671
543	ENSG00000173588	CEP83	-1.155074173	0.046831416

544	ENSG00000244256	RN7SL130P	-2.913507222	0.046831416
545	ENSG00000197057	DTHD1	-3.054957021	0.046831416
546	ENSG00000226822	AL390036.1	-2.389688667	0.046831416
547	ENSG00000136931	NR5A1	-4.876929927	0.046831416
548	ENSG00000278829	AC099811.5	-2.309731054	0.046928635
549	ENSG00000103064	SLC7A6	-1.121785135	0.047104441
550	ENSG00000082269	FAM135A	-1.721460591	0.047155006
551	ENSG00000204103	MAFB	-2.765970514	0.047319323
552	ENSG00000227212	PFN1P6	-5.257006544	0.047599178
553	ENSG00000211662	IGLV3-21	-4.755881774	0.04814759
554	ENSG00000162367	TAL1	-2.183641647	0.04814759
555	ENSG00000058668	ATP2B4	-1.76811476	0.04822025
556	ENSG00000107551	RASSF4	-2.114371202	0.048222125
557	ENSG00000244300	GATA2-AS1	-1.729832879	0.048239092
558	ENSG00000270184	AC018695.4	-1.510753126	0.048239092
559	ENSG00000233261	FAM238A	-1.948659303	0.048239092
560	ENSG00000205084	TMEM231	-2.034785968	0.048468983
561	ENSG00000135905	DOCK10	-1.71008699	0.048473564
562	ENSG00000182197	EXT1	-1.830891709	0.048718772
563	ENSG00000100320	RBFOX2	-4.929970701	0.048718772
564	ENSG00000132694	ARHGEF11	-1.723548566	0.048718772
565	ENSG00000168916	ZNF608	-1.958328471	0.048718772
566	ENSG00000236278	PEBP1P3	-2.281502947	0.048718772
567	ENSG00000238086	PPP1R26P1	-3.347910636	0.048718772
568	ENSG00000157193	LRP8	-1.745349252	0.048718772
569	ENSG00000181963	OR52K2	-3.210494423	0.048834444
570	ENSG00000143850	PLEKHA6	-2.283657849	0.048999847
571	ENSG00000152377	SPOCK1	-3.752227374	0.049054145
572	ENSG00000005471	ABCB4	-3.331744768	0.0490663
573	ENSG00000233974	AC138951.1	-4.162180392	0.049088618
574	ENSG00000150637	CD226	-1.984657257	0.049872605

Supplementary Table 11. Crisis vs. Stable: significantly upregulated genes

#	Gene ID	Gene Name	log2FoldChange	padj
1	ENSG00000144681	STAC	5.074236637	8.06E-13
2	ENSG00000105376	ICAM5	1.832202447	1.57E-09
3	ENSG00000099998	GGT5	3.334946735	3.58E-07
4	ENSG00000109099	PMP22	2.38082297	4.44E-06
5	ENSG00000170017	ALCAM	1.573384236	1.54E-05
6	ENSG00000272398	CD24	1.586262429	1.54E-05
7	ENSG00000158292	GPR153	1.296352114	5.43E-05
8	ENSG00000176834	VSIG10	2.414284296	0.000291287
9	ENSG00000106565	TMEM176B	2.332386469	0.000972717

10	ENSG00000274736	CCL23	1.943245837	0.001404547
11	ENSG00000152778	IFIT5	1.501947269	0.002199907
12	ENSG00000163053	SLC16A14	1.945592027	0.003217548
13	ENSG00000002933	TMEM176A	2.035468817	0.004758901
14	ENSG00000124019	FAM124B	1.591791214	0.005324176
15	ENSG00000184347	SLIT3	7.343298156	0.005438631
16	ENSG00000157045	NTAN1	1.029658611	0.005844163
17	ENSG00000176105	YES1	1.518992581	0.006484226
18	ENSG00000174950	CD164L2	2.381849138	0.006764773
19	ENSG00000270972	AC136475.9	2.049829848	0.006958617
20	ENSG00000157303	SUSD3	1.214921746	0.007027369
21	ENSG00000175920	DOK7	1.885435918	0.010748313
22	ENSG00000134755	DSC2	1.277685124	0.011889335
23	ENSG00000220586	TUBBP9	7.388483535	0.012591097
24	ENSG00000057294	PKP2	1.792425364	0.012915431
25	ENSG00000177098	SCN4B	1.801695159	0.012915431
26	ENSG00000051382	PIK3CB	1.247087331	0.014243704
27	ENSG00000161714	PLCD3	1.21324801	0.015210294
28	ENSG00000168461	RAB31	1.098091004	0.016048412
29	ENSG00000054983	GALC	1.334948	0.019138154
30	ENSG00000176871	WSB2	1.223774114	0.019138154
31	ENSG00000265735	RN7SL5P	4.461041797	0.019860433
32	ENSG00000229926	AL591424.1	6.301400792	0.020306771
33	ENSG00000111328	CDK2AP1	1.15418774	0.020756461
34	ENSG00000261812	TUBB8P7	5.029857603	0.024933042
35	ENSG00000165434	PGM2L1	1.076802364	0.027389035
36	ENSG00000166664	CHRFAM7A	1.610600746	0.027934245
37	ENSG00000160180	TFF3	1.512392095	0.029941679
38	ENSG00000225690	TREML5P	7.001437236	0.030029511
39	ENSG00000175352	NRIP3	1.896282179	0.030278383
40	ENSG00000185897	FFAR3	2.560527983	0.030387207
41	ENSG00000002726	AOC1	1.440464005	0.030800744
42	ENSG00000082153	BZW1	1.067094508	0.032209359
43	ENSG00000140030	GPR65	1.35881587	0.041078216
44	ENSG00000104043	ATP8B4	1.03466944	0.042833695
45	ENSG00000111275	ALDH2	1.05288589	0.044886747
46	ENSG00000142606	MMEL1	1.687194631	0.044886747
47	ENSG00000145491	ROPN1L	1.094644691	0.047285045
48	ENSG00000050820	BCAR1	4.33227081	0.048120723

Supplementary Table 12. Crisis vs. stable: significantly downregulated genes

#	Gene ID	Gene Name	log2FoldChange	padj
1	ENSG00000124107	SLPI	-3.5338641	1.54E-05
2	ENSG00000124102	PI3	-7.2633323	1.94E-05
3	ENSG00000162645	GBP2	-1.4934434	2.45E-05
4	ENSG00000169429	CXCL8	-3.038461	2.86E-05
5	ENSG00000196735	HLA-DQA1	-3.3099735	3.89E-05
6	ENSG00000198353	HOXC4	-4.4038364	3.92E-05
7	ENSG00000163421	PROK2	-2.9919339	0.00059611
8	ENSG00000211753	TRBV28	-3.7929114	0.00097272
9	ENSG00000011028	MRC2	-3.4855553	0.00097272
10	ENSG00000133048	CHI3L1	-3.736711	0.00108352
11	ENSG00000109956	B3GAT1	-2.3963437	0.00127856
12	ENSG00000176083	ZNF683	-3.251779	0.00202702
13	ENSG00000277117	FP565260.3	-4.3881105	0.00219991
14	ENSG00000172508	CARNS1	-2.3299591	0.00298201
15	ENSG00000251301	LINC02384	-3.1876634	0.00349904
16	ENSG00000153898	MCOLN2	-2.786404	0.0047589
17	ENSG00000182782	HCAR2	-2.3986434	0.00532418
18	ENSG00000105011	ASF1B	-1.4723731	0.00534492
19	ENSG00000119535	CSF3R	-2.9192169	0.00676477
20	ENSG00000115523	GNLY	-2.8021614	0.00702737
21	ENSG00000115507	OTX1	-4.4413096	0.00772014
22	ENSG00000197165	SULT1A2	-2.3780362	0.00906359
23	ENSG00000196247	ZNF107	-1.6507298	0.00938611
24	ENSG00000116299	KIAA1324	-2.6052488	0.00938611
25	ENSG00000149328	GLB1L2	-2.9224998	0.00959544
26	ENSG00000117226	GBP3	-1.7595067	0.00977423
27	ENSG00000185650	ZFP36L1	-2.3376437	0.00977423
28	ENSG00000165338	HECTD2	-2.8177549	0.01007608
29	ENSG00000104970	KIR3DX1	-4.196773	0.0107197
30	ENSG00000171596	NMUR1	-2.4593455	0.0107197
31	ENSG00000182253	SYNM	-2.2271732	0.01157854
32	ENSG00000154451	GBP5	-1.8699632	0.01225817
33	ENSG00000261596	AC005632.2	-1.7856034	0.01253159
34	ENSG00000173200	PARP15	-2.2748564	0.01291543
35	ENSG00000162551	ALPL	-2.517902	0.01292165
36	ENSG00000179299	NSUN7	-4.3614869	0.01436508
37	ENSG00000282508	LINC01002	-2.0990318	0.01595275
38	ENSG00000248099	INSL3	-3.2961189	0.01626492
39	ENSG00000186265	BTLA	-2.4795217	0.01639627
40	ENSG00000067798	NAV3	-6.7451184	0.0173484
41	ENSG00000170873	MTSS1	-2.0781189	0.01901012

42	ENSG00000197879	MYO1C	-1.7327277	0.01913815
43	ENSG00000174171	AC020659.1	-2.4347913	0.01913815
44	ENSG00000137101	CD72	-2.1457126	0.01913815
45	ENSG00000213809	KLRK1	-2.2065528	0.01960274
46	ENSG00000279884	AC010894.5	-1.2191753	0.01960274
47	ENSG00000248323	LUCAT1	-4.1866001	0.01975082
48	ENSG00000124731	TREM1	-2.0832965	0.01986043
49	ENSG00000166965	RCCD1	-1.9838196	0.01986043
50	ENSG00000180549	FUT7	-1.3564518	0.0200678
51	ENSG00000113721	PDGFRB	-3.2511021	0.02075646
52	ENSG00000282420	TRBJ1-2	-4.8436341	0.02520692
53	ENSG00000134539	KLRD1	-1.9653195	0.02609478
54	ENSG00000234883	MIR155HG	-3.8467691	0.02649335
55	ENSG00000183844	FAM3B	-3.8606475	0.02755401
56	ENSG00000162069	BICDL2	-2.3456825	0.02755401
57	ENSG00000175003	SLC22A1	-3.5771838	0.03002951
58	ENSG00000185436	IFNLR1	-3.0118773	0.03002951
59	ENSG00000196411	EPHB4	-2.4772805	0.03014148
60	ENSG00000245648	AC022075.1	-2.9612171	0.03086619
61	ENSG00000123689	G0S2	-1.6359448	0.03365862
62	ENSG00000018280	SLC11A1	-2.2068708	0.03383923
63	ENSG00000156463	SH3RF2	-4.9409164	0.03409334
64	ENSG00000211898	IGHD	-2.7184762	0.03880602
65	ENSG00000125485	DDX31	-1.6193206	0.04563796
66	ENSG00000237803	LINC00211	-3.0227868	0.04563796
67	ENSG00000197124	ZNF682	-2.4091714	0.04563796
68	ENSG00000174885	NLRP6	-1.776311	0.04563796
69	ENSG00000111696	NT5DC3	-2.1880613	0.04646364
70	ENSG00000127561	SYNGR3	-3.0812381	0.04655824
71	ENSG00000107331	ABCA2	-1.4973295	0.04726484
72	ENSG00000170956	CEACAM3	-2.2979094	0.04728505
73	ENSG00000183762	KREMEN1	-4.1238355	0.04728505
74	ENSG00000148926	ADM	-2.9322236	0.0495805

Supplementary Table 13. Stable vs. healthy: significantly upregulated genes

#	Gene ID	Gene Name	log2FoldChange	padj
1	ENSG00000169397	RNASE3	3.539237953	1.66E-07
2	ENSG00000188536	HBA2	7.831872846	7.96E-06
3	ENSG00000267452	LINC02073	5.06070514	7.96E-06
4	ENSG00000267174	AC011472.2	2.375251757	4.45E-05
5	ENSG00000206172	HBA1	9.639428292	0.000101737
6	ENSG00000244734	HBB	7.231411501	0.000104977
7	ENSG00000169385	RNASE2	3.082005839	0.000104977

8	ENSG00000108244	KRT23	3.442417369	0.000984996
9	ENSG00000152463	OLAH	5.98829794	0.001533146
10	ENSG00000187837	HIST1H1C	1.57564963	0.007474928
11	ENSG00000157856	DRC1	3.292423487	0.007474928
12	ENSG00000274902	AC004241.2	3.114444723	0.008292006
13	ENSG00000251533	LINC00605	3.75360284	0.009228302
14	ENSG00000257108	NHLRC4	1.549744061	0.009418625
15	ENSG00000179933	C14orf119	1.431793248	0.009418625
16	ENSG00000100985	MMP9	2.054517812	0.009771747
17	ENSG00000101335	MYL9	2.98901478	0.010483472
18	ENSG00000244459	AC147067.1	1.663366261	0.011951849
19	ENSG00000274173	AL035661.1	3.692761826	0.012556981
20	ENSG00000223891	OSER1-DT	1.673618786	0.012773078
21	ENSG00000276747	PADI6	5.784834698	0.013594382
22	ENSG00000234477	AC004231.1	6.081647937	0.016125576
23	ENSG00000183844	FAM3B	4.580573188	0.017315227
24	ENSG00000225873	C3orf86	1.398542755	0.017315227
25	ENSG00000163993	S100P	1.870503875	0.018642162
26	ENSG00000180573	HIST1H2AC	1.521985338	0.020675439
27	ENSG00000152904	GGPS1 PSMB8-	1.286418312	0.020712334
28	ENSG00000204261	AS1	1.128872944	0.024515274
29	ENSG00000229320	KRT8P12	1.107969733	0.025381963
30	ENSG00000256706	AC005342.2	1.798137238	0.028017298
31	ENSG00000244041	LINC01011	1.399734659	0.03065616
32	ENSG00000153485	TMEM251	1.373634355	0.032396215
33	ENSG00000008438	PGLYRP1	1.374685401	0.032396215
34	ENSG00000180549	FUT7	1.51227012	0.033506352
35	ENSG00000135116	HRK	2.189016571	0.036700406
36	ENSG00000234506	LINC01506	1.489504386	0.036700406
37	ENSG00000272799	AC006238.1	1.444339882	0.037705944
38	ENSG00000272456	AC087045.2	4.482419195	0.037739185
39	ENSG00000270049	AC009061.2	1.337910473	0.038120442
40	ENSG00000244273	PGBD4P1	5.892949861	0.038258765
41	ENSG00000079337	RAPGEF3	2.003251823	0.041469835
42	ENSG00000143546	S100A8	1.526190959	0.041469835
43	ENSG00000140932	CMTM2	2.619885269	0.044078653
44	ENSG00000182484	WASH6P	1.528173327	0.04469229
45	ENSG00000162069	BICDL2	2.602034678	0.045798939
46	ENSG00000232671	AL391069.2	1.638081359	0.046075082
47	ENSG00000137806	NDUFAF1	1.402153164	0.047760663
48	ENSG00000102010	BMX	2.399519157	0.047900708
49	ENSG00000170160	CCDC144A	4.140493045	0.048132531

50 ENSG00000131203 IDO1 1.865296171 0.048132531

Supplementary Table 14. Stable vs. healthy: significantly downregulated genes

#	Gene ID	Gene Name	log2FoldChange	padj
1	ENSG00000161681	SHANK1	-8.131060036	1.03E-05
2	ENSG00000267519	AC020916.1	-1.922427927	5.33E-05
3	ENSG00000125740	FOSB	-3.583329817	6.94E-05
4	ENSG00000050628	PTGER3	-3.900876918	0.00016007
5	ENSG00000231329	AL031772.1	-3.077889861	0.00016007
6	ENSG00000086570	FAT2	-3.44250843	0.000546389
7	ENSG00000257629	AC006199.1	-7.256430913	0.000546389
8	ENSG00000251158	AC131392.2	-3.217294668	0.00073733
9	ENSG00000258136	AC007622.2	-2.559109185	0.000811702
10	ENSG00000250138	AC139495.3	-3.029759599	0.000811702
11	ENSG00000206633	SNORA80B	-6.91334056	0.000811702
12	ENSG00000230666	CEACAM22P	-7.122346103	0.000811702
13	ENSG00000174776	WDR49	-8.030309769	0.000927992
14	ENSG00000207205	RNVU1-15	-7.229350302	0.001626462
15	ENSG00000174010	KLHL15	-1.678966725	0.004228066
16	ENSG00000136244	IL6	-6.67868009	0.004228066
17	ENSG00000169855	ROBO1	-7.703061014	0.004268039
18	ENSG00000283208	AC001226.2	-2.940637857	0.004268039
19	ENSG00000154447	SH3RF1	-2.765780423	0.004334758
20	ENSG00000269737	AL691432.1	-2.430406901	0.004336956
21	ENSG00000198794	SCAMP5	-2.829560267	0.004680341
22	ENSG00000204524	ZNF805	-2.084861705	0.005119867
23	ENSG00000258302	AC025034.1	-2.896018101	0.005458954
24	ENSG00000120210	INSL6	-2.174812518	0.005458954
25	ENSG00000125538	IL1B	-3.974257029	0.005458954
26	ENSG00000114923	SLC4A3	-2.319200765	0.005672082
27	ENSG00000281021	AL078581.3	-2.43915695	0.007968297
28	ENSG00000277610	RNVU1-4	-6.147299364	0.008272658
29	ENSG00000230225	MTND5P14	-6.12211922	0.008292006
30	ENSG00000249661	TNRC18P1	-7.371986855	0.008301548
31	ENSG00000219881	GAPDHP42	-4.394301742	0.009411171
32	ENSG00000249791	AC008494.2	-7.138786348	0.009418625
33	ENSG00000200985	RNA5SP493	-6.713325763	0.009418625
34	ENSG00000223503	AL353807.1	-7.220113889	0.009715898
35	ENSG00000105855	ITGB8	-2.767238823	0.010008386
36	ENSG00000166736	HTR3A	-6.062443507	0.010050088
37	ENSG00000119865	CNRIP1	-3.120935437	0.010540568
38	ENSG00000273018	FAM106A	-2.288012913	0.01099141
39	ENSG00000163635	ATXN7	-3.195292338	0.011230904

40	ENSG00000243243	AC073130.2	-4.120305057	0.011349753
41	ENSG00000224207	AC018797.1	-6.515705733	0.011487046
42	ENSG00000282936	AC004706.3	-2.493508806	0.011487046
43	ENSG00000204103	MAFB	-3.606377811	0.012045218
44	ENSG00000238057	ZEB2-AS1	-2.284775973	0.012045218
45	ENSG00000167601	AXL	-4.260929423	0.012045218
46	ENSG00000047634	SCML1	-2.171532617	0.012164369
47	ENSG00000249835	VCAN-AS1	-7.145549626	0.012556981
48	ENSG00000220494	YAP1P1	-4.648839599	0.012556981
49	ENSG00000162924	REL	-1.891829302	0.012556981
50	ENSG00000241175	RN7SL494P	-6.179289271	0.012773078
51	ENSG00000170113	NIPA1	-1.101724331	0.012773078
52	ENSG00000124882	EREG	-1.772902914	0.014514103
53	ENSG00000202014	RF00019	-6.449932987	0.015225246
54	ENSG00000224690	UBE2D3P3	-6.210600644	0.015415415
55	ENSG00000163359	COL6A3	-2.859230629	0.015802009
56	ENSG00000081041	CXCL2	-3.06374182	0.015857114
57	ENSG00000167034	NKX3-1	-3.841824991	0.016112084
58	ENSG00000123411	IKZF4	-1.372567454	0.017315227
59	ENSG00000258928	AL358333.2	-3.998984435	0.020022775
60	ENSG00000279192	PWAR5	-3.627734536	0.020675439
61	ENSG00000135976	ANKRD36	-1.65499139	0.020805469
62	ENSG00000235674	LDHAP2	-2.120546226	0.02163778
63	ENSG00000111249	CUX2	-3.409684607	0.02163778
64	ENSG00000085552	IGSF9	-3.866526526	0.02191516
65	ENSG00000279853	AC004453.2	-6.080060432	0.023027562
66	ENSG00000263847	AP005899.1	-1.891521495	0.023246883
67	ENSG00000261560	AC007216.3	-2.791772721	0.024515274
68	ENSG00000226756	AC007365.1	-6.221721749	0.024515274
69	ENSG00000225690	TREML5P	-7.439662572	0.026219984
70	ENSG00000260007	AC107871.1	-1.929005925	0.028978919
71	ENSG00000178127	NDUFV2	-1.704287895	0.029256213
72	ENSG00000006459	KDM7A	-1.131521925	0.029803347
73	ENSG00000271913	AL035530.2	-1.82331192	0.032396215
74	ENSG00000231165	TRBV26OR9-2	-6.246324571	0.032396215
75	ENSG00000188056	TREML4	-3.333783574	0.033506352
76	ENSG00000229391	HLA-DRB6	-2.793691856	0.033506352
77	ENSG00000060749	QSER1	-1.528420063	0.033506352
78	ENSG00000140836	ZFH3	-3.086479535	0.033506352
79	ENSG00000234945	GTF3C2-AS1	-2.446114351	0.033633665
80	ENSG00000269235	ZNF350-AS1	-1.500978651	0.033846293
81	ENSG00000222345	RF00569	-6.006835592	0.03394982

82	ENSG00000128016	ZFP36	-1.031807145	0.033971871
83	ENSG00000145246	ATP10D	-1.721753642	0.033971871
84	ENSG00000169224	GCSAML	-2.533838113	0.033971871
85	ENSG00000181722	ZBTB20	-1.952634312	0.034317404
86	ENSG00000214960	ISPD	-2.347924327	0.034317404
87	ENSG00000143751	SDE2	-1.161573138	0.034317404
88	ENSG00000106034	CPED1	-3.139473511	0.034317404
89	ENSG00000181800	CELF2-AS1	-2.944697634	0.035855716
90	ENSG00000165507	DEPP1	-3.129647953	0.036700406
91	ENSG00000080603	SRCAP	-1.290840246	0.037168864
92	ENSG00000187862	TTC24	-2.426853322	0.037168864
93	ENSG00000175920	DOK7	-1.78061769	0.037168864
94	ENSG00000246477	AF131216.1	-1.513526613	0.037168864
95	ENSG00000234925	ATP5PDP4	-2.999635603	0.038120442
96	ENSG00000228366	AL592437.1	-4.573544548	0.038120442
97	ENSG00000229961	AL357143.1	-3.173116623	0.038128937
98	ENSG00000136826	KLF4	-3.190253979	0.038391606
99	ENSG00000233974	AC138951.1	-5.532357791	0.039963159
100	ENSG00000273254	AF129075.2	-5.404059824	0.04081162
101	ENSG00000145975	FAM217A	-5.881121742	0.04081162
102	ENSG00000228323	AC008440.1	-1.80135558	0.043168476
103	ENSG00000145012	LPP	-1.192215593	0.043914762
104	ENSG00000260318	COX6CP1	-5.629943665	0.044029158
105	ENSG00000153234	NR4A2	-1.603616765	0.044146026
106	ENSG00000153914	SREK1	-1.11200775	0.044146026
107	ENSG00000261573	AL157402.2	-2.95391205	0.044146026
108	ENSG00000124766	SOX4	-2.5427079	0.044626106
109	ENSG00000213779	AC107956.2	-5.360967043	0.04467224
110	ENSG00000083168	KAT6A	-1.069741247	0.04467224
111	ENSG00000198265	HELZ	-1.234608454	0.045948074
112	ENSG00000278986	AC091060.1	-1.791345642	0.046075082
113	ENSG00000165655	ZNF503	-1.734767999	0.048132531
114	ENSG00000118257	NRP2	-3.269020433	0.04856188
115	ENSG00000127081	ZNF484	-1.141773041	0.04865893
116	ENSG00000119725	ZNF410	-1.56027786	0.048917347
117	ENSG00000261114	AC012181.1	-1.563274952	0.049085224

Supplementary Table 15. GO enrichment analysis: Crisis vs. healthy – upregulated

Category	GOID	Description	BgRatio	padj	Gene Name
BP	GO:0042743	hydrogen peroxide metabolic process	43/14707	0.002084034	HBA2/DUOX2/HBA1/HBB/DUOX2/PRDX5/EPX/RAC2SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/NDUFA2/UQCR11/NDUFAF1/COX6B1
	GO:0006119	oxidative phosphorylation	129/14707	0.002084034	SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/UQCR11/NDUFAF1/COX6B1
BP	GO:0042775	coupled electron transport	93/14707	0.002084034	SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/UQCR11/NDUFAF1/COX6B1
	GO:0042773	ATP synthesis coupled electron transport	94/14707	0.002084034	SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/UQCR11/NDUFAF1/COX6B1
BP	GO:0042744	hydrogen peroxide catabolic process	22/14707	0.002152703	HBA2/DUOX2/HBA1/HBB/PRDX5/EPX
	GO:0098754	detoxification	104/14707	0.003760795	HBA2/DUOX2/HBA1/HBB/TXN/PRDX5/GSR/EPX/SELENOX/ADH5/MT2A
BP	GO:0022900	electron transport chain	172/14707	0.004151956	IDO1/SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/GSR/NDUFC1/ADH5/NDUFA2/UQCR11/NDUFAF1/COX6B1
	GO:0022904	respiratory electron transport chain	111/14707	0.005288201	SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/UQCR11/NDUFAF1/COX6B1
BP	GO:1990748	cellular detoxification	93/14707	0.00547901	HBA2/DUOX2/HBA1/HBB/TXN/PRDX5/GSR/EPX/SELENOX/ADH5
	GO:0017001	antibiotic catabolic process	42/14707	0.00547901	HBA2/DUOX2/HBA1/HBB/PRDX5/EPX/ADH5
BP	GO:0016999	antibiotic metabolic process	128/14707	0.014650088	HBA2/DUOX2/HBA1/HBB/DUOX2/SDHAF2/PRDX5/EPX/AKR1C1/ADH5/RAC2
	GO:0034109	homotypic cell-cell adhesion	68/14707	0.015720114	HBB/CD9/MYL12A/PKP2/CSR1P/MYL9/CLIC1/DSC2
BP	GO:0098869	cellular oxidant detoxification	89/14707	0.016944895	HBA2/DUOX2/HBA1/HBB/TXN/PRDX5/GSR/EPX/SELENOX
	GO:0046034	ATP metabolic process	267/14707	0.021476323	WSDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/TMSB4X/NDUFA2/UQCR11/NDUFAF1/FBP1/TP1/COX6B1
BP	GO:0009144	purine nucleoside triphosphate metabolic process	303/14707	0.02722323	DGUOK/SDHAF2/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/TMSB4X/NDUFA2/UQCR11/NDUFAF1/FBP1/TP1/COX6B1
	GO:0070469	respiratory chain	96/15513	0.004926204	HIGD1A/NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/UQCR11/NDUFAF1
CC	GO:0098800	inner mitochondrial membrane protein complex	139/15513	0.004926204	ATP5MD/NDUFB6/APOO/NDUFA1/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/NDUFA2/NDUFAF1
	GO:0005743	mitochondrial inner membrane	485/15513	0.006179956	MPC2/HIGD1A/ATP5MD/SLC25A1/TIMM8B/NDUFB6/APOO/NDUFA1/MRPL13/SLC25A2/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/NDUFA2/BCL2L1/UQCR11/NDUFAF1/RSAD2/COX6B1/ACAA2/MRPS18C
CC	GO:0060205	cytoplasmic vesicle lumen	314/15513	0.015637872	S100P/HBA2/HBA1/HBB/COTL1/FTL/RNASE2/GMFG/EPX/CFD/B2M/FOLR3/TMSB4X/S100A11/RNASE3/CLU/CDA
	GO:0031983	vesicle lumen	315/15513	0.015637872	S100P/HBA2/HBA1/HBB/COTL1/FTL/RNASE2/GMFG/EPX/CFD/B2M/FOLR3/TMSB4X/S100A11/RNASE3/CLU/CDA
CC	GO:0098798	mitochondrial protein complex	260/15513	0.015637872	ATP5MD/TIMM8B/NDUFB6/APOO/NDUFA1/MRPL13/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/NDUFA2/NDUFAF1/MRPS18C
	GO:0098803	respiratory chain complex	83/15513	0.015637872	NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/NDUFAF1
CC	GO:0044455	mitochondrial membrane part	215/15513	0.021199354	MPC2/ATP5MD/NDUFB6/APOO/NDUFA1/UQCR10/COX5B/COX8A/ATP5F1E/NDUFC1/ATP5MF/NDUFA2/NDUFAF1
	GO:0005746	mitochondrial respiratory chain	90/15513	0.021199354	1NDUFB6/NDUFA1/UQCR10/COX5B/COX8A/NDUFC1/NDUFA2/NDUFAF1
MF	GO:0016209	antioxidant activity	79/14759	0.001678696	HBA2/DUOX2/HBA1/HBB/TXN/ALOX5AP/PRDX5/GSR/EPX/SELENOX
	GO:0004601	peroxidase activity	41/14759	0.003528128	HBA2/DUOX2/HBA1/HBB/ALOX5AP/PRDX5/EPX
MF	GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	45/14759	0.004440317	HBA2/DUOX2/HBA1/HBB/ALOX5AP/PRDX5/EPX
	GO:0048306	calcium-dependent protein binding	54/14759	0.011182562	S100P/CPLX2/VLDLR/ANXA1/S100A11/S100A6/CHP1
MF	GO:0098632	cell-cell adhesion mediator activity	30/14759	0.028692834	PDLIM1/PKP2/ANXA1/S100A11/DSC2

Supplementary Table 16. GO enrichment analysis: Crisis vs. healthy – downregulated

Category	GOID	Description	BgRatio	padj	Gene Name
MF	GO:0022804	active transmembrane transporter activity	310/14759	0.01180186	SLC4A3/ABCA13/ABCA9/ABCC6/SLC8A1/SLC9A7/SLC23A2/SLC24A4/SLC5A3/SLC8A3/CLCN6/SLC11A1/ANKH/CLCN5/SLC16A11/ABCA2/TIMM23B/SLC7A6/ATP2B4/ABCB4
MF	GO:0001076	transcription factor activity, RNA polymerase II transcription factor binding	171/14759	0.01180186	KLF4/RERE/PPARGC1B/FOS/JUN/POU2AF1/RORA/TCF4/SOX4/CLOCK/ARHGAP5/PPRC1/PARP15/TCERG1
MF	GO:0015297	antiporter activity	76/14759	0.01180186	SLC4A3/SLC8A1/SLC9A7/SLC24A4/SLC8A3/CLCN6/SLC11A1/CLCN5/SLC7A6
MF	GO:0001046	core promoter sequence-specific DNA binding	78/14759	0.01180186	FOS/POU2AF1/RORA/ESR1/SOX4/CLOCK/ZFH3/POU2F1/PRMT5
MF	GO:0000982	transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding	343/14759	0.01572755	KLF4/FOSB/EBF1/ERG/FOS/JUN/POU2F2/MYEF2/BCL11A/TCF4/ESR1/SOX4/CLOCK/ZFH3/SNAI1/ETS2/ATF3/IRF4/MEIS1/MAFB
MF	GO:0001047	core promoter binding	128/14759	0.01572755	NKX3-1/KLF4/FOS/POU2AF1/RORA/ESR1/SOX4/CLOCK/ZFH3/POU2F1/PRMT5
MF	GO:0001190	transcriptional activator activity, RNA polymerase II transcription factor binding	53/14759	0.01572755	KLF4/RERE/FOS/JUN/POU2AF1/SOX4/CLOCK
MF	GO:0034185	apolipoprotein binding	14/14759	0.01572755	LRP1/LRP6/SCARB1/LRP8
MF	GO:0005201	extracellular matrix structural constituent	54/14759	0.01572755	MATN1/COL19A1/CHI3L1/COL4A3/FBLN2/ENAM/ELN
MF	GO:0001228	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific DNA binding	331/14759	0.01696716	KLF4/FOSB/EBF1/ERG/FOS/JUN/POU2F2/LHX4/RORA/TCF4/ESR1/SOX4/ZNF175/TRPS1/ATF3/IRF4/MEIS1/HOXC4/MAFB
MF	GO:0030228	lipoprotein particle receptor activity	16/14759	0.02240567	LRP1/LRP6/SCARB1/LRP8
MF	GO:0015491	cation:cation antiporter activity	29/14759	0.02431989	SLC8A1/SLC9A7/SLC24A4/SLC8A3/SLC11A1
MF	GO:0000987	proximal promoter sequence-specific DNA binding	377/14759	0.02510061	KLF4/FOSB/ERG/FOS/JUN/POU2F2/TCF7L2/MYEF2/BCL11A/TCF4/ESR1/CLOCK/USP3/ZFH3/ETS2/ATF3/IRF4/MEIS1/MAFB/TAL1
MF	GO:0015291	secondary active transmembrane transporter activity	197/14759	0.03055006	SLC4A3/SLC8A1/SLC9A7/SLC23A2/SLC24A4/SLC5A3/SLC8A3/CLCN6/SLC11A1/ANKH/CLCN5/SLC16A11/SLC7A6
MF	GO:0015298	solute:cation antiporter activity	32/14759	0.03115694	SLC8A1/SLC9A7/SLC24A4/SLC8A3/SLC11A1
MF	GO:0001085	RNA polymerase II transcription factor binding	129/14759	0.03199517	KLF4/RERE/FOS/JUN/TCF7L2/TCF4/ESR1/TRPS1/TCERG1/TAL1
MF	GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	365/14759	0.0328566	FOSB/ERG/FOS/JUN/POU2F2/TCF7L2/MYEF2/BCL11A/TCF4/ESR1/CLOCK/USP3/ZFH3/ETS2/ATF3/IRF4/MEIS1/MAFB/TAL1
MF	GO:0015081	sodium ion transmembrane transporter activity	132/14759	0.03394622	SLC4A3/SLC8A1/ASIC4/SLC9A7/SLC23A2/SLC24A4/SLC5A3/SLC8A3/SCN9A/ASIC1
MF	GO:0097110	scaffold protein binding	54/14759	0.04599566	TREM1/SHANK1/SHANK3/AKAP5/DSP/ATP2B4

Supplementary Table 17. GO enrichment analysis: Crisis vs. stable – upregulated

Category	GOID	Description	BgRatio	padj	geneName
BP	GO:0086091	regulation of heart rate by cardiac conduction	31/14423	0.04622252	DSC2/PKP2/SCN4B
BP	GO:0086002	cardiac muscle cell action potential involved in contraction	39/14423	0.04622252	DSC2/PKP2/SCN4B
BP	GO:0086065	cell communication involved in cardiac conduction	42/14423	0.04622252	DSC2/PKP2/SCN4B
BP	GO:0086003	cardiac muscle cell contraction	47/14423	0.04622252	DSC2/PKP2/SCN4B
BP	GO:2001198	regulation of dendritic cell differentiation	10/14423	0.04622252	TMEM176B/TMEM176A
BP	GO:0086001	cardiac muscle cell action potential	54/14423	0.04826277	DSC2/PKP2/SCN4B
BP	GO:0086069	bundle of His cell to Purkinje myocyte communication	12/14423	0.04826277	DSC2/PKP2
BP	GO:0098911	regulation of ventricular cardiac muscle cell action potential	13/14423	0.04982516	DSC2/PKP2
CC	GO:0014704	intercalated disc	42/15187	0.0190972	DSC2/PKP2/SCN4B
CC	GO:0044291	cell-cell contact zone	59/15187	0.02619264	DSC2/PKP2/SCN4B
CC	GO:0030057	desmosome	21/15187	0.04722077	DSC2/PKP2
MF	GO:0044325	ion channel binding	100/14473	0.01418447	STAC/YES1/PKP2/SCN4B

Supplementary Table 18. GO enrichment analysis: Stable vs. healthy – upregulated

Category	GOID	Description	BgRatio	padj	Gene Name
BP	GO:0015669	gas transport	11/14763	0.00052398	HBA2/HBA1/HBB
BP	GO:0042744	hydrogen peroxide catabolic process	22/14763	0.00154578	HBA2/HBA1/HBB
BP	GO:0043312	neutrophil degranulation	473/14763	0.00154578	RNASE3/HBB/RNASE2/MMP9/S100P/PGLYRP1/S100A8
		neutrophil activation involved in			
BP	GO:0002283	immune response	476/14763	0.00154578	RNASE3/HBB/RNASE2/MMP9/S100P/PGLYRP1/S100A8
BP	GO:0042119	neutrophil activation	485/14763	0.00154578	RNASE3/HBB/RNASE2/MMP9/S100P/PGLYRP1/S100A8
BP	GO:0002446	neutrophil mediated immunity	487/14763	0.00154578	RNASE3/HBB/RNASE2/MMP9/S100P/PGLYRP1/S100A8
BP	GO:0036230	granulocyte activation	491/14763	0.00154578	RNASE3/HBB/RNASE2/MMP9/S100P/PGLYRP1/S100A8
BP	GO:0015701	bicarbonate transport	37/14763	0.002992	HBA2/HBA1/HBB
BP	GO:0017001	antibiotic catabolic process	41/14763	0.00351589	HBA2/HBA1/HBB
BP	GO:0042743	hydrogen peroxide metabolic process	42/14763	0.00351589	HBA2/HBA1/HBB
BP	GO:0051187	cofactor catabolic process	51/14763	0.00573746	HBA2/HBA1/HBB
BP	GO:0019730	antimicrobial humoral response	68/14763	0.01240798	RNASE3/PGLYRP1/S100A8
BP	GO:0002544	chronic inflammatory response	16/14763	0.01792431	S100A8/IDO1
BP	GO:0000302	response to reactive oxygen species	209/14763	0.02123369	HBA2/HBA1/HBB/MMP9
BP	GO:0098869	cellular oxidant detoxification	88/14763	0.02123369	HBA2/HBA1/HBB
BP	GO:1990748	cellular detoxification	92/14763	0.02267468	HBA2/HBA1/HBB
BP	GO:0098754	detoxification	104/14763	0.03051629	HBA2/HBA1/HBB
BP	GO:0051291	protein heterooligomerization	112/14763	0.03573708	HBA2/HBA1/HBB
BP	GO:0010035	response to inorganic substance	460/14763	0.03701679	HBA2/HBA1/HBB/MMP9/S100A8
BP	GO:0042737	drug catabolic process	118/14763	0.03701679	HBA2/HBA1/HBB
		positive regulation of release of			
BP	GO:0090200	cytochrome c from mitochondria	29/14763	0.03701679	MMP9/HRK
BP	GO:0042542	response to hydrogen peroxide	125/14763	0.03929557	HBA2/HBA1/HBB
BP	GO:0016999	antibiotic metabolic process	126/14763	0.03929557	HBA2/HBA1/HBB
BP	GO:0046677	response to antibiotic	287/14763	0.04109342	HBA2/HBA1/HBB/S100A8
CC	GO:0071682	endocytic vesicle lumen	17/15574	1.06E-06	HBA2/HBA1/HBB/PGLYRP1
CC	GO:0060205	cytoplasmic vesicle lumen	312/15574	1.06E-06	RNASE3/HBA2/HBA1/HBB/RNASE2/S100P/PGLYRP1/S100A8
CC	GO:0031983	vesicle lumen	313/15574	1.06E-06	RNASE3/HBA2/HBA1/HBB/RNASE2/S100P/PGLYRP1/S100A8
CC	GO:1904724	tertiary granule lumen	55/15574	0.0023239	HBB/MMP9/PGLYRP1
CC	GO:0034774	secretory granule lumen	298/15574	0.0023239	RNASE3/RNASE2/S100P/PGLYRP1/S100A8
CC	GO:0072562	blood microparticle	91/15574	0.00696934	HBA2/HBA1/HBB
CC	GO:0030139	endocytic vesicle	270/15574	0.01254876	HBA2/HBA1/HBB/PGLYRP1
CC	GO:0045111	intermediate filament cytoskeleton	123/15574	0.01254876	KRT23/PADI6/S100A8
CC	GO:0070820	tertiary granule	159/15574	0.02324545	HBB/MMP9/PGLYRP1
CC	GO:0022627	cytosolic small ribosomal subunit	47/15574	0.02445208	HBA2/HBA1
CC	GO:0015935	small ribosomal subunit	72/15574	0.04818403	HBA2/HBA1
CC	GO:0005902	microvillus	73/15574	0.04818403	S100P/RAPGEF3
MF	GO:0019825	oxygen binding	32/14810	0.00058011	HBA2/HBA1/HBB
MF	GO:0140104	molecular carrier activity	34/14810	0.00058011	HBA2/HBA1/HBB
MF	GO:0020037	heme binding	105/14810	0.00058011	HBA2/HBA1/HBB/IDO1
MF	GO:0046906	tetrapyrrole binding	115/14810	0.00058011	HBA2/HBA1/HBB/IDO1
MF	GO:0004601	peroxidase activity	40/14810	0.00058011	HBA2/HBA1/HBB
		oxidoreductase activity, acting on			
MF	GO:0016684	peroxide as acceptor	44/14810	0.00064515	HBA2/HBA1/HBB
MF	GO:0050786	RAGE receptor binding	10/14810	0.0012558	S100P/S100A8
MF	GO:0016209	antioxidant activity	78/14810	0.00267611	HBA2/HBA1/HBB
MF	GO:0005506	iron ion binding	141/14810	0.01333154	HBA2/HBA1/HBB
MF	GO:0048037	cofactor binding	447/14810	0.0450865	HBA2/HBA1/HBB/IDO1

Supplementary Table 19. GO enrichment analysis: Stable vs. healthy – downregulated

Category	GOID	Description	BgRatio	padj	Gene Name
BP	GO:0042035	regulation of cytokine biosynthetic process	88/14763	0.01118387	IL6/IL1B/EREG/ZFP36/KLF4
BP	GO:0042089	cytokine biosynthetic process	97/14763	0.01118387	IL6/IL1B/EREG/ZFP36/KLF4
BP	GO:0042107	cytokine metabolic process	98/14763	0.01118387	IL6/IL1B/EREG/ZFP36/KLF4
BP	GO:0032755	positive regulation of interleukin-6 production	65/14763	0.02336312	IL6/IL1B/EREG/ZBTB20
BP	GO:0002675	positive regulation of acute inflammatory response	24/14763	0.02336312	PTGER3/IL6/IL1B
BP	GO:0042036	negative regulation of cytokine biosynthetic process	27/14763	0.02793129	IL6/ZFP36/KLF4
CC	GO:0005667	transcription factor complex	298/15574	0.03885292	MAFB/REL/ZFHX3/KLF4/NR4A2/SOX4
CC	GO:0043235	receptor complex	319/15574	0.03885292	SHANK1/IL6/ITGB8/HTR3A/AXL/NRP2
MF	GO:0000982	transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding	342/14810	0.00343316	FOSB/MAFB/CUX2/ZFHX3/ZBTB20/KLF4/NR4A2/SOX4
MF	GO:0001228	transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific DNA binding	329/14810	0.04775542	FOSB/MAFB/REL/KLF4/NR4A2/SOX4
MF	GO:0001077	transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding	223/14810	0.04775542	FOSB/MAFB/KLF4/NR4A2/SOX4
MF	GO:0001047	core promoter binding	130/14810	0.04775542	NKX3-1/ZFHX3/KLF4/SOX4

BgRatio: In background GO database, the ratio of all genes concerning this GO term to all genes.

9 Bibliography

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