A BENCH TO BEDSIDE APPROACH TO FACTORS INFLUENCING OUTCOMES IN COMMUNITY ACQUIRED PNEUMONIA

by

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ABSTRACT

Community acquired pneumonia (CAP) is the leading cause of death from infectious disease in the UK, with more than 250,000 hospitalised episodes in adults each year. CAP predominantly affects older adults who frequently experience high burdens of mortality and morbidity in both short and long-term.

CAP research thus far has focussed on antimicrobial therapy, or in people with CAP admitted to intensive care (ICU), only 5% of episodes in the UK involve an ICU admission. Older adults bear the brunt of CAP related morbidity and mortality, but they are underrepresented in research.

This thesis aimed to explore factors influencing outcomes from CAP by using a multi-modal approach with clinical data, statistical modelling and immunology with integrated bioinformatics.

Chapter Two demonstrates that the pandemic significantly reduced hospitalisation with CAP in Birmingham, but those who were admitted had 50% increased mortality compared to the pre-pandemic period. During the pandemic hospitalised CAP was more severe, with increased rates of sepsis, but importantly care quality was not altered.

Chapter Three explores severity assessment in CAP, demonstrating that although CAP specific scores are more accurate at predicting adverse outcomes, CURB65 is impaired in people with multimorbidity. A novel score BCAPS was generated, BCAPS provides greater accuracy at prediction of 30-day mortality compared to CURB65.

CAP requires a carefully orchestrated immune response, and particularly in older adults the neutrophil response is altered, which has been linked with adverse outcomes. Chapters 4-6 explore the role of neutrophils in CAP.

Chapter Four describes optimisation and validation of extracellular flux technology to assess *ex-vivo* metabolism in isolated neutrophils. Given the short lifespan and propensity for activation of neutrophils this work was crucial for Chapter Five. This study also demonstrated that assessment of mitochondrial respiration in neutrophils is not possible using extracellular flux.

Chapter Five is an observational cohort study which recruited 25 older adults with CAP and sepsis and 32 age and frailty matched controls. This study hypothesised that the altered neutrophil function seen in older adults with infection was due to impaired cellular energetics. Aberrant neutrophil function was demonstrated with altered migration, oxidative burst and degranulation in CAP participants, but *ex-vivo* rates of glycolysis were not changed. However, glycolysis was significantly different in younger adults compared to older adults with or without infection.

Chapter Six demonstrates that RNAsequencing is feasible in isolated neutrophils and resulted in 760 differentially expressed genes, these genes offer novel targets for mechanistic investigation of neutrophil function. This data also demonstrated no difference in RNA expression of glycolytic enzymes or pathways.

This thesis demonstrates an interdisciplinary approach to CAP. In future CAP research should focus on the needs of older adults, streaming together research disciplines to bring rapid impact by testing clinical interventions in accurately identified subgroups beside the pipeline of discovery science to provide the therapeutic targets of the future.

COVID-19 DECLARATION

My fellowship study has been significantly interrupted by COVID-19, impacting my ability to recruit participants and undertake laboratory assays.

My fellowship commenced in November 2019 and main study approved to open in March 2020, I recruited the first participant in early March. All non-essential studies were then closed by the NHS trust until September 2020. During this time, I undertook fulltime NHS work as part of the frontline pandemic response. The study was then closed again December 2020-Febuary 2021, again I undertook fulltime NHS work during this period. These periods of NHS work facilitated a four-month extension to my fellowship.

To mitigate the reduced ability to recruit participants and undertake experimental assays the following work was completed:

1. Chapter Two: Epidemiology of CAP during the pandemic
2. Chapter Three: Severity assessment in CAP
3. Chapter Six: Neutrophil transcriptome in CAP

Despite the challenges, I believe that the work presented in this thesis represents a significant contribution to the field of CAP research. Advancing understanding of how the pandemic impacted non COVID-19 respiratory infections, developing novel tools for CAP severity assessment, and exploring both neutrophil metabolism and transcriptome in older adults with CAP.

This thesis is in memory of two inspirational women: Mum and Lauren.

But also, to the next generation of women: Lydia, Ella, Delilah and Elowyn.

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List of Abbreviations

2-DG	2 Deoxy-glucose
AAT	alpha-1 antitrypsin
ADP	Adenosine diphosphate
AHRF	Acute Hypoxaemic respiratory failure
AI	Artificial Intelligence
AKI	Acute Kidney Injury
AMPK	Adenosine monophosphate activated protein kinase
AMU	Acute Medical Unit
ANOVA	Analysis of variance
AnV	Annexin V
ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine triphosphate
ATS	American Thoracic Society
AUC	Area under curve
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BTS	British Thoracic Society
CAP	Community acquired pneumonia
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CCI	Charlson comorbidity index
CD	Cluster of differentiation
CFS	Clinical Frailty Scale
COPD	Chronic Obstructive Pulmonary Disease
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
CT	Computed tomography
CVS	Cardiovascular
CXR	Chest radiograph
DEG	Differentially expressed gene
DHR-123	Dihydrohodamine-123
DNAR	Do not attempt resuscitation.
ECAR	Extracellular acidification rate
ED	Emergency Department
EDTA	Ethylenediaminetetraacetic acid
ELR	Glutamate-leucine-arginine motif
EPR	Electronic patient record
ERS	European Respiratory Society
FDR	False discovery rate
fMLP	N-Formylmethionine-leucyl-phenylalanine
G-CSF	Granulocyte- colony stimulating factor
G6PD	Glucose-6 phosphate deficiency

GCS	Glasgow Coma Scale
GM-CSF	Granulocyte macrophage- colony stimulating factor
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HK2	Hexokinase
ICD	International Disease Classification
ICU	Intensive Care Unit
IDSA	Infectious Diseases Society of America
IL8	Interleukin 8
IMD	Index of multiple deprivation
IMV	Invasive Mechanical Ventilation
IQR	Interquartile range
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kPa	Kilopascal
LIF	Leukaemia inhibitory factor
LOS	Length of stay
LPS	Lipopolysaccharide
LR	Likelihood ratio
MHC	Major Histocompatibility complex
mmHg	Milimetres of mercury
MPO	Myeloperoxidase
mtDAMPS	Mitochondrial damage associated molecular patterns
NAD	Nicotinamide adenine dinucleotide
ncRNA	noncoding RNA
NE	Neutrophil elastase
NETS	Neutrophil Extracellular Traps
NEWS2	National Early Warning Score 2
NGAL	Neutrophil gelatinase associated lipocalin
NH	Nursing home
NTHI	Non typeable *Haemophillus influenzae*
OCR	Oxygen consumption rate
OR	Odds Ratio
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCT	Procalcitonin
PCV 13/15	Pneumococcal conjugate vaccine
PER	Proton efflux rate
PFKM	Phosphofructokinase
PI	Propidium iodide
PI3K	Phosphoinositol 3 kinase

PKA	Protein kinase A
PKM	Pyruvate kinase
PMA	Phorbol 12-myristate 13-acetate
PPP	Pentose Phosphate Pathway
PPV23	Pneumococcal polysaccharide vaccine
PR3	Proteinase-3
PSI	Pneumonia severity index
PTM	Post-translational modification
QEHB	Queen Elizabeth Hospital Birmingham
qSOFA	quick Sequential Organ Failure Assessment
RCT	Randomised control trial
REDCap	Research Electronic Data Capture
RH	Residential home
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RR	Respiratory Rate
RSU	Respiratory Support Unit
SAM	Society of Acute Medicine
SAPK	Stress activated protein kinase
SEM	Standard error of mean
SOFA	Sequential organ failure assessment
SP	*Streptococcus pneumoniae*
TNF	Tumour necrosis factor
UHB	University Hospital Trust Birmingham
UK	United Kingdom
UPS	Ubiquitin proteasomal system
URA	Upstream regulator analysis
VE	Vaccine efficacy
WHO	World Health Organisation
XF	Extracellular flux

1. INTRODUCTION

Parts of this chapter are published in a review article:

1. "**Neutrophils in community-acquired pneumonia: parallels in dysfunction at the extremes of age**" Thorax, 2019. (Grudzinska et al., 2019b)

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Authors contribution: FG conceived and designed the review, undertook literature searches, and wrote the initial manuscript and completed revisions. MB, BS, TJ, AS, DT and ES designed and planned the review and revised the manuscript and approved final version.

1.1. Community acquired pneumonia.

Pneumonia was first described by Hippocrates during 5th century (Stefanakis et al., 2020), in the 19th century physicians further developed the concept of pneumonia using the terms bronchopneumonia and lobar pneumonia. Since the early 20th century pneumonia has been a leading cause of death from infectious disease (Collaborators, 2017).

Pneumonia is an umbrella term covering a broad range of clinico-pathological scenarios from neonatology to gerontology. Thus, pneumonia is typically described according to the environment in which it is acquired as this influences the likely causative pathogen and therefore the treatment indicated. This thesis focuses on community acquired pneumonia (CAP) which is pneumonia not acquired in a healthcare setting. The British Thoracic Society (BTS) definition of CAP is in Table 1-1 and used throughout this thesis.

Table 1-1: BTS CAP Definition

Symptoms of an acute lower respiratory illness (cough and at least one other lower respiratory tract symptom), new focal chest signs on examination, at least one systemic feature (either a symptom complex of sweating, fevers, shivers, aches, and pains and or temperature of 38°C or more and no other explanation for the illness which is treated as CAP with antibiotics" (Lim et al., 2009).

Pneumonia is infection of the pulmonary parenchyma and can be caused by a wide range of pathogens. Bronchopneumonia describes a patchy process where smaller bronchioles and adjacent alveoli are affected across one or multiple lobes, lobar pneumonia describes inflammation of an entire lobe or lung. In the acute phase the

pathological changes are characterised by an influx of neutrophils which then orchestrate arrival of other immune cells leading to a resolution, organisation or scarring phase (Cagle, 2018). The four histopathological stages of bacterial pneumonia are described in Figure 1-1 (Robbins and Contran, 1979).

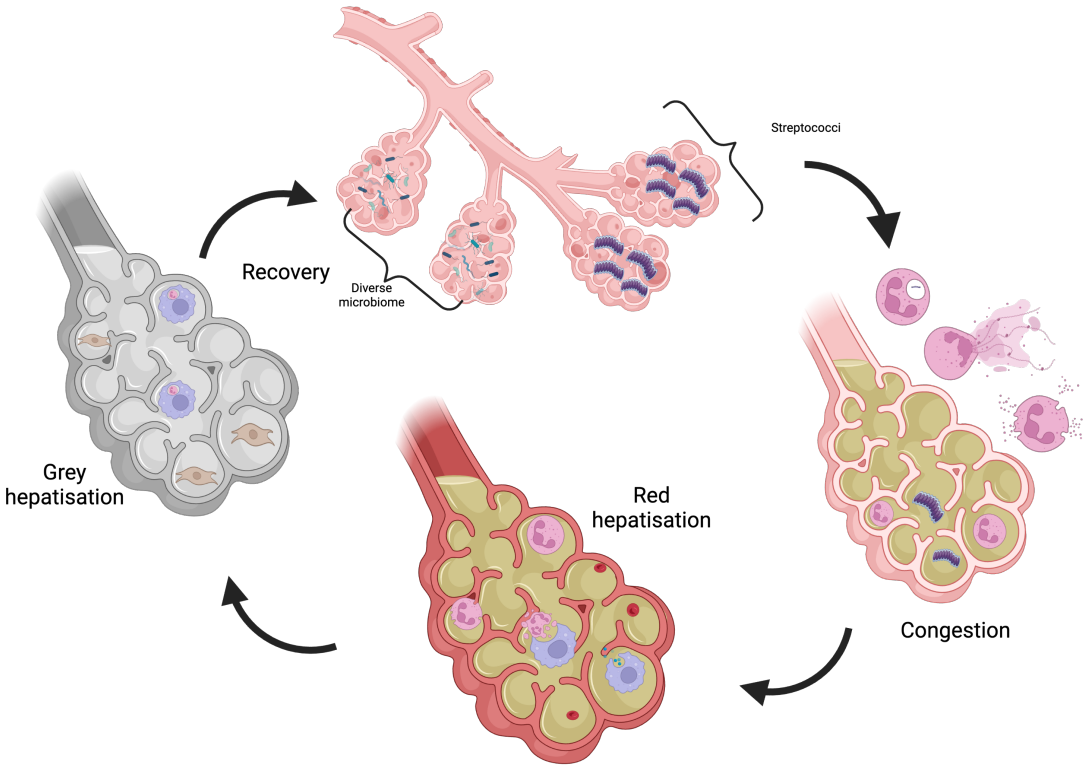

Figure 1-1: Expected Resolution of Pneumonia

In health the pulmonary microbiome is diverse and remains stable. *Streptococcus pneumoniae* is the most common causative organism of pneumonia. When the microbiome is disrupted by a pathogenic organism pneumonia can result. The first stage is termed congestion where the alveolar sac is filled with exudate rich in neutrophils and macrophages which orchestrate the immune response. Following this there is an influx of macrophages, red blood cells and fibrin termed red hepatisation. Macrophages contribute by efferocytosing apoptotic neutrophils and performing phagocytosis. Moving towards resolution there are increasing numbers of macrophages and fibroblasts which work together to clear debris and heal the alveolar sac. Created with BioRender.com.

1.2. Epidemiology

In 2019 there were an estimated 489 million episodes of respiratory tract infection worldwide, which contributed to an estimated 2.49 million deaths and 97 million

disability adjusted life years (Abbafati et al., 2020) . In 2017/8 there were 245,620 episodes of hospitalised CAP in England (Public Health England, 2019) and most deaths occurred in older people (Healthdata.org, 2019). The overall incidence of CAP is 1-1.7/1000 person years, climbing to 14 per 1000 person years in people aged over 65 years (Torres et al., 2013).

Overall, 17% of all CAP episodes require hospitalisation (Theilacker et al., 2021) but in older adults hospitalisation rates are between 52-95% with those over 85 years having the greatest risk of hospitalisation (Cillóniz et al., 2013, 2012; Theilacker et al., 2021). Rates of hospitalisation are increasing (Quan et al., 2016; Grajales Beltrán et al., 2023), although the drivers of increased hospitalisation are unclear.

1.2.1. Short-term Outcomes in CAP

In-patient mortality was 10.4% rising to 13.6% at 30-days, with 22% 30-day mortality in severe CAP in the national BTS CAP audit (W. S. Lim and Lawrence, 2019), this data is in keeping with evidence from similar healthcare systems (Cillóniz et al., 2013). There are multiple factors which influence mortality in CAP, in particular increasing age is associated with significantly higher mortality (Cillóniz et al., 2013). In adults <65 years of age the most socio-economically deprived patients have an increased risk of mortality, but this is not true for older adults (Lawrence, McKeever and Lim, 2022). Older adults with CAP are more likely to develop serious complications such as acute respiratory distress syndrome (ARDS), sepsis and have higher mortality associated with these complications (Martin, Mannino and Moss, 2006). Presence of co-morbidities, especially severe co-morbidities increases mortality risk (Cillóniz et al., 2013). Frailty and inability to complete activities of daily living impacts CAP mortality independent of age (Kosai et al., 2014).

Respiratory failure and sepsis lead to intensive care (ICU) admission in around 5% of all episodes in the UK. Of those admitted to ICU in the UK 15% required invasive mechanical ventilation (IMV), 38% received non-invasive ventilatory support and 21% required cardiovascular support (W. S. Lim and Lawrence, 2019), BTS audit data demonstrates a trend of falling ICU admissions and associated need for organ support. Older adults are less likely to be admitted to ICU, and less likely to receive IMV if admitted (Cillóniz et al., 2013).

Cardiovascular (CVS) complications are common (14-18%) and independently increase mortality risk (Tralhão and Póvoa, 2020; Corica et al., 2023). Incident heart failure and arrythmia are the most common events (Corrales-Medina et al., 2011; Tralhão and Póvoa, 2020). CVS complications are more common in older adults and people with existing co-morbidities (Corrales-Medina et al., 2011).

Admission with CAP is commonly associated with functional impairment at discharge. Risk factors for functional impairment are increased age, dementia, reduced consciousness and CAP severity. Functional impairment at discharge is associated with increased mortality, length of stay (LOS) and higher healthcare costs (Chen et al., 2022)

Ongoing symptoms are common following CAP with 65% of hospitalised adults seeking further healthcare consultation within a month of discharge. Persistent respiratory symptoms and slow recovery were the most common reasons to seek advice (Daniel et al., 2018). At 4-6 weeks days following discharge 70% report ongoing symptoms, the most common symptoms are fatigue (45-72%), cough (35-69%) and dyspnoea (34-67%) (Pick et al., 2019).

There are multiple factors influencing LOS for CAP: increasing age, presence of multimorbidity, multiple clinical parameters indicating more severe disease, discharge to a nursing home and complications during treatment. There is a wide range of inter-hospital and health system variability in LOS, but failure to adhere to treatment guidelines is associated with increased LOS (Welte, Torres and Nathwani, 2012). In the BTS CAP audit median LOS for survivors was five days, LOS is higher in more severe CAP, increased age and has decreased in recent years (Lim and Lawrence, 2019a; Guo et al., 2023).

1.2.2. Long-term outcomes in CAP

People treated for CAP have increased mortality for up to nine years following the episodes compared to matched controls (Koivula, Stén and Mäkelä, 1999). This study and others attribute the increased rate of death due to incident CVS disease in CAP (Koivula, Stén and Mäkelä, 1999; Corrales-Medina et al., 2015). However, other studies have attributed the increased mortality to pre-existing co-morbidities, this is supported by the observation that risk of chronic obstructive pulmonary disease (COPD) or heart failure exacerbations is heightened in the year following an episode of pneumonia (Bornheimer et al., 2017; Wagenvoort et al., 2017).

In addition to the physical health burden, impaired quality of life has been identified in people up to 18 months after an episode of CAP, and this was more pronounced in people who had existing co-morbidities. Respiratory symptoms had mainly resolved by 6 months, but general wellbeing remained poor (El Moussaoui et al., 2006).

30-day readmission following hospitalisation for CAP occurs in 14% and rates of readmission are steadily increasing over time, readmission rates are highest (19%) in those with severe pneumonia (W. S. Lim and Lawrence, 2019). High levels of

deprivation are associated with increased risk of readmission, as are increasing age (Lawrence, McKeever and Lim, 2022). In those readmitted, pneumonia is the leading cause of admission, of which 23% of cases had hospital acquired infection. In people readmitted with pneumonia mortality was significantly higher than those admitted with a non- pneumonia diagnosis (Lawrence, McKeever and Lim, 2023). Episodes of secondary infection are associated with increased length of stay, mortality, and further readmissions (Toledo et al., 2018). Risk factors for hospitalised recurrent pneumonia show significant overlap with those first episodes of CAP (smoking, age, male gender, increasing deprivation and presence of co-morbidities) (Baskaran, Lim and McKeever, 2022).

1.3. Economic burden

In the United Kingdom (UK) there were >250,000 hospital admissions for CAP in 2019, associated with a direct cost of £730 million considering inpatient treatment costs only (Campling et al., 2022). Increasing age, multi-morbidity and severe pneumonia significantly increase CAP costs (Welte, Torres and Nathwani, 2012; Campling et al., 2022; Haessler et al., 2022).

Healthcare utilisation is high following an episode of CAP, 65% of adults have a further healthcare consultation within 28-days of discharge (Daniel et al., 2018) and at 4 weeks following discharge 35% of working participants had not yet returned to work (Daniel et al., 2018).

1.4. Factors influencing CAP susceptibility and outcomes.

There are well-established risk factors that partially explain the high incidence of CAP in older adults. Advanced age alone is a significant risk factor for CAP (Ewig *et al*. 2009) and during ageing the human host and respiratory system undergoes

structural, physiological and pathological changes which can lower resilience to infection, as described in Figure 1-2. Sociodemographic factors such as alcohol abuse, smoking and household overcrowding also influence susceptibility to CAP (Samokhvalov, Irving and Rehm, 2010; Torres et al., 2013; Baskaran, Lim and McKeever, 2022).

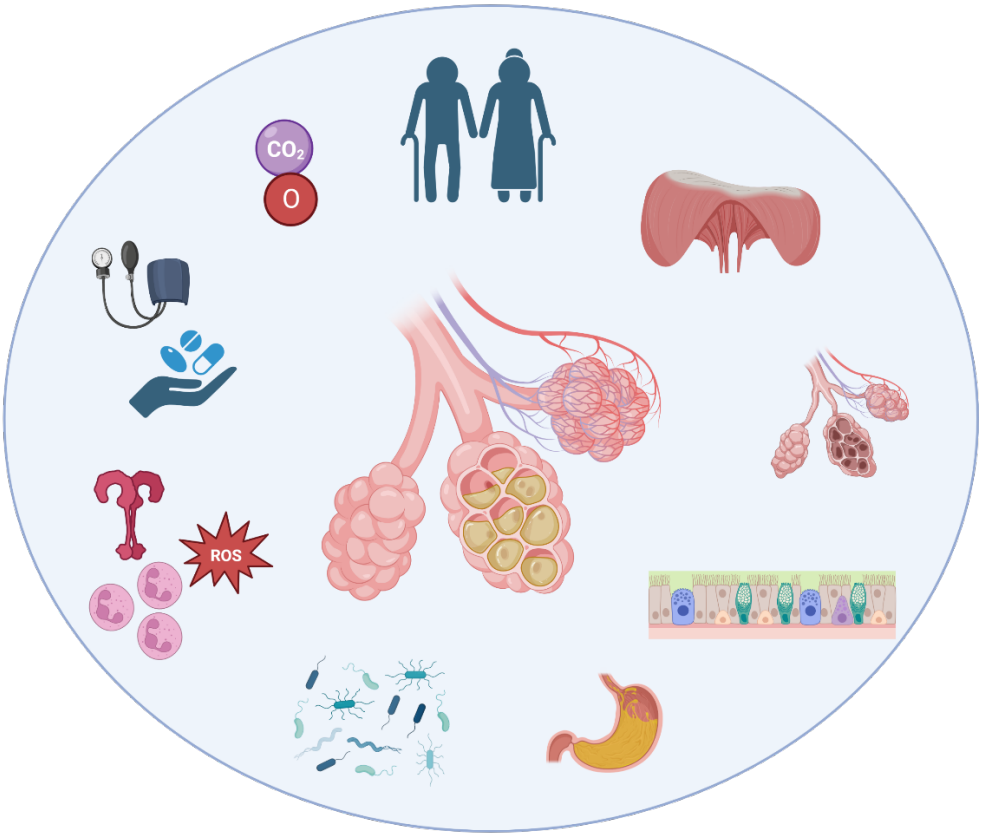

Figure 1-2: Factors Increasing Susceptibility to CAP.

Factors present in older adults (starting at the top and moving clockwise): (1) age alone is associated with an increased burden of CAP(Ewig et al., 2009); (2) the mechanics of ventilation are impaired with age, the thoracic cage is less compliant and the diaphragm is weaker(Mittman C Norris AH, 1965); (3) the lung parenchyma loses elasticity, leading to senile emphysema(Gillooly and Lamb, 1993); (4) the mucociliary escalator is less efficient in older adults, reducing the clearance of bacteria and microparticles from the lung; (5) microaspiration is common (6) the lung microbiome alters with age(Stearns et al., 2015); (7) ageing is associated with a low-grade pulmonary inflammation with increased neutrophilic invasion, increased bacterial receptors and reduced bacterial peptides(Hopp et al., 1985; Meyer et al., 1996; Hinojosa, Boyd and Orihuela, 2009; Boyd and Orihuela, 2011; Moliva et al., 2014); (8) multimorbidity and polypharmacy are common in old age and CAP; and (9) older adults exhibit reduced responsiveness to hypoxia and hypercapnia(Kronenberg and Drage, 1973). Created with BioRender.com, adapted from (Grudzinska et al., 2019b).

Many of the factors which increase susceptibility to CAP also influence outcome in CAP. Presence of co-morbid disease impacts on both risk of CAP and outcomes following CAP. Co-morbidities are common in CAP, especially in older adults where 80% have at least one co-morbidity (Welte, Torres and Nathwani, 2012; Cillóniz et al., 2013; Jain et al., 2015). The most common co-morbidities are respiratory disease (68%), cardiovascular disease (47%), diabetes mellitus (33%), stroke (33%) and dementia (33%) (Cillóniz et al., 2013; Welte et al., 2012; Jain et al., 2015). One third of people hospitalised with CAP have had a prior episode of CAP (Torres et al., 2013). Frailty is associated with increasing susceptibility but also risk of hospitalisation and poor outcomes (Iwai-Saito et al., 2021).

Polypharmacy is common in older adults and is associated with increased mortality in CAP (Maher, Hanlon and Hajjar, 2014), although this an association rather than causation, it may inform that people who take high numbers of medications are more complex (increased number +/- severity of co-morbidities) and therefore have increased risk of poor outcome.

Older adults are often more challenging to diagnose with CAP. They commonly present late with atypical features such as delirium and reduced mobility while lacking classical signs and symptoms of pneumonia such as fever and cough with less elevated inflammatory markers (Metlay et al., 1997; Bruunsgaard et al., 1999; Guo et al., 2023). This may lead to delayed diagnosis and implementation of treatment which has been demonstrated to be associated with adverse outcomes (Lim et al., 2003)

The host immune response to infection also changes with age and frailty; termed immunosenescence. Immunosenescence is discussed further in Chapter 1.8.

1.5. Aetiology of CAP

CAP can be caused by bacteria, viruses and fungi. The leading causative organism in Europe is *Streptococcus pneumoniae (S. pneumoniae)* identified in between 11-68% cases of CAP, other common causative agents are *Haemophillus influenzae*, *Staphylococcus aureus*, Gram negative bacilli and viral causes (Table 1-2). Although even in prospective studies aiming to identify a causative pathogen, 35-60% of cases have no pathogen identified (Welte, Torres and Nathwani, 2012). In older adults a causative organism is less likely to be identified and viral infections are more common in adults >85 years compared to 65-75, but no other significant differences in pathogen burden according to age have been reported (Cillóniz et al., 2013; Jain et al., 2015; Haessler et al., 2022). The reduced ability to detect pathogens in older adults may be due to the challenges of sputum sampling in older adults, where in a real-world study only 36% of participants were able to produce a sputum sample, and this was significantly less likely in older adults (Ewig et al., 2002).

A microbiological cause was more likely to be identified in patients with severe CAP than non-severe in a retrospective US study. Respiratory cultures were positive in 34% of severe compared to 21% of non-severe, and blood cultures positive in 10% of severe compared to 5% of non-severe (Haessler et al., 2022) however this may reflect sampling bias in severe cases. Respiratory samples from severe CAP were more likely to isolate pathogens with resistance to typical CAP antimicrobials (Haessler et al., 2022).

Molecular testing such as BioFire array may have an important role in patients who have received anti-microbials prior to hospitalisation where traditional culture is limited (Niederman and Torres, 2022). Molecular testing is also much faster than

traditional culture techniques, which may offer to the opportunity to rapidly rationalise antibiotics to avoid under/overtreatment. These tests are typically polymerase chain reaction (PCR) based tests to detect pathogen genomic material to inform semi-quantitative bacterial identification, common viruses and some antimicrobial resistance genes using sputum, endotracheal aspirates or bronchoalveolar lavage (BioFire, 2022).

Table 1-2: Aetiology of Hospitalised CAP in Europe

Causative pathogen	Frequency (%)
Bacterial pathogens	0-47
Streptococcus pneumoniae	11-68
Haemophilus influenza	5.3-12.3
Legionella pneumophilia	0-12.8
Staphylococcus aureus	0- 11.8
Moraxella catarrhalis	0-5.4
Gram negative bacteria	0-24.2
Mycoplasma pneumoniae	0.7-32
Chlamydia pneumoniae	1-26.5
Viruses	0-34
Influenza viruses	6-15
Rhinoviruses	9-12
Respiratory Syncytial Virus	3
Parainfluenza	3
Metapneumovirus	3-4
Adenoviruses	2
Bacterial-viral co-infection	23-31
No pathogen identified	35-62

The most common causative organisms for CAP are listed above with frequency expressed as a percentage. Adapted from (Lim et al., 2001; Ewig et al., 2009; Welte and Kohnlein, 2009; Torres et al., 2014; Holter et al., 2015; Jain et al., 2015)

1.6. Changing epidemiology due to COVID-19

In late 2019 a novel coronavirus emerged in northern China. Over the following months COVID-19 caused a worldwide healthcare emergency and was declared a pandemic in March 2020 by the World Health Organisation (WHO). In the first months of the pandemic the lack of directed therapies or vaccination mandated public health approach of lockdowns and social distancing to limit transmission and avoid

overload of healthcare services while protecting the most vulnerable. Throughout 2020-2022 social distancing guidelines varied, but older adults and people with co-morbidities were shielded and took precautionary measures. This group represent some of the highest risk for acquisition of CAP. As these measures protected against most respiratory pathogens it was anticipated that the epidemiology of non-COVID-19 respiratory infections would change. In addition, delivery of planned healthcare was interrupted, this may have influenced disease (co-morbidity) control and therefore risk of CAP. In early 2020 non-COVID-19 hospital admissions fell by 50% (Riley et al., 2020). This pattern has been replicated in both adult and paediatric populations across the world. (Yeoh et al., 2021; Zhang, Cao and Meng, 2022).

1.7. CAP Diagnosis and Management

1.7.1. Clinical Diagnosis

CAP is a clinical diagnosis, where according to the BTS definition patients must have a combination of clinical signs, symptoms, laboratory, and radiological findings to meet the CAP definition (Lim et al., 2009). However, CAP can be challenging to diagnose, especially in older adults who present atypically (Metlay et al., 1997). CAP is a leading cause of sepsis, which is defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection"(Seymour et al., 2016).

Signs and symptoms

The BTS definition uses the following signs and symptoms: "cough with at least one other lower respiratory tract symptom, new focal chest signs on examination and at least one systemic feature such as fever, sweats, shivers" (Lim et al., 2009). A meta-analysis of the predictive accuracy of signs and symptoms to diagnose CAP (where radiological CAP was used as the reference) demonstrated that individual signs and

symptoms were unhelpful, but overall clinical impression was the best predictor (Ebell et al., 2020). The most predictive clinical symptoms and signs are in Table 1-3. Interestingly cough and purulent sputum were not significant predictors of a radiological diagnosis of CAP, despite being among the most common symptoms quoted in guidance (NICE, 2014; BMJ Best Practice, 2023).

Table 1-3: Diagnostic accuracy of clinical signs and symptoms in CAP.

Feature	Diagnostic Odds Ratio (95% CI)
Overall clinical impression	11.5 (6.7-18.5)
Symptoms	
Fever/chills	2.1 (1.48-2.87)
Absence of coryzal symptoms	2.07 (1.31-3.13)
Dyspnoea	1.75 (1.28-2.34)
Chest pain	1.41 (1.13-1/74)
Signs	
Egophony	6.46 (1.36-18.9)
Any abnormal chest sign	3.18 (1.83-2.08)
Any abnormal vital sign	6.01 (3.03-10.6)

CI: confidence interval

Laboratory investigations

BTS guideline recommends the following laboratory investigations on admission to hospital: arterial blood gas if hypoxaemic, urea and electrolytes, C-reactive protein (CRP), full blood count and liver function tests (Lim et al., 2009). CRP and procalcitonin (PCT) have been demonstrated to be the most accurate predictors of CAP diagnosis, where PCT is also able to predict severity of CAP (Müller et al., 2007). People with CAP experience high rates of acute kidney injury (17%) (Akram et al., 2010).

Radiological investigations

Chest radiographs (CXR) have long been used as the reference standard for diagnosis in CAP, but thoracic imaging is not infallible. The sensitivity of CXR to detect pneumonia is between 32-82% (Self et al., 2013; Bourcier et al., 2014; Karimi, 2019). The studies testing accuracy of CXRs use computed tomography (CT) imaging as gold standard, but also importantly use radiologist interpretation rather than bedside clinician interpretation, inter-observer variability in reporting of plain CXRs is significant (Hopstaken et al., 2004). Despite the clear utility of CT imaging in diagnosis of CAP it's practical use has been limited by concerns over radiation exposure and increased cost (Kroft et al., 2019). A randomised control trial (RCT) of ultra-low dose CT versus CXR in diagnosis of pulmonary disease in the emergency department demonstrated increased diagnosis of CAP in the emergency department, but this had no effect on clinical management, outcomes, or health status at 28 days. They also showed increased rates of incidental findings (of no clinical significance), and increased radiologist burden in the CT group (van den Berk et al., 2023).

The role of artificial intelligence (AI) has been investigated in improving the sensitivity of CXR in diagnosis of CAP (Kermany et al., 2018; Becker et al., 2022), and AI has been used to develop prognostic indicators from CXRs for CAP outcomes (Quah et al., 2021). In time, and with refinement of process AI is likely to play role in CXR reporting (Oren, Gersh and Bhatt, 2020).

1.7.2. Severity assessment in CAP

CAP guidelines advocate for early severity assessment using CURB65 or pneumonia severity index (PSI) (Lim et al., 2009; NICE, 2014; Martin-Loeches et al., 2023). Severity assessment results should be used to inform clinical decision making around

antimicrobials and place of treatment, certainly there is evidence that timely antibiotics and early consideration of mechanical ventilation compared to late intervention impact outcomes (Delclaux et al., 2000; Priya Daniel et al., 2016). Documentation of severity assessment is also used as a marker of care quality in the BTS CAP audit (W. S. Lim and Lawrence, 2019).

In clinical practice severity scoring is infrequently completed (5.2%) (Karmakar and Wilsher, 2010) and even when completed does not clearly impact outcome (Baker et al., 2022), however, use of CURB65 score has been shown to reduce inappropriate antimicrobial use (Chalmers et al., 2011).

Prognosis prediction is helpful to support clinical decision making around place of treatment and rationalising antimicrobials but can also be helpful in discussing prognosis with patients and their families to improve communication and supportive care. Severity assessment tools also benefit clinical research by accurate stratification at an early stage.

Commonly used tools in current practice were designed to be easy to use and remember in an era before widespread use of smartphones and paper-free hospitals. With this computational power in mind there is a clear opportunity to develop severity assessment tools that have greater discrimination and consider other outcomes.

1.7.3. Management of CAP

Vaccination to prevent CAP.

There are national vaccination programmes against common CAP pathogens: *S. pneumoniae*, influenza, and COVID-19. Vaccination was the success story of the COVID-19 pandemic. Over 90% of eligible people in the UK have received a dose of a COVID-19 vaccination (Office for National Statistics, 2023). In 2022 82.3% and

70.6% of eligible adults received influenza and pneumococcal vaccinations respectively (UK Health Security Agency, 2021, 2023a).

Vaccination can prevent infection, but the predominant effect is to prevent severe disease including hospitalisation. The pneumococcal vaccination programme is the most established and has the most complete data regarding responses in older adults. In the UK adults ≥65 years are offered pneumococcal polysaccharide vaccine-23 (PPV23), pneumococcal vaccination is also offered to adults with long-term health conditions regardless of age. In these groups PPV23 is offered, however in those with asplenia or significant immunosuppression pneumococcal conjugate vaccine (PCV13/15) is indicated (Health Security Agency, 2023).

Antibody responses to PPV23 have been investigated in older adults, where differential antibody titres were detected according to age and sex. Men had higher antibody titres, and antibody titre fell in both sexes with increased age, however, this study only assessed antibody response at a median of 35 days following vaccination (Sankilampi et al., 1996), and despite the differences shown most demonstrated an acceptable response to vaccination. Ridda *et al* investigated PPV23 response in frail older adults at 6 months following vaccination and identified that frailty but not age was predictive of poor antibody response, as was female sex. There was some variation in response according to pneumococcal serotype (Ridda et al., 2009). However, an optimal antibody response has not been well defined in older adults, and these studies are conducted over short time frames, limiting the real-world application of this data.

The clinical efficacy and duration of protection incurred from PPV23 in older adults is controversial with very limited RCT data, and observational studies offerings subtly

different outcomes. A study using routine primary care data demonstrated that PPV23 was effective at reducing pneumococcal CAP for two years following vaccination, with the greatest protection for adults ≥80 years compared to ≤65years, however this study contained no data on serotype and relied on ICD coding for diagnosis (Streeter et al., 2022). Crude vaccine efficacy (VE) against hospitalised PPV23 serotype pneumococcal CAP has been estimated to be 23%, this study did not show any significant impact of age or time since vaccination on vaccine efficacy, although did show a trend to reduced efficacy in those ≥75 years but not reaching statistical significance (Lawrence et al., 2020). However, an observational study of older adults demonstrated a significant protective effect against CAP in older adults who had received vaccination in the previous five years, but no protection beyond that (Ochoa-Gondar et al., 2014). Waning of PPV23 protection after 5 years is further supported from US data where vaccination ≥5 years ago increased risk of hospitalised CAP in people ≥65 compared those vaccinated ≤5 years ago (Hsiao et al., 2022). Together these data suggest that the long-term efficacy of PCV23 in older adults may wane over time, but lack of involvement of frail older adults in RCTs of pneumococcal vaccination have limited the conclusions that can be drawn.

Seasonal influenza vaccination is offered to adults ≥65 years and those in clinical risk groups. Given the seasonal variation in circulated influenza strains, each year the WHO makes recommendations on which strains should be used for vaccines. Influenza vaccines used in the UK are quadrivalent containing two Influenza A strains and two Influenza B strains (UKHSA, 2022). Given the seasonal variation in vaccine components overall VE is hard to estimate, ranging from -3 to 78% between 2010-2017 in adults ≥65 years. Overall, VE against Influenza A/B across the entire study

period was significantly lower in adults ≥75 years (Pebody et al., 2018). These data suggest that influenza vaccination is not effective at preventing influenza infection in older adults, however, there are data suggesting that vaccination reduces hospitalisation and mortality (when well matched to circulating strains) in older adults (Nichol et al., 2007).

COVID-19 vaccination was introduced to the UK in late 2020, where it was introduced in a stepwise fashion to those most at risk of severe disease- the oldest old and those with high-risk medical conditions. COVID-19 vaccines have demonstrated exceptionally high VEs, with VE of 88% reported after three doses (Public Health England, 2023). A meta-analysis of the short-term immunogenicity of a wide range of COVID-19 vaccinations identified no difference between responses in those <60 years and ≥60 years (Zhang et al., 2022). The clinical efficacy has been explored in a systematic review, where COVID-19 vaccination in older adults reduced rates of infection and subsequent death from infection, but no benefit was seen in hospitalisation or ICU admission in adults ≥60 years (Xu et al., 2023). This was an international study encompassing a wide range of vaccines, and eligibility criteria in terms of age. UK based studies have demonstrated benefits in hospitalisation for older adults, and older adults living in long-term care facilities (Hyams et al., 2021; Shrotri et al., 2021). It is important to note that the VEs reported for COVID-19 vaccines are likely to have been impacted by social distancing, where older adults were especially advised to take precautions and care home visits etc were strictly controlled, thus limiting exposure to virus.

Vaccination against common causes of CAP is recommended for older adults, however there are limited high quality RCTs which recruited frail older adults

including those who reside in long-term care facilities: the population most at risk of CAP and severe outcomes.

Treatment

CAP guidelines predominantly focus on use of anti-microbials with limited evidence for other interventions. BTS, NICE and ERS guidelines advocate for severity assessment and then anti-microbial choice dependent on severity of infection with local pathogens and resistance patterns considered (Lim et al., 2009; NICE, 2014; Martin-Loeches et al., 2023). Empirical antibiotics are recommended for seven days, with consideration for ten days in high severity or in presence of complications (Lim et al., 2009).

Recently the ERS severe CAP guidelines have endorsed the use of steroids in severe CAP (Martin-Loeches et al., 2023). This is primarily supported by the CAPE COD trial where early hydrocortisone treatment in severe CAP without septic shock demonstrated a mortality benefit in addition to reduced length of stay reduced duration of IMV (Dequin et al., 2023). Steroids are widely used in refractory septic shock regardless of aetiology (Evans et al., 2021). The history of steroid trials in CAP demonstrates the importance of clearly defining the risk population, there have been signals of benefit according to sex and inflammatory markers (Metlay and Waterer, 2023). Prior to the CAPE COD study the largest study was a US study based in a veteran's hospital where 96% participants were male. Participants were randomised to methylprednisolone (40mg tapering dose) within 96 hours of meeting the eligibility criteria. They did not demonstrate any benefit in 60-day mortality (primary outcome), or any secondary outcomes (Meduri et al., 2022). Important differences in these studies are:

1. Timing of intervention: in the Dequin study all participants received the study drug within 24h of meeting criteria (median 15 hours), whereas in the Meduri study the median time to start treatment was 40 hours.
2. Few women were enrolled in the Meduri study (4%), whereas Dequin enrolled ~30% women and in a secondary analysis demonstrated increased benefit in women compared to men.
3. Influenza: Dequin excluded cases with influenza, whereas in the Meduri study 10% had influenza.
4. Subgroup analysis of the Dequin study demonstrated increased benefit in those ≥65 years, increased benefit was also seen in those with CRP >150mg/L, those not receiving mechanical ventilation, and in whom no pathogen had been identified.

Sex differences in steroid response have been seen in septic shock studies (Thompson et al., 2021), although the effect was in the opposite direction. The CAPE COD study has shown that early intervention in those with very elevated CRP are likely to benefit most.

Respiratory failure is common in CAP and failure to maintain PaO2 ≥8kPa has been used as an indicator for critical care. If refractory respiratory failure is present IMV is recommended as use of continuous positive airway pressure (CPAP) has been associated with increased mortality (Delclaux et al., 2000), some groups advocate for a trial of CPAP in a highly monitored environment with early intubation if failure to improve (Martin-Loeches et al., 2023). Management of acute hypoxaemic respiratory failure (AHRF) has changed following the COVID-19 pandemic where ward-based CPAP and high flow nasal oxygen therapies were used to treat patients who would

have been traditionally managed with IMV in critical care units (Perkins et al., 2022). These changes were borne out of necessity but suggest that management of AHRF in CAP should be revisited, although with caution as early COVID-19 displayed a unique pattern of organ failure in a different cohort of patients.

Evidence regarding supportive care in CAP such as nutrition, intravenous fluids, mobilisation/physiotherapy is limited and often based on expert opinion and extrapolation (Lim et al., 2009; NICE, 2014).

1.8. Immunosenescence and relevance to CAP

Immunosenescence is age associated immune dysfunction, thought to occur due to four central causes of tissue damage: genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis (Weiskopf, Weinberger and Grubeck-Loebenstein, 2009). The clinical impact of immunosenescence can be observed in the burden of infectious disease in older adults. The cellular features of immunosenescence are outlined in Table 1-4. Alongside immunosenescence the concept of “inflamm-ageing” (Franceschi et al., 2000) underpins the principle that older adults experience constant low-grade inflammation caused by the same four causes of age-associated damage leaving their immune system continuously activated. This has been demonstrated by elevated levels of pro-inflammatory cytokines in older adults which correlate with mortality (Kim et al., 2011; Adriaensen et al., 2014; Puzianowska-Kuznicka et al., 2016).

Table 1-4: Cellular Features of Immunosenescence

Neutrophils - Increased incidence of neutropenia**(Chatta et al., 1993)** - Altered cytokine production **(Weiskopf, Weinberger and Grubeck-Loebenstein, 2009)** - Impaired migration **(Sapey et al., 2017)** - Reduced pathogen killing ability**(Hazeldine et al., 2014)** - Increased apoptosis **(Fulop Jr. et al., 1985)**
Macrophages/ Monocytes - Reduced phagocytosis and production of free radicals **(Gomez et al., 2008)** - Possible reduced efferocytosis **(Albright et al., 2016)** - Decreased ability to antigen present due to reduced expression of MHC class II **(Villanueva et al., 1990)**
Dendritic Cells Function maintained in healthy older adults **(Lung et al., 2000)**, however impaired in frail older adults **(Uyemura, Castle and Makinodan, 2002)**
Natural Killer Cells - Increased numbers but reduced cytotoxicity **(Le Garff-Tavernier et al., 2010)**
Adaptive Immunity (Martín, Pérez and Aldecoa, 2017) - Reduced numbers of naïve T cells - T cell exhaustion - Decreased capacity to respond to novel antigens**.** - Lower affinity antibodies - Reduced numbers of B cells

MHC: major histocompatibility complex. Taken from (Grudzinska et al., 2019b).

1.9. Neutrophil Responses

1.9.1. Classical neutrophil response

Neutrophils are among the first line effector cells to be recruited in inflammation, they also have a role in the resolution of inflammation and angiogenesis; and thus, require significant plasticity (Kubes, 2018). The importance of neutrophils in infection and inflammation is demonstrated clinically by neutropenia or specific congenital neutrophil deficits which predispose to severe, recurrent, and potentially fatal infections (Tamayo et al., 2012) but also by conditions where neutrophils responses

are poorly contained, such as Alpha 1 Anti-trypsin deficiency, where a functional lack of anti-proteinase can cause severe early onset emphysema.

Neutrophils are generated in the bone marrow and retained by high expression of CXCR4 a marker of immaturity (Janicova and Relja, 2021). Granulocyte colony stimulating factor (G-CSF) supresses CXCR4 and therefore promotes extravasation of neutrophils from the bone marrow (Hyun et al., 2006). Figure 1-3 provides a schematic overview of major neutrophil effector functions.

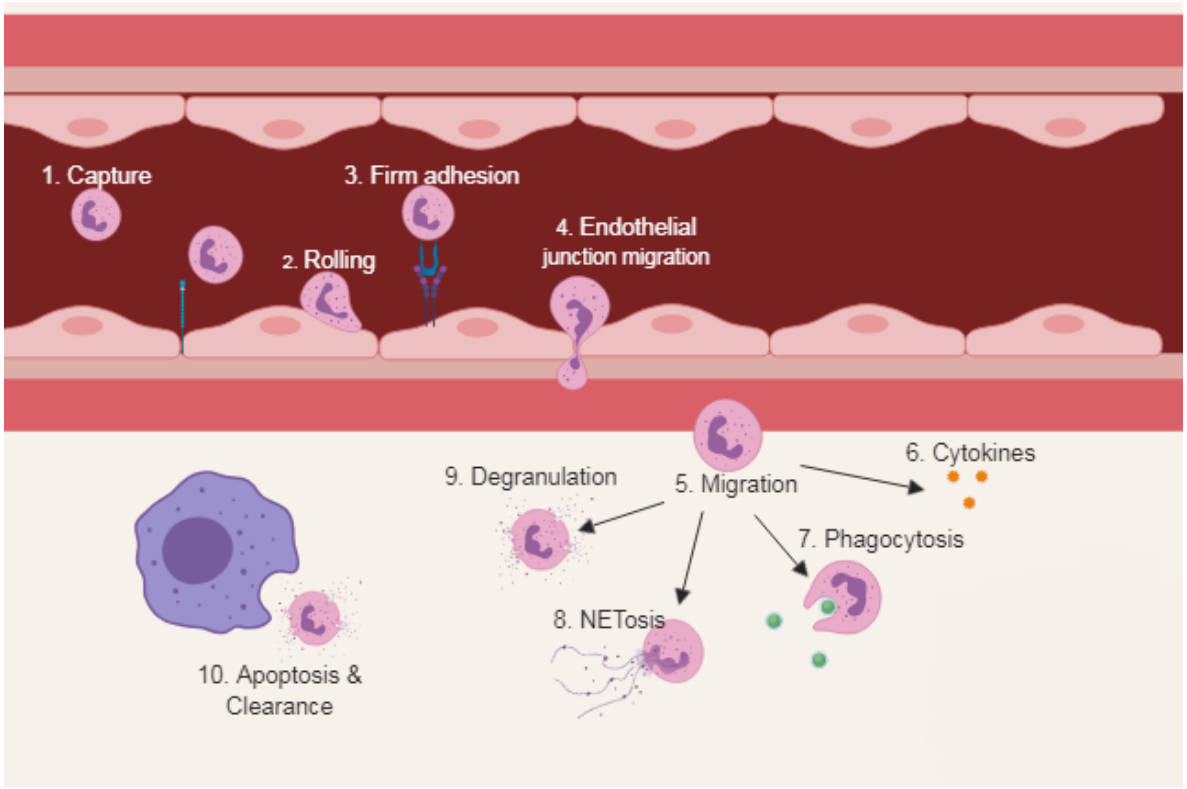

Figure 1-3: Classical Neutrophil Functions

1) Neutrophils actively patrol the circulation, they can detect host and pathogen derived inflammatory signals via interaction with endothelial cells, 2) neutrophils become tethered and roll along the endothelial wall in a process mediated by selectins, 3) activation of integrins causes firm adhesion and then 4) cytoskeletal rearrangement allows migration through the endothelial junction. 5) Once in the interstium neutrophils are exposed to a medley of chemoattractants and begin to migrate to the site of injury, 6) neutrophils secrete a range of cytokines which participate in orchestrating the immune response, 7) bacterial killing is achieved through phagocytosis, exposing entrapped bacteria to anti-microbial proteins and reactive oxygen species (ROS) within the phagolysosome, 8) NETosis is a process by which decondensed chromatin is extruded into the extracellular space covered with an array of neutrophil-derived anti-microbial protein, 9) To migrate through dense extracellular matrices, neutrophils utilise granules containing high concentrations of proteinases. 10) Clearance of an apoptotic neutrophil by a macrophage. Once an infection is contained, apoptosis and clearance of apoptotic neutrophils are central to the resolution of inflammation. Persistence of a pro-inflammatory neutrophilic response is associated with greater tissue damage. Created with BioRender.com, taken from (Grudzinska et al., 2019b).

1.9.2. Neutrophil priming

Neutrophils have the potential to cause significant tissue damage due to their highly cytotoxic contents and therefore are maintained in three main states: quiescent, primed and activated. Neutrophil priming is defined as exposure to primary agonist which enhances response to a secondary agonist (Haslett et al., 1985). Neutrophil priming was initially identified *in-vitro* (Haslett et al., 1985), but was quickly also

identified in patients with sepsis (Bass et al., 1986). Neutrophil priming *in-vivo* is challenging to study due to the wide array of potential agents and broad mechanisms engaged.

Priming impacts neutrophil rheology, adhesion markers, morphology, and survival (Vogt et al., 2018). These changes therefore have direct influence on endothelial transit, migration, effector functions and apoptosis. Neutrophil priming is not a binary outcome, but more a spectrum with the response driven by the combination, timing, concentration, and duration of exposure to priming agents.

Neutrophil priming is a reversible process, however there is limited understanding of this. Reversal of priming has been demonstrated in humans; *ex-vivo* neutrophils primed with G-CSF were returned to the circulation, these neutrophils are trapped in the pulmonary circulation initially but then gradually return to the systemic circulation (Summers et al., 2014) this observation has been further evidenced by mechanical deformation studies using *ex-vivo* neutrophils, showing that neutrophils are mechanically sensitive, and deformation can cause both priming and depriming (Ekpenyong et al., 2017). Priming is thought to exist as a safety mechanism to prevent inadvertent tissue damage, but in lung disease such as CAP de-priming in the lungs is impaired, potentially leading to the sustained activation of neutrophils with resulting widespread activation of the endothelium (Summers et al., 2014).

1.9.3. Neutrophil migration

Once trafficked from the bone marrow, neutrophils can detect bacterial, and host derived inflammatory signals through interactions with endothelial cells. In larger blood vessels, such as in the bronchial circulation, neutrophils become tethered and roll along the endothelium in the direction of blood flow in a process mediated by

selectins (Ley et al., 2007). Activation of β_2 integrins causes firm adhesion and at this point cytoskeleton rearrangement allows the process of migration through the endothelial junction. In the pulmonary circulation, neutrophils traverse narrow capillary beds largely independent of selectins (Wagner and Roth, 2000). Due to the narrow diameter of the pulmonary microcirculation neutrophils are required to elongate rather than roll (Doerschuk et al., 1993) but this mechanical stress and cytoskeletal rearrangement only slightly slows their passage through the pulmonary circulation (Summers et al., 2014). Once in the interstitium neutrophils are exposed to an inflammatory medley of chemoattractants and bacterial products and they begin a process of migration. During migration neutrophils follow complex and shallow chemoattractant gradients by amplifying small external signals through intracellular pathways. Chemotaxis is the guided cell movement towards a chemical signal at a distant site according to a concentration gradient (Petri and Sanz, 2018).

There are four main groups of chemoattractants which can be grouped as intermediate or end targets. Intermediate chemoattractants are lipid factors and chemokines, end target chemoattractants are N-Formylmethionine-leucyl-phenylalanine (fMLP) and complement factors.

Signalling pathways following induction of intermediate or end targets are distinct; end targets signal through phospholipase A2 and or MAP kinase p38, whereas intermediate signals track through PI3K (Heit et al., 2008).

This thesis discusses migration towards CXCL8 (IL8) a chemokine, therefore further discussion will focus on chemokines and related pathways.

Chemokines are low molecular weight cytokines which can be grouped as CCL or CXC with their receptors termed CCR and CXCR respectively (Rajarathnam et al.,

2019), however there is evidence of promiscuity between receptors and their ligands. The CXC group is divided into chemokines which express the glutamate-leucine-arginine (ELR) motif and those which lack the ELR motif. CXCIL1-3 and 5-8 express the ELR motif, are most relevant to neutrophil migration and bind the CXCR1 and CXCR2 receptors (Baggiolini, 2001; Weathington et al., 2006; Sanz and Kubes, 2012).

There is diversity in receptor and ligand affinity and downstream signalling. Chemokines have similar affinity for CXCR2, whereas IL8 has the highest affinity for CXCR1 (Jones et al., 1996a). There is further bias in signalling dependent on the receptor/ligand combination, where for example IL8 binding to CXCR1 but not CXCR2 primes for oxidative burst (Jones et al., 1996a).

As discussed, regarding priming neutrophils have safety mechanisms to limit unnecessary tissue damage by migration. Chemokines can be nitrated by peroxynitrate a reactive oxygen species generated by neutrophils. Nitrated IL8 is an ineffective chemokine compared to IL8, and nitrated IL8 can be identified in bronchoalveolar lavage from patients with ventilator acquired pneumonia but not controls (Thompson et al., 2023). These data suggest that neutrophils have effective inbuilt mechanisms to limit inflammation.

1.9.4. Degranulation

Neutrophils contain four types of granules, named in the order in which they are formed: azurophilic (primary), specific (secondary), gelatinase (tertiary) and secretory granules. Primary and secondary granules contain a wealth of bactericidal proteins including myeloperoxidase (MPO), proteinases, defensins and NADPH oxidase which form ROS. These degranulate at the site of injury while tertiary and secretory

granules (which contain gelatinases and collagenases and form a store of surface membrane-bound receptors) assist with mobilisation through the tissues (Amulic et al., 2012). To enable migration through dense extracellular matrices, neutrophils utilise granules containing high concentrations of proteinases such as neutrophil elastase (NE) and proteinase 3 (PR3). NE and PR3 have significant proteolytic activity and the radius of tissue damage is up to eight times greater than that of the granule itself for NE (Liou and Campbell, 1996) and even more for PR3 (Crisford, Sapey and Stockley, 2018)

1.9.5. Reactive Oxygen Species

Reactive oxygen species (ROS) vary in their stability, reactivity, and membrane permeability (Hampton, Kettle and Winterbourn, 1998), however despite this diversity, all ROS are capable of influencing signalling pathways and have direct antimicrobial and cytotoxic effects. The nicotinamide adenine dinucleotide + hydrogen (NADH) oxidase machinery in the plasma and phagosomal membranes starts the ROS cascade by reducing molecular oxygen to superoxide, which is a weak oxidant, but can reacts with other molecules in the inflammatory environment to generate stronger oxidants. Superoxide rapidly dismutates to hydrogen peroxide which can react with MPO in the phagosome to produce other reactive species (Amulic et al., 2012). MPO catalyses the formation of hypochloric acid from hydrogen peroxide and chloride. Hypochloric acid is strongly anti-microbial and thought to be the major contributor to the anti-microbial effect of reactive oxygen species(Winterbourn et al., 2006).

1.9.6. Neutrophil extracellular traps

Neutrophil extracellular traps (NETS) are decondensed chromatin extruded into the extracellular space covered with an array of neutrophil-derived anti-microbial proteins

(Brinkmann et al., 2004). NETs are thought to entrap bacteria while exposing them to high concentrations of anti-microbial peptides, but their release also has been associated with tissue damage from these toxic proteins. NETosis can occur by divergent pathways dependent on stimuli. Both nitric oxide (NOX) dependent and independent pathways have been described (Fuchs et al., 2007; Kenny et al., 2017). NETosis also requires actin polymerisation (Sprenkeler et al., 2022). Neutrophils from donors with actin polymerisation defects but normal ROS and degranulation are unable to generate NETs to a wide range of stimuli, and pharmacological inhibition of actin polymerisation in *ex-vivo* neutrophils also prevent NETosis (Sprenkeler et al., 2022; Kuhns et al., 2016).

1.9.7. Phagocytosis

Bacterial killing is achieved through phagocytosis, exposing entrapped bacteria to anti-microbial proteins and ROS within the phagolysosome. Phagocytosis is a key effector of neutrophil function and can occur via two pathways; direct interaction of a pathogen recognition receptor with a pathogen associated molecular pattern or via indirect interaction with a pathogen via an opsonin (Amulic et al., 2012). The mechanisms of phagocytosis are best characterised for Fcγ receptors for opsonised particles. There is increasing awareness of other receptors which engage phagocytosis, although there is significant overlap in the downstream signalling. CD13 mediated phagocytosis has been shown to occur at similar levels to Fcγ mediated phagocytosis. Using sheep erythrocytes with labelled with specific antibodies to CD13 Perez-Figueroa *et al*. demonstrated that neutrophil phagocytosis with subsequent ROS generation and NETosis mediated by CD13 occurred at similar levels to Fcγ mediated phagocytosis. CD13 mediated phagocytosis was mediated

through actin rearrangement and pan PI3K inhibition or cytochalasin D inhibited phagocytosis in both CD13 and Fcγ mediated responses demonstrating significant overlap in downstream signalling but highlighting the diverse array of neutrophil receptors and responses to pathogens (Pérez-Figueroa, Álvarez-Carrasco and Ortega, 2022).

1.9.8. Reverse transmigration

Reverse transmigration is return of activated neutrophils to the circulation rather than apoptosis and efferocytosis at the site of inflammation. Neutrophils defined to have reverse transmigrated ($CD54^{low}/CXCR1^{high}$) have been detected *ex-vivo* in human samples at low levels in health but at increased levels in inflammatory disease (Buckley et al., 2006). *In-vitro* models of neutrophil reverse transmigration have demonstrated phenotypic changes with increased ROS generation in response to fMLP (Buckley et al., 2006). However, the phenotypic change has not been replicated effectively in humans. These neutrophils are likely to be efficiently removed by the reticulo-endothelial cell systems of spleen and liver, but when combined with the reduced deformability of neutrophils in infection or inflammation this suggests these pro-inflammatory neutrophils may reverse migrate and drive distant organ dysfunction.

1.9.9. Apoptosis

Once an infection is contained, apoptosis and clearance of apoptotic neutrophils are central to the resolution of inflammation. Persistence of a pro-inflammatory neutrophilic response is associated with greater tissue damage (Butcher et al., 2003; Sadiku et al., 2021) and adverse outcomes in acute lung injury (Ware and Matthay, 2000). Neutrophil apoptosis can be delayed by interwoven environmental (such as

hypoxia), host (the presence of certain diseases such as severe asthma) and pathogen related (as seen in *Legionella pneumophilia* infections) factors (Fulop Jr. et al., 2002; Walmsley et al., 2005, 2011). Apoptotic cells are typically removed from the lung by sputum clearance and by efferocytosis (phagocytosis of neutrophils by macrophages). Failure of either of these mechanisms leads to ongoing inflammation and secondary necrosis (Amulic et al., 2012). Apoptotic neutrophils amplify anti-inflammatory signals by inhibiting further neutrophil recruitment to the area of infection and efferocytosis of apoptotic neutrophils by macrophages induces macrophages to adopt an anti-inflammatory phenotype to support inflammation resolution (Kennedy and DeLeo, 2009). Neutrophils also participate in resolution of inflammation by switching synthesis of lipid mediators from pro-inflammatory such as leukotrienes and prostaglandins in early inflammation to pro-resolving lipid mediators which can inhibit neutrophil functions (Krishnamoorthy et al., 2010). Pro-repair subsets of neutrophils have been identified in murine and human models (Massena et al., 2015).

Neutrophils are effective and efficient leukocytes; they have plasticity of response to a wide array of environments and stimuli. Due to their potentially toxic contents, they have multiple in-built safety measures to prevent inadvertent tissue damage. Neutrophils must demonstrate ultimate restraint with a professional and rapid approach to danger but also display selectivity and rapid de-escalation when appropriate. Neutrophil responses in ageing and infection are discussed further in Chapter 5.2.2.

1.10. Neutrophil Metabolism

Immunometabolism is a rapidly developing field which describes the interface between immunology and cellular metabolism. Immunometabolism can describe how nutrients or environment influences immune cell function, but also how immune cells can influence tissue homeostasis. Cellular metabolism is a central regulator of function and activation of immune cells. This was first observed in macrophages by Vats *et al*. who identified that metabolism influenced cytokine production in macrophages (Vats et al., 2006), subsequently this effect has been widely demonstrated across innate and adaptive immune cells (Kumar, 2019). There is increasing evidence for the role of immunometabolism in infections, however there is very limited data regarding neutrophils, in a review of immunometabolism and pulmonary infections the role of neutrophils was not discussed (M. Rao et al., 2019). Neutrophil metabolic pathways and impact on effector function are described further in Chapter 5.2.5.

1.11. Summary

CAP is a leading cause of hospitalisation associated with high mortality and morbidity. An episode of CAP negatively influences health status for many years after resolution of the acute illness placing high burden on healthcare services. Current guidelines advocate for severity assessment and anti-microbial therapy. Most people receive timely and appropriate antibiotics; however, outcomes remain poor for older adults suggesting that antibiotics alone are not the answer.

CAP disproportionately impacts older adults, especially those with existing co-morbidities. Modelling suggests significant increases in the prevalence of multi-morbidity over the next decade, particularly in older adults, who will spend longer with

multi-morbidity (Kingston et al., 2018a). This suggests that the incidence of CAP is likely to increase.

The large burden of CAP both at an individual level and societal level combined with the expectation of increasing population ageing, increased prevalence of multi-morbidity means that we urgently need strategies to improve outcomes from CAP. The change in mortality in CAP demonstrated in the BTS audit is reassuring, but we still have much to achieve, especially for those most vulnerable to CAP (W. S. Lim and Lawrence, 2019). There is significant variation in CAP outcomes across published studies (Lawrence, Lim and McKeever, 2020) demonstrating that better care for people with CAP is possible.

Heterogenous populations have plagued CAP research, recent advances have shown that improved mortality is possible, but with accurate identification of the right patient population. Developing risk stratification tools which are validated in a pragmatic cohort of CAP participants with recent changes in population demographics and changes in CAP care considered will allow both researchers to stratify for studies, but also support individual decision making.

Alterations in neutrophil function have been consistently shown in ageing and then further impacted by acute infection, especially CAP. Older adults experience a high burden of complications and poor outcomes which may be neutrophil driven. However, the mechanism underlying these changes remain undefined. Metabolism is a core cellular function, and neutrophil functions are highly energy dependent. Exploring the metabolism of neutrophils in CAP offers a promising therapeutic strategy.

To achieve better care for people with CAP a multi-faceted approach is needed, by understanding the population who are hospitalised, defining accurate risk stratification and explain the immunological changes seen.

1.12. Thesis Aim

CAP is a leading cause of emergency hospitalisation and associated with high rates of adverse outcomes. This thesis aimed to investigate factors associated with poor outcomes in CAP using multimodal approach will contribute to enhanced care and outcomes for people with CAP.

1.12.1. Structure of thesis

The studies presented in this thesis were performed to investigate factors which influence outcomes in community acquired pneumonia, with a particular focus on older adults, severity assessment, changes in healthcare during the COVID-19 pandemic and neutrophils.

Chapter Two describes the changes in CAP epidemiology in Birmingham during 2019-2022 at time of unprecedented public health measures to prevent transmission of COVID-19.

Chapter Three describes development and validation of a novel severity assessment tool in CAP, designed to address the limitations of existing severity assessment tools.

Chapter Four is a published manuscript describing assessment of neutrophil metabolism using extracellular flux.

Chapter Five investigates neutrophil function and metabolism in older adults with CAP and matched controls.

Chapter Six describes the neutrophil transcriptome in CAP compared to controls.

2. EPIDEMIOLOGY OF HOSPITALISED COMMUNITY ACQUIRED PNUEMONIA BETWEEN 2019 AND 2022 IN BIRMINGHAM

2.2. Abstract

Community acquired pneumonia is a leading cause of emergency hospitalisation during the winter months. In late 2019 the COVID pandemic emerged as a global threat. A lack of specific treatments meant that the focus was on public health interventions to slow the spread of the virus. These interventions were anticipated to impact the incidence of non-COVID-19 respiratory infections. Therefore, this study aimed to quantify hospitalisation with CAP during the pandemic compared to the pre-pandemic period. Routinely recorded data was accessed through PIONEER, a health research hub to compare hospitalised CAP before and during the pandemic at a single hospital site in Birmingham.

This study identified significant falls in hospitalisation due to CAP during the pandemic which was sustained into the late pandemic (Winter 2021/22). 30-day mortality was 56% higher in winter 2020/21 and 47% higher in winter 2021/22 compared to 2019/20. This increased mortality was seen alongside increased burden of comorbidity, higher rates of severe CAP and sepsis. However, processes of care remained unchanged over time.

This study suggests that during the pandemic people were presenting later to hospital, with more severe disease. The reasons for this delay are unclear, however in the event of a future pandemic public health campaigns should encourage people to seek medical attention and reassure the public that accessing healthcare remains safe.

2.3. Introduction

Epidemiology is the study of the determinants, occurrence and distribution of health and disease in a defined population (Coggon, Barker and Rose, 2009). Epidemiology enables researchers to understand patterns of disease, identify at risk populations for incident disease and identify people with the disease who are at high risk of adverse outcomes.

Epidemiology of hospitalised CAP changes over time and according to geographical location. The BTS CAP audit has demonstrated a 50% reduction in mortality between 2009-2019 with associated falls in ICU admission, but with increasing readmission rates. The BTS audit also examines care processes and has demonstrated improvements in proportions of people receiving antibiotics within four hours and increased senior clinical review within twelve hours of admission (W. Lim and Lawrence, 2019). A further UK based study demonstrated that incidence of CAP increased over time, the study also showed that the case mix changed significantly over time with median age increasing to 80 from 74 years. In keeping with the BTS audit data they identified reduced mortality, length of stay and increased rates of readmission over time. There were no significant shifts in identified pathogen burden (Quan et al., 2016). In contrast to this, a longitudinal study between 2007-2017 examined the epidemiology of hospitalised CAP in Spain and demonstrated small shifts in the age and sex of participants, while CAP severity and outcomes were unchanged. The study demonstrated no shift in bacterial aetiology (however testing rates were not reported), although bacteriaemic CAP was less common, which was not associated with a change in rate of pneumococcal vaccination (Sellarès-Nadal et al., 2022).

Together this data demonstrates clear shifts in the epidemiology of CAP which may reflect altered demographics of participants, or changes in care. As new treatment strategies emerge the epidemiology of disease should be revisited to assess their impact.

2.3.1. COVID-19 Restrictions

In early 2020 COVID-19 emerged as a global pandemic. Early in the pandemic a lack of targeted therapies mandated introduction of public health interventions such as social distancing, face coverings and isolation for infected cases. These interventions were aimed at slowing the spread of the virus to prevent healthcare systems from being overwhelmed. Some interventions such as closing hospitality or non-essential shops are not viable long-term options due to the financial and social costs involved, however some more moderate interventions such as face coverings, avoiding contact with others when unwell or hand washing are potentially sustainable interventions which could substantially impact epidemiology of CAP in addition to epidemic viruses. Figure 2-1 shows a timeline of restrictions in place across the UK between March 2020 and March 2022.

In May 2023 the world health organisation (WHO) declared that COVID-19 was no longer a global health emergency and was deemed to be endemic signalling the end of the pandemic, although many governments removed COVID-19 restrictions in 2022.

2.3.2. Impact of restrictions on non-pandemic diseases

The restrictions to limit spread of COVID-19 aimed to limit transmission of virus by all possible routes, including droplet spread, air-borne spread, surface transmission and faecal-oral spread (Freeman et al., 2021). These routes of transmission have

significant overlap with common CAP pathogens (Torres et al., 2021), and are therefore expected to influence other respiratory pathogens capable of causing CAP, therefore influencing CAP epidemiology. However, it is important to note that common CAP pathogens such as *S. pneumoniae* or *Haemophillus influenzae* are also capable of colonisation, particularly in people with chronic lung disease (Weiser, Ferreira and Paton, 2018), so preventing transmission may not completely abolish risk of CAP.

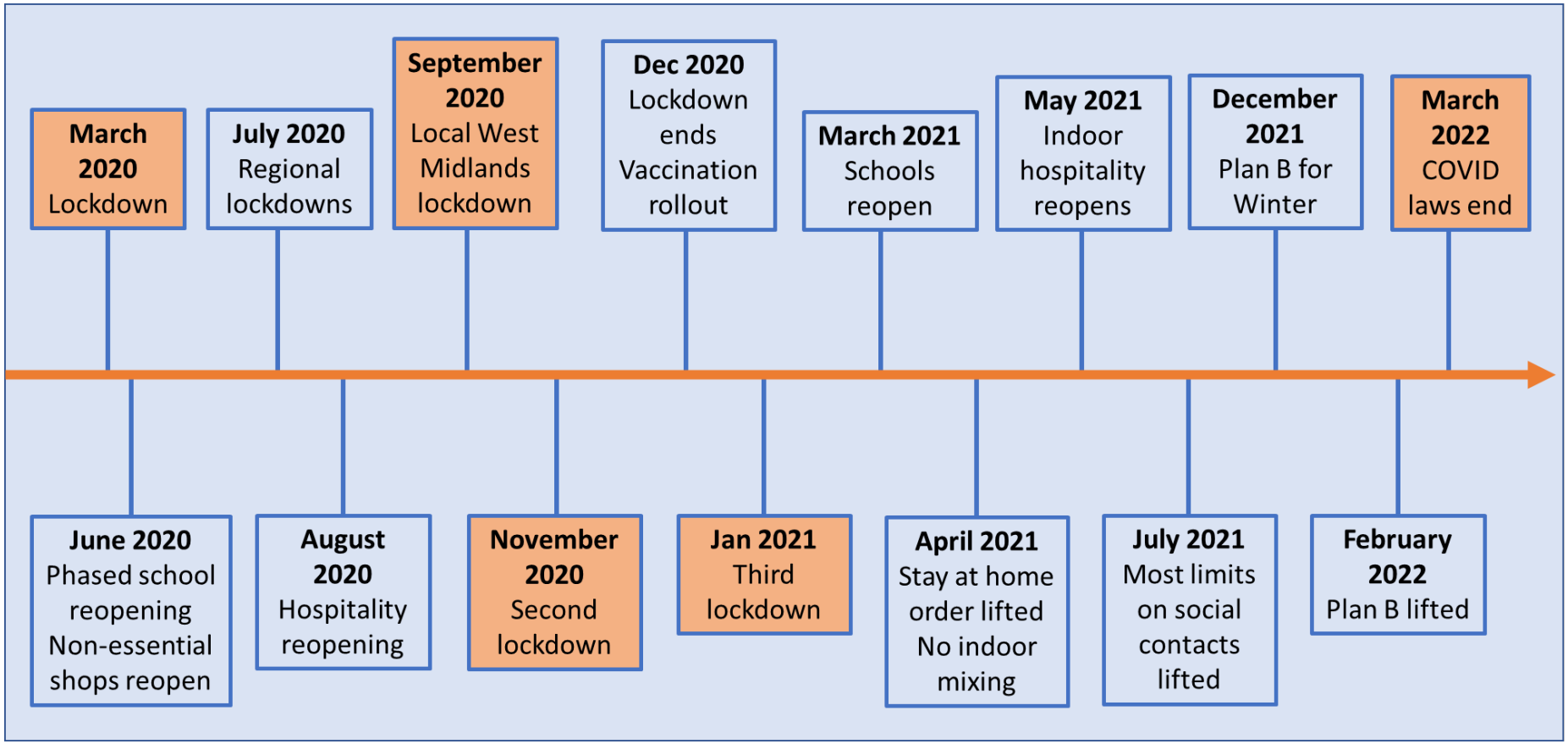

Figure 2-1: Timeline of COVID-19 restrictions in the UK

Plan B rules: widespread testing, vaccination, NHS COVID pass and mask use to prevent transmission. Work from home where possible. Data taken from (CDC, n.d.; Hale et al., 2021; Institute for Government analysis, 2022)

2.3.3. Impact of COVID-19 restrictions on healthcare contacts

Following the introduction of COVID-19 restrictions across the world, non-COVID-19 hospital admissions fell by between 30-50% (Baum and Schwartz, 2020; Riley et al., 2020; Blecker et al., 2021; Bodilsen et al., 2021), more significant falls were seen in geographies under greater pandemic strain (Blecker et al., 2021). In general, by late summer 2020 non-COVID-19 admissions started to climb but did not reach pre-pandemic levels (Blecker et al., 2021; Bodilsen et al., 2021).

Primary care contacts were also altered during the pandemic with some studies reporting a 50% fall in in-person consultations, not fully replaced by virtual consultations (Bodilsen et al., 2021; Mansfield et al., 2021). Altered primary care consultations disproportionately affected those under four years of age and those over seventy years of age (Bodilsen et al., 2021), the populations at highest risk of CAP. The fall in consultations was not uniform across disease types, consultations for alcohol related problems and mental health issues rose sharply during the early pandemic period in both primary and secondary care (Riley et al., 2020; Mansfield et al., 2021).

To examine the impact of altered consultation rates and styles, prescription data for anti-hypertensives, cholesterol lowering drugs and anti-diabetic drugs were examined across the UK. During the pandemic there were significant reductions in prescriptions for incident and prevalent disease, suggesting that screening for important health problems fell during the pandemic (Dale et al., 2023). The impact of these missed screening opportunities may not be observed for years as cardiovascular disease is indolent and usually asymptomatic until presentation with organ-damage.

An international study of non-COVID-19 ICU admissions during the pandemic identified a 9% fall in ICU admission globally, with increased rates of ICU and in-hospital mortality. However, they showed that the increased mortality was only seen in middle income countries, whereas in high income countries, ICU and in-hospital mortality fell. The study identified no relationship between stringency of COVID-19 restriction and ICU mortality, even when countries of similar income level were compared (McLarty et al., 2023).

2.3.4. Impact of COVID-19 restrictions on Community Acquired Pneumonia

Japan was an early adopter of widespread public health campaigns regarding COVID-19 which suggested self-isolation, limiting social contacts, hand washing and face coverings, prior to the introduction of mandatory laws. Tashiro *et al* demonstrated that these moderate and unenforced guidelines resulted in a fall in CAP admissions prior to the introduction of enforced rules, but there was no associated fall in episodes of pyelonephritis or biliary tract infection suggesting that without adversely impacting healthcare delivery or large financial penalties the incidence of CAP can be reduced (Tashiro et al., 2023). Other studies demonstrated a fall in CAP hospitalisation during the pandemic, associated with increased mortality (Serigstad et al., 2023; Bodilsen et al., 2021).

2.3.5. Care Quality Markers used in CAP.

The BTS CAP audit uses four quality indicators to assess CAP care:

1. Chest radiograph within four hours of admission.
2. Antibiotics administered within four hours of admission.
3. Oxygen requirement assessed and prescribed appropriately.

4. CURB65 score calculated and used to guide antibiotic therapy.

Quality indicators for Acute Medical Units (AMU) are defined by the Society of Acute Medicine (SAM), targets relevant to this study are:

1) Senior medical review ≤6 hours if admitted during the daytime.
2) Senior medical review ≤14 hours if admitted between 1800-0800.

This study is of particular importance to Birmingham. Birmingham experienced some of the highest rates of COVID-19 infection and highest proportions of ventilated patients nationally placing the region and hospitals under significant strain (Atkin et al., 2021). WHO have declared COVID-19 should be considered endemic, however, there is an estimated probability of 47-57% of a global pandemic similar in scale to COVID-19 by 2050 (Financing the Global Commons for Pandemic Preparedness and Response, 2021). Therefore, understanding the indirect effects of public health measures to limit pathogen spread is important for future pandemic preparedness, but also to understand whether moderate interventions such as hand washing, or face masks can influence risk of CAP acquisition and or outcomes.

2.4. Hypothesis

Social distancing restrictions designed to limit transmission of COVID-19 reduced hospitalisation due to CAP but increased short and long-term mortality due to alterations in CAP severity.

2.4.1. Aims:

1. Describe demographics of people hospitalised with CAP in the winter seasons of 2019/20, 2020/2021 and 2021/22.
2. Describe CAP severity in the winter seasons of 2019/20, 2020/2021 and 2021/22.
3. Compare markers of care quality for people hospitalised with CAP in the winter seasons of 2019/20, 2020/2021 and 2021/22.
4. Evaluate mortality and readmission rates for people with CAP in the winter seasons of 2019/20, 2020/2021 and 2021/22.

2.5. Methods

This was a retrospective single centre study of patients admitted to Queen Elizabeth Hospital, Birmingham (QEHB) with CAP (non-COVID-19) from before (01/09/2019 to 31/01/20) termed cohort 1, during (01/09/20 to 31/01/21)- termed cohort 2 and in the late pandemic (01/09/21 to 31/01/22) termed cohort 3. The baseline period was selected to end in January as the first hospitalised COVID-19 infection in Birmingham was identified in February 2020 and this correlates with peak CAP hospitalisations in December and January in the Northern Hemisphere (Murdoch et al., 2014).

QEHB is a large hospital in central Birmingham with 1215 inpatient beds and the largest single site ICU in Europe. QEHB also experienced the highest number of COVID-19 admissions across the UK in the early phase of the pandemic with the greatest proportion of ventilated patients nationally (Atkin et al., 2021).

Data was sought through PIONEER, a Health Data Research Hub in Acute Care with ethical approval for use of unconsented anonymised routine healthcare data to be accessed in a trusted research environment (Gallier et al., 2021). Ethical approval was granted by: East Midlands–Derby REC (20/EM/0158) and Confidentiality Advisory Group (20/CAG/0084). PIONEER curates routinely recorded healthcare data from University Hospitals Birmingham (UHB), this data is updated weekly from the electronic patient record (EPR). Data includes physiological measurements, laboratory testing, measures of acuity, comorbidities, medications and outcome data. All data is anonymised and accessed in a trusted research environment. In this work only structured data such as

physiological indices were accessed, free text data such as radiology or microbiology reports were not accessible in this study.

Inclusion criteria were broadly based on those used by the BTS CAP audit (Table 2-1). However, thoracic imaging reports were not analysed to confirm radiological changes in keeping with CAP as these reports are free text and this study only included structured, coded health data.

Table 2-1: Study Inclusion Criteria

Inclusion Criteria	Exclusion Criteria
ICD codes J09-J18	ICD code Y95 (hospital acquired infection
≥18 years old	Transferred from another hospital.
Negative COVID-19 PCR test at admission.	Readmitted within ten days of a prior admission.
Thoracic imaging within 24 hours of admission	Elective admission, or ED attendance only

ICD: international classification of disease, ED: emergency department.

Data were collected on demographics, physiological and laboratory indices, markers of care processes at admission including time to antibiotic administration, time to chest radiograph and time to senior medical review. This study was limited to structured, coded data only from the electronic patient record (EPR). Outcomes were also collected: ICU admission, mortality at 30, 90 and 360-days following admission and 30-day readmission to University Hospitals Birmingham (UHB) NHS trust.

Observations are the first recorded on admission to hospital (either in Emergency Department (ED) or AMU dependent on route of admission). Confusion was defined as Glasgow coma scale (GCS) <15, or any score less than alert on ACVPU score. Severity scores were calculated according to their derivation studies (Lim et al., 2003; Seymour et al., 2016). Charlson comorbidity index was calculated according to the derivation study using ICD coding disease states (Charlson et al., 1987). Usual working hours were defined as 0800-1800 (Society for Acute Medicine, 2019). Acute kidney injury was defined as increase in serum creatinine by ≥26μmol/L within 48 hours, or increase ≥1.5x baseline which is known or presumed to have occurred within 7 days, or urine output ≤0.5ml/kg/hour for six hours (Kellum et al., 2012).

Complete case analysis was undertaken with cases with missing data excluded on a case-by-case basis.

Data were analysed using Stata version 17.0 (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC.). Data were tested for normality using Shapiro-wilk test where *p*-value ≥0.05 indicates normally distributed data. For continuous data Kruskal-Wallis test was used for non-parametric data and t-test used for normally distributed data. Categorical variables were compared using Chi-squared test. In all cases *p*-value <0.05 was considered significant.

2.6. Results

2.6.1. Admission rates

Using the winter of 19/20 as a baseline, admissions with CAP to QEHB fell by 44% in the winter of 2020/21. Admissions in winter 21/22 were still 19% less than the baseline period.

2.6.2. Demographics and comorbidities of hospitalised CAP

Age, gender, ethnicity, index of multiple deprivation (IMD), and body mass index were stable over the three cohorts (Table 2-2). Charlson comorbidity index (CCI) was significantly higher during the second cohort as seen in Figure 2-2. There were no other significant differences in co-morbidities between the cohorts. The most common comorbidities were renal disease, COPD, and diabetes. The percent of patients in whom full clinical escalation including use of cardiopulmonary resuscitation was deemed to be an appropriate treatment remained stable over time.

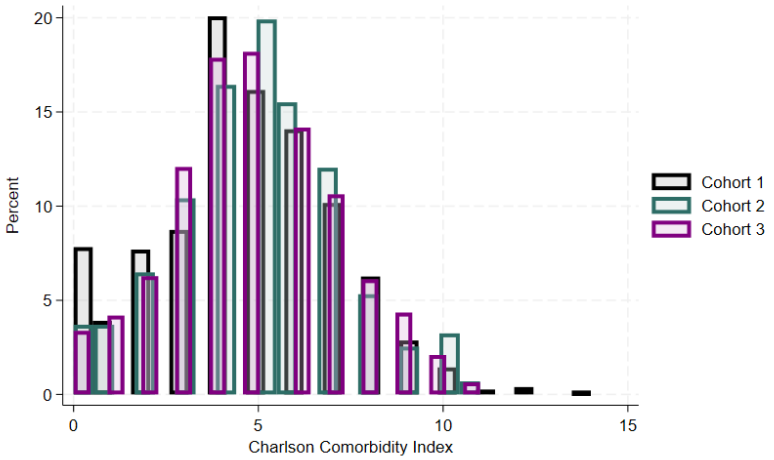

Figure 2-2: Charlson Comorbidity Index across cohorts

Overlaid histogram demonstrating Charlson Comorbidity Index (CCI) between cohorts. Cohort 1 shown in black outline, cohort 2 in green outline and cohort 3 in purple outline. Charlson comorbidity index was calculated according to (Charlson et al., 1987). CCI was significantly higher in cohorts 2 and 3, *p*-value=0.0456 by Kruskal-Wallis).

Table 2-2: Demographics and comorbidities across cohorts

	Cohort 1	Cohort 2	Cohort 3	*p*-value
Number of cases	767	432	621	
% change in number of cases	n/a	- 44	- 19	
Age (Median, IQR)	78 (65-86)	77 (65-86)	77 (67-85)	0.819
Female Sex (Count, %)	407 (53)	201 (46.5)	324 (52.1)	0.079
White British Ethnicity (Count, %)	571 (74.5)	317 (73.4)	481 (77.5)	0.560
IMD (Median, IQR)	2 (1-3)	1 (1-3)	2 (1-3)	0.185
CCI (Median, IQR)	***5 (3-6)***	***5 (4-6)***	***5 (3-6)***	***0.045***
BMI (Median, IQR)	25 (21-30)	25 (22-30)	26 (22-31)	0.121
IHD (Count, %)	104 (13.6)	81 (18.6)	100 (16.1)	0.056
Solid malignancy (Count, %)	74 (9.7)	48 (11.1)	76 (12.2)	0.300
Cerebrovascular disease (Count, %)	69 (9.0)	55 (12.7)	67 (10.8)	0.123
Heart failure (Count, %)	61 (8.0)	36 (8.3)	71 (11.4)	0.064
Dementia (Count, %)	83 (10.8)	54 (12.5)	52 (8.4)	0.085
Diabetes mellitus (Count, %)	242 (31.6)	141 (32.6)	212 (34.1)	0.593
Liver disease (Count, %)	23 (3.0)	12 (3.0)	18 (2.9)	0.992
PVD (Count, %)	37 (4.8)	30 (7.0)	48 (7.7)	0.072
COPD (Count, %)	159 (20.7)	108 (25.0)	154 (24.8)	0.116
CKD (Count, %)	177 (23.1)	106 (24.6)	135 (21.8)	0.567
For full escalation including CPR (Count, %)	53 (16)	40 (14)	55 (14.5)	0.789

Significant factors in bold italics. IQR: interquartile range. IMD: index of multiple deprivation. BMI: body mass index, IHD: ischaemic heart disease, PVD: peripheral vascular disease, COPD: chronic obstructive pulmonary disease, CKD: chronic kidney disease and CPR: cardiopulmonary resuscitation. Comparisons made using Kruskal-Wallis test for continuous variables and Chi^2 for categorical variables. % change in admissions was calculated using cohort 1 as the reference.

2.6.3. Admission Factors

Most patients were admitted on weekdays during working hours, and this was unchanged across the cohorts (Table 2-3). Significantly fewer patients were admitted from nursing or residential homes during the pandemic. Most patients arrived at hospital by ambulance, there were no changes in mode of arrival across cohorts.

Table 2-3: Admission type by cohort

	Cohort 1	**Cohort 2**	**Cohort 3**	***p*-value**
Weekday admission	557 (72.6)	320 (74.0)	450 (72.5)	0.822
Admitted in usual working hours	477 (62.2)	265 (61.3)	370 (59.6)	0.607
Admitted from RH/NH	***96 (12.5)***	***21 (4.9)***	***35 (5.7)***	***<0.0001***
Admitted from own home	643 (83.8)	402 (93.1)	578 (93.1)	
Arrived via Ambulance	604 (85.2)	359 (90.5)	528 (87.6)	0.159

Significant factors in bold italics RH: residential home, NH: nursing home. All comparisons made using Chi^2 test.

2.6.4. CAP severity at point of admission.

CAP severity as judged by CURB65 was significantly higher during the pandemic, in Figure 2-3 and Table 2-4.

Figure 2-4 demonstrates a significant shift to higher qSOFA scores in cohort 2 and 3. Sepsis as defined by qSOFA ≥2 was significantly more common in the pandemic.

National early warning score 2 (NEWS2) was significantly higher in cohorts 2 and 3 compared to cohort 1 (Figure 2-5).

Table 2-4: CAP severity scores across cohorts

	Cohort 1	Cohort 2	Cohort 3	*p*-value
CURB65 (Median, IQR)	***2 (1-2)***	***2 (1-2)***	***2 (1-2)***	***0.008***
Severe CAP (Count, %)	***126 (16.9)***	***93 (22.2)***	***134 (22.1)***	***0.019***
NEWS2 (Median, IQR)	***4 (2-7)***	***5 (2-7)***	***5 (3-7)***	***<0.0001***
qSOFA (Median, IQR)	***0 (0-1)***	***1 (0-1)***	***0 (0-1)***	***<0.0001***
Sepsis (Count, %)	***60 (7.8)***	***54 (12.5)***	***73 (11.8)***	***0.012***

Significant factors in bold italics. Severe CAP: CURB65≥3. Sepsis: qSOFA ≥2. NEWS2: national early warning score 2, qSOFA: quick sequential organ failure assessment score. Comparisons made using Kruskal-Wallis test for continuous variables and Chi^2 for categorical variables.

Respiratory rate (RR) was significantly higher during the pandemic. Significantly more patients had abnormal ACVPU scores in cohort 2 compared to cohort 1. Other physiological parameters associated with adverse outcome in critical illness were unchanged (Table 2-5).

Table 2-5: Physiological Parameters of CAP Severity

	Cohort 1	Cohort 2	Cohort 3	*p*-value
Required oxygen at admission (Count, %)	329 (43.0)	257 (59.6)	328 (53.0)	0.087
Respiratory Rate (Median, IQR)	***19 (17-23)***	***20 (18-25)***	***20 (18-25)***	***0.0001***
Systolic BP (Median, IQR)	125 (111-142)	127 (110-149)	127 (112-147)	0.342
Diastolic BP (Median, IQR)	72 (64-83)	74 (66-84)	74 (66-85)	0.266
Heart rate (Median, IQR)	93 (79-108)	94 (79-112)	93 (80-110)	0.467
Alert on ACVPU score (Count, %)	***718 (93.8)***	***368 (85.4)***	***552 (89.2)***	***<0.0001***

Significant factors in bold italics Observations are the first recorded on admission to hospital. BP: blood pressure, IQR: interquartile range. Comparisons made using Kruskal-Wallis test for continuous variables and Chi^2 for categorical variables.

Laboratory investigations demonstrated a significantly higher urea in cohort 2 compared to cohorts 1 and 3. Median CRP was significantly lower in cohort 2 (62) than cohorts 1 and 3. Serum lactate was significantly higher in cohort 2. Other factors which have previously been demonstrated to influence mortality in CAP such as neutrophil lymphocyte ratio (NLR) and incidence of AKI were not altered between cohorts (Table 2-6).

Table 2-6: Laboratory tests associated with CAP severity.

	Cohort 1	Cohort 2	Cohort 3	*p*-value
Urea (mmol/L, Median, IQR)	***7.1 (5-10.8)***	***8 (5.2-12.3)***	***7 (5-10.7)***	***0.013***
Creatinine (μmol/L, Median, IQR)	85 (63-117)	87 (63-127)	84 (62-117)	0.245
CRP (mg/L, Median, IQR)	***103 (39.5-192)***	***62 (18-137)***	***88 (36-179)***	***0.0001***
WCC (10^9/L, Median, IQR)	12.0 (8.9-16.8)	12.3 (9.0-15.7)	12.3 (8.9-17.1)	0.621
Lymphocytes (10^9/L, Median, IQR)	1 (0.60-1.5)	1 (0.62-1.6)	1 (0.7-1.6)	0.102
Neutrophils (10^9/L, Median, IQR)	9.9 (6.9-14.5)	9.9 (6.8-13.4)	9.9 (6.8-14.6)	0.575
Lactate (mmol/L, Median, IQR)	***2.0 (1.5-2.8)***	***2.2 (1.6-3.1)***	***1.8 (1.3-2.5)***	***0.0001***
pH (Median, IQR)	7.38 (7.34-7.42)	7.38 (7.34-7.42)	7.39 (7.34-7.42)	0.699
NLR (Median, IQR)	10.5 (5.5-18.4)	8.7 (5.1-18.2)	9.6 (5.2-17.1)	0.113
AKI (Count, %)	150 (19.6)	109 (25.3)	143 (23.0)	0.059

Significant factors in bold italics. IQR: interquartile range, CRP: C reactive protein, NLR: neutrophil lymphocyte ratio, WCC: white cell count and AKI: acute kidney injury. Comparisons made using Kruskal-Wallis test for continuous variables and Chi^2 for categorical variables.

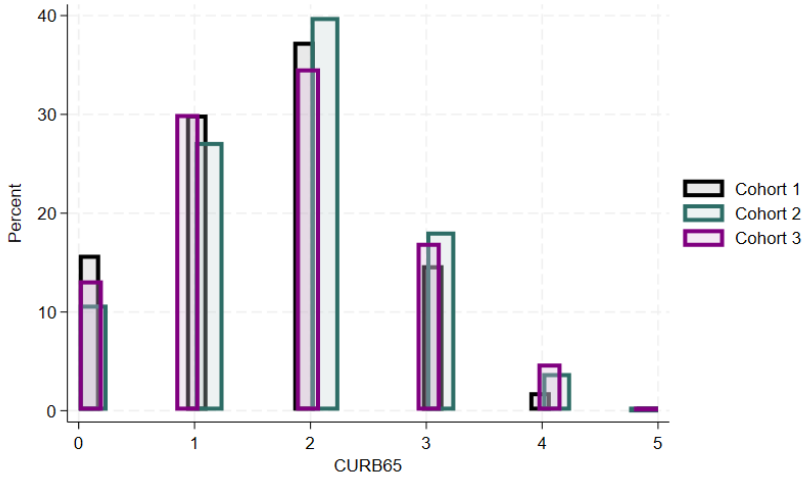

Figure 2-3: Distribution of CURB65 scores across cohorts

CURB65 was calculated according to the derivation study. Cohort 1 shown in black outline, cohort 2 in green outline and cohort 3 in purple outline. Histogram demonstrates a significant shift to increased CURB65 score in later cohorts (*p*-value: 0.0078 by Kruskal-Wallis).

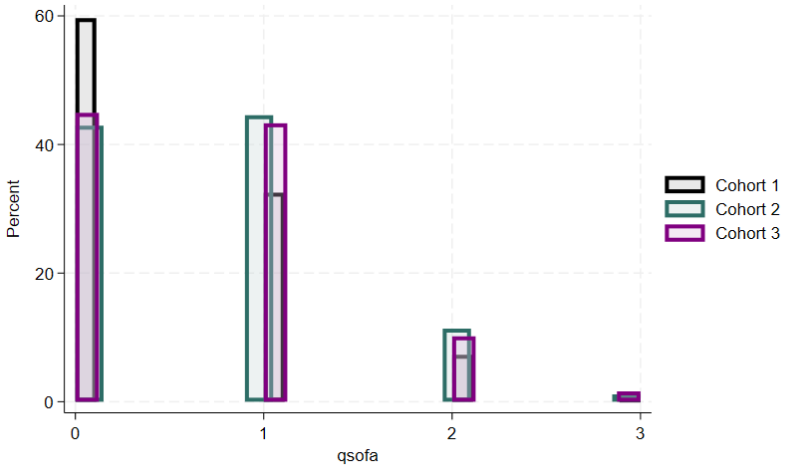

Figure 2-4: Distribution of qSOFA score across cohorts.

qSOFA was calculated according to the derivation study. Cohort 1 shown in black outline, cohort 2 in green outline and cohort 3 in purple outline. Histogram demonstrates a significant shift to increased qSOFA score in later cohorts (*p*-value: 0.0001 by Kruskal-Wallis). qSOFA: quick sequential organ failure assessment score.

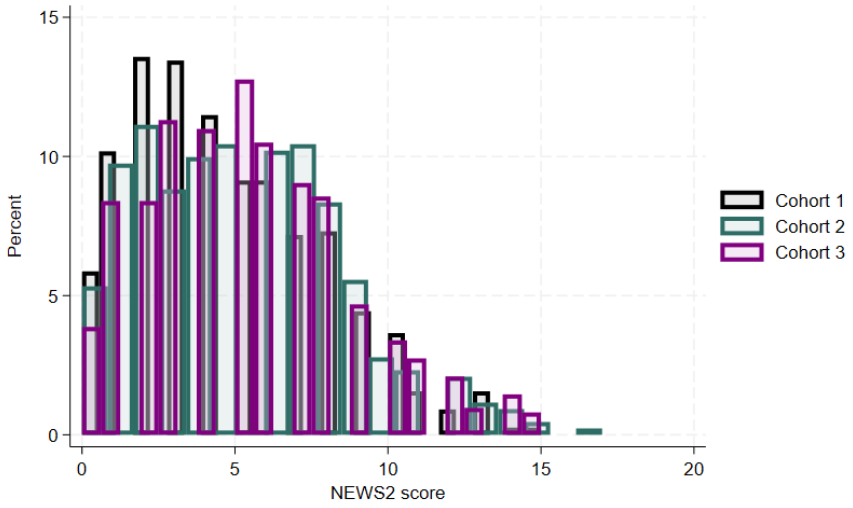

Figure 2-5: Distribution of NEWS2 score across cohorts.

NEWS2 was calculated according to the derivation study. Cohort 1 shown in black outline, cohort 2 in green outline and cohort 3 in purple outline. Histogram demonstrates a significant shift to increased NEWS2 score in later cohorts (*p*-value: 0.0001 by Kruskal-Wallis). NEWS2: national early warning score 2.

2.6.5. Processes of care

Processes of care are shown in Table 2-7. There were no differences in proportions of patients receiving CXR or antibiotics in under 4 hours. Significantly more patients had senior medical review within prescribed indicators in during the pandemic compared to cohort 1.

Table 2-7: Processes of CAP Care

	Cohort 1	Cohort 2	Cohort 3	*p*-value
Had CXR ≤ four hours (Count, %)	590 (76.9)	345 (79.9)	460 (74.0)	0.090
Had antibiotics in ≤ four hours (Count, %)	472 (62.0)	288 (67.5)	374 (60.3)	0.055
Senior medical review in ≤ 14 hours if admitted OOH (Count, %)	***204 (72.1)***	***149 (89.8)***	***237 (94.2)***	***<0.0001***
Senior medical review ≤6 hours if admitted in daytime (Count, %)	***146 (26.5)***	***219 (61.5)***	***255 (55.4)***	***<0.0001***
Had oxygen prescription (Count, %)	640 (83.4)	375 (86.8)	531 (85.5)	0.262

Significant factors in bold italics. OOH: out of hours, CXR: chest radiograph, ICU: intensive care admission. Comparisons made using Kruskal-Wallis test for continuous variables and Chi^2 for categorical variables.

2.6.6. Microbiological testing

Patterns of microbiological testing and positivity rates varied across the cohorts. Overall testing rates were low. Significantly more sputum samples were sent in cohort 3 compared to 1 and 2, and rates of positive sputum sample (if sample sent) were significantly lower in cohort 2 compared to 1 and 3 (

Table 2-8). There were no changes in pattern of blood culture sampling, or positivity rates. Pleural fluid results were not analysed due to low number of samples, across the entire cohort there were 41 pleural fluid samples sent.

There were no cases of hospital acquired COVID-19 infection in cohort 1, there were significantly more cases of hospital acquired COVID-19 infection in cohort 2 compared

to cohort 3, however, nosocomial COVID-19 was not associated with increased risk of 30-day mortality (p=0.599 by Chi^2).

Table 2-8: Microbiological testing and results

	Cohort 1	Cohort 2	Cohort 3	*p*-value
Sputum microbiology sent (Count, %)	***120 (15.7)***	***46 (10.7)***	***107 (17.3)***	***0.011***
Blood culture sent (Count, %)	359 (46.8)	200 (46.3)	293 (47.2)	0.961
Positive sputum sample (Count, %)	***27 (3.5)***	***11 (2.5)***	***42 (6.8)***	***0.001***
Positive blood culture (Count, %)	51 (6.7)	29 (6.7)	31 (5.0)	0.364
Sputum positivity rate (%)	***22.5***	***23.9***	***39.3***	***0.015***
Blood culture positivity rate (%)	14.2	14.5	10.6	0.306
Hospital-acquired COVID-19 infection	***0***	***18 (4.2)***	***11 (1.8)***	***<0.0001***

Significant factors in bold italics Positivity rate was calculated as number tests positive/number of tests sent. Hospital acquired COVID-19 defined as negative PCR test at admission and positive PCR test ≥3 days after admission. Comparisons made using Chi^2.

2.6.7. Outcomes

Mortality at 30, 90, 180 and 360 days was significantly increased during the pandemic compared to the baseline cohort (Table 2-9). The increase in mortality was highest at early time points and fell at later time points both cohorts compared to the baseline period. Readmission within 30 days to UHB fell in both during the pandemic. There were no differences in rates of ICU admission, or length of stay. Overall length of stay was also unchanged across the cohorts.

Table 2-9: CAP Outcomes

	Cohort 1	Cohort 2	Δ	Cohort 3	Δ	*p*-value
30-day mortality	***116***	***102***	***56.3***	***138***	***47.0***	***<0.0001***
(Count, %)	***(15.1)***	***(23.6)***		***(22.2)***		
90-mortality	***184***	***140***	***35.0***	***184***	***22.5***	***0.004***
(Count, %)	***(24)***	***(32.4)***		***(29.6)***		
180-day mortality	***232***	***162***	***23.8***	***218***	***15.8***	***0.024***
(Count, %)	***(30.3)***	***(37.5)***		***(35.1)***		
360-day mortality	***300***	***199***	***17.9***	***245***	***1.0***	***0.042***
(Count, %)	***(39.1)***	***(46.1)***		***(39.5)***		
30-day readmission	***111***	***46***	***-30.5***	***73***	***-23.4***	***0.033***
(Count, %)	***(15.4)***	***(10.7)***		***(11.8)***		
Admitted to ICU	42	16		29		0.381
(Count, %)	(5.5)	(3.7)	-	(4.7)	-	
ICU LoS	4.3 (2.3-8.5)	4.3 (1.5-8.9)		3.9 (2.8-9.3)		0.492
(Days, median, IQR)			-		-	
LOS	7.1 (3.3-14.2)	7.7 (3.8-13.9)		7.1 (3.8-12.8)		0.367
(Days, median, IQR)			-		-	

Significant factors in bold italics. ICU: intensive care unit. Readmission to UHB trust only. Mortality and readmission rates for cohorts 2 and 3 were compared to the baseline cohort (1), Δ change in rates presented a percent.

2.6.8. Performance of severity assessment tools over time

Given the increase in mortality it was prudent to consider whether the severity assessment tools in use were performing similarly, as these tools are used to support treatment decisions around microbial testing strategy, anti-microbial therapy and level of care needed. Both CURB65 and qSOFA performed similarly at predicting 30-day mortality over all three cohorts (Figure 2-6). For CURB65 area under curve (AUC) was

0.708, 0.679 and 0.674 in cohorts 1,2 and 3 respectively, *p*=0.571 by DeLongs test. For qSOFA AUC was 0.640,0.590 and 0.652 in cohorts 1,2 and 3 respectively, *p*= 0.283 by DeLongs test.

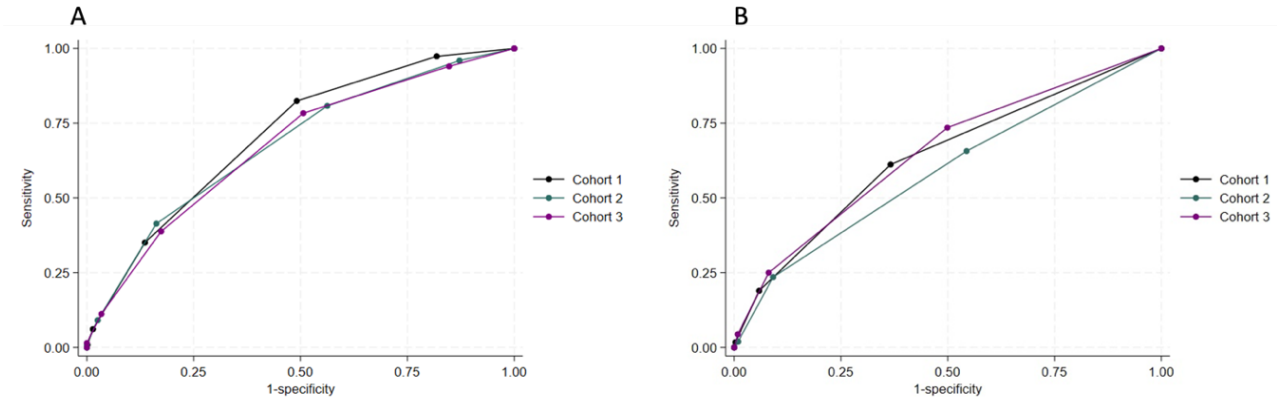

Figure 2-6: ROC comparing accuracy of CURB65 and qSOFA.

(A) CURB65 and (B) qSOFA were calculated according to their derivation studies and receiver operator curve analysis performed to generate area under the curve (AUC). AUCs were similar across all three cohorts for both scores.

2.6.9. Factors associated with 30-day mortality.

Logistic regression was performed in the whole dataset to identify factors associated with increased risk of 30-day mortality. Given that this a pragmatic cohort of all admissions, this study was not powered to detect factors associated with 30-day mortality, particularly in the individual cohorts, therefore analysis should be considered exploratory for further testing. Three models containing baseline characteristics, physiological observations and care processes were generated using published data regarding variables established to impact outcome in CAP. Factors were excluded from models if collinearity was identified.

Factors associated with increased risk of 30-day mortality in the overall cohort.

Demographic characteristics

Demographic characteristics associated with 30-day mortality were increasing age, male sex and presence of a solid malignancy (Table 2-10). Presence of COPD was associated with reduced risk of 30-day mortality (OR 0.684, p=0.032).

Physiological markers

Physiological markers at the point of hospital admission associated with increased risk of 30-day mortality were reduced systolic blood pressure, increased urea, neutrophil count, lactate, CURB65, NEWS2 and presence of AKI (Table 2-11) Raised creatinine was associated with a modest reduced risk of mortality (OR 0.994, p<0.0001).

Processes of care

Microbiological testing and other markers of care quality were also significantly associated with mortality (Table 2-12). Having a blood culture sent for analysis (regardless of result) was associated with increased risk of mortality (OR 1.4, p= 0.007), conversely having a sputum sample sent for analysis was associated with reduced risk of mortality (OR 0.65, p=0.021), but if the sputum sample was positive there was an increased risk of mortality (OR 2.2, p=0.049). Having antibiotics administered within 4 hours and senior medical review within 14 hours of admission were also associated with increased risk of mortality.

Table 2-10: Logistic regression using demographic factors.

	Odds ratio	95% Confidence Interval	*p*-value
Age	***1.02***	***1.01-1.04***	***0.001***
IMD score	0.97	0.92-1.02	0.202
CCI	1.10	0.94-1.28	0.238
Male sex	***1.35***	***1.06-1.72***	***0.015***
IHD	0.96	0.67-1.38	0.815
Solid malignancy	***1.94***	***1.22-3.08***	***0.005***
Heart failure	1.21	0.80-1.83	0.370
Dementia	1.16	0.78-1.72	0.461
Diabetes Mellitus	0.72	0.51-1.0	0.053
COPD	***0.68***	***0.48-0.97***	***0.032***
CKD	0.96	0.62-1.5	0.863
Nosocomial COVID-19 infection	1.06	0.44-2.53	0.904

IMD: index multiple deprivation, CCI: Charlson-comorbidity index, IHD: ischaemic heart disease, COPD: chronic obstructive pulmonary disease and CKD: chronic kidney disease. Nosocomial infection defined as not present at admission, and positive test ≥3 days after admission. Significant variables are in bold italics.

Table 2-11: Logistic regression using physiological factors.

	Odds ratio	95% Confidence Interval	*p*-value
Respiratory rate	1.02	0.98-1.05	0.314
Systolic blood pressure	***0.99***	***0.98-1.0***	***0.046***
Diastolic blood pressure	1.01	1.00-1.02	0.052
Heart rate	0.99	0.99-1.00	0.161
Urea	***1.11***	***1.07-1.14***	***<0.0001***
CRP	1.00	1.00-1.00	0.107
Creatinine	***0.99***	***0.99-1.00***	***<0.0001***
WCC	0.99	0.96-1.02	0.554
Lymphocyte count	0.90	0.76-1.07	0.233
Neutrophil count	1.01	0.99-1.03	0.47
Lactate	***1.14***	***1.04-1.24***	***0.004***
pH	0.57	0.08-4.02	0.573
NLR	0.99	0.98-1.01	0.338
NEWS2	***1.19***	***1.10-1.28***	***<0.0001***
CURB65	***1.28***	***1.05-1.57***	***0.015***
qSOFA	0.72	0.51-1.01	0.058
AKI	***1.90***	***1.35-2.67***	***<0.0001***

Significant variables are in bold italics. CRP: c-reactive protein, WCC: white cell count, NLR: neutrophil: lymphocyte ratio, NEWS2: national early warning score 2, qSOFA: quick sequential organ failure assessment and AKI: acute kidney injury.

Table 2-12: Logistic regression using processes of care.

	Odds ratio	95% Confidence Interval	*p*-value
CXR ≤4 hours	1.01	0.72-1.41	0.974
Antibiotics ≤4 hours	***1.48***	***1.10-2.10***	***0.011***
Senior medical review ≤14 hours	***1.41***	***1.07-1.85***	***0.015***
Sputum culture sent	***0.67***	***0.47-0.95***	***0.027***
Blood culture sent	***1.42***	***1.11-1.81***	***0.005***
Positive blood culture (if sent)	1.09	0.32-3.74	0.892
Positive sputum culture (if sent)	***2.26***	***1.00-5.09***	***0.049***

Significant factors in bold italics. CXR: chest radiograph.

Differences in factors associated with mortality across cohorts.

The same logistic regression models were applied to each of the three cohorts. The variation in variables identified as significant is demonstrated in Figure 2-7. Across the cohorts the only consistent findings were risk of mortality associated with increasing urea and NEWS2 score. Full results of logistic regression by cohort are in

Table 2-13,14 and 15. Factors associated with reduced risk of mortality were increasing creatinine, qSOFA, sputum sample being sent for analysis, presence of COPD and increasing systolic blood pressure.

Factor	Whole cohort	Cohort 1	Cohort 2	Cohort 3
Number of cases	1816	764	432	619
Increasing age	↑	↑	↔	↔
Presence of solid tumour	↑	↑	↔	↔
Male sex	↑	↔	↔	↑
Increasing diastolic blood pressure	↔	↑	↔	↔
Increasing urea	↑	↑	↑	↑
Increasing NEWS2 score	↑	↑	↑	↑
Increasing CURB65 score	↑	↑	↔	↔
Presence of AKI	↑	↑	↔	↑
Increasing creatinine	↓	↔	↓	↔
Increasing qSOFA score	↔	↔	↓	↔
Increasing CRP	↔	↔	↔	↑
Increasing lactate	↑	↔	↔	↑
Blood culture sent	↑	↔	↑	↑
Sputum sent	↓	↔	↓	↔
COPD	↓	↔	↔	↔
Increasing systolic blood pressure	↓	↔	↔	↔
Antibiotics administered <4 hours	↑	↔	↔	↔
Senior medical review <14 hours	↑	↔	↔	↔
Sputum sample positive if sent	↑	↔	↔	↔

Figure 2-7: Factors associated with 30-day mortality in the three cohorts.

Logistic regression with three separate models was performed in the whole dataset, and individual cohorts as described in section xx. Increased risk of mortality is denoted by red cell and ↑, reduced mortality risk denoted by green cell with ↓. Non-significant factors are shown by a yellow cell with ↔. Only factors which were statistically significant in at least one cohort are shown.

Table 2-13: Logistic regression using demographic factors between cohorts.

	Cohort 1		Cohort 2		Cohort 3	
	Odds ratio	***p*-value**	**Odds ratio**	***p*-value**	**Odds ratio**	***p*-value**
Age	***1.05***	***0.001***	1.01	0.37	1.02	0.23
IMD	0.92	0.089	0.97	0.481	1.02	0.657
CCI	1.03	0.849	1.12	0.438	1.11	0.436
Male sex	1.36	0.15	1.07	0.788	***1.58***	***0.026***
IHD	1.09	0.794	0.72	0.356	1.03	0.931
Solid malignancy	***2.40***	***0.039***	1.36	0.49	2.05	0.067
Heart failure	1.33	0.446	1.29	0.556	1.17	0.645
Dementia	1.14	0.695	1.09	0.819	1.33	0.443
Diabetes Mellitus	0.66	0.172	0.74	0.361	0.79	0.398
COPD	0.65	0.192	0.68	0.247	0.72	0.245
CKD	1.17	0.68	0.87	0.734	0.99	0.979
Nosocomial COVID-19	1.00	0.15	0.59	0.419	1.63	0.453

IMD: index multiple deprivation, CCI: Charlson-comorbidity index, IHD: ischaemic heart disease, COPD: chronic obstructive pulmonary disease and CKD: chronic kidney disease. Significant variables are in bold italics.

Table 2-14: Logistic regression using physiological factors between cohorts.

	Cohort 1		Cohort 2		Cohort 3	
	Odds ratio	***p*-value**	**Odds ratio**	***p*-value**	**Odds ratio**	***p*-value**
Respiratory rate	0.99	0.627	1.04	0.188	1.03	0.284
SBP	0.99	0.23	0.99	0.186	0.99	0.184
DBP	***1.02***	***0.009***	0.99	0.556	1.01	0.254
Heart rate	0.99	0.276	1.00	0.846	0.99	0.063
Urea	***1.11***	***<0.001***	***1.09***	***0.002***	***1.12***	***<0.001***
CRP	1.00	0.911	1.00	0.552	***1.003***	***0.012***
Creatinine	1.00	0.078	***0.99***	***0.026***	***0.99***	***0.005***
WCC	1.00	0.908	1.35	0.179	0.98	0.569
Lymphocyte count	0.81	0.259	0.55	0.061	1.00	0.998
Neutrophil count	1.01	0.388	0.70	0.151	1.01	0.362
Lactate	1.06	0.466	1.18	0.054	***1.23***	***0.013***
pH	3.51	0.442	0.26	0.522	0.14	0.263
NLR	0.99	0.273	1.00	0.779	0.99	0.628
NEWS2	***1.25***	***<0.001***	1.16	0.054	***1.21***	***0.004***
CURB65	***1.51***	***0.016***	1.42	0.095	1.02	0.922
qSOFA	0.81	0.453	***0.45***	***0.022***	0.75	0.353
AKI	***2.01***	***0.019***	1.85	0.053	**2.06**	**0.027**

Significant variables are in bold italics. CRP: c-reactive protein, WCC: white cell count, NLR: neutrophil: lymphocyte ratio, NEWS2: national early warning score 2, qSOFA: quick sequential organ failure assessment and AKI: acute kidney injury. SBP: systolic blood pressure, DBP: diastolic blood pressure.

Table 2-15: Logistic regression using care quality factors between cohorts.

	Cohort 1		Cohort 2		Cohort 3	
	Odds ratio	***p*-value**	**Odds ratio**	***p*-value**	**Odds ratio**	***p*-value**
CXR ≤4 hours	1.02	0.994	0.83	0.588	1.26	0.388
Antibiotics ≤4 hours	1.7	0.057	***1.74***	***0.07***	1.27	0.326
Senior medical review ≤14 hours	***1.45***	***0.009***	0.79	0.488	0.98	0.937
Sputum culture sent	0.59	0.100	***0.32***	***0.022***	0.87	0.87
Blood culture sent	1.06	0.792	***1.66***	***0.034***	***1.53***	***0.037***
Positive blood culture (if sent)	2.76	0.182	*	*	*	*
Positive sputum culture (if sent)	2.10	0.298	3.8	0.201	2.00	0.356

Significant factors in bold italics. CXR: chest radiograph. * excluded due to co-linearity.

2.6.10. Missing data

Rates of missing data are presented in Table 2-16. CFS and GCS had extremely high missing rates and were therefore not used in data analysis. There were large differences in CFS missingness between cohorts, this corresponded to introduction of a mandatory assessment in the EPR which included CFS.

Table 2-16: Missing data by cohort.

	Whole dataset (%)	Cohort 1 (%)	Cohort 2 (%)	Cohort 3 (%)
CFS	80.4	99.9	51.2	78.4
GCS	50.3	53.5	42.1	52.2
BMI	24.8	22.4	21.5	30.0
Lactate	17.1	17.7	16.4	16.9
pH	15.4	16.6	13.2	15.5
Bilirubin	7.5	1.2	0.5	20.1
CRP	6.8	0.9	0.5	18.4
Creatinine	6.2	0.0	0.2	18.0
WCC	6.2	0.0	0.2	18.0
Timestamps	1.2	2.7	0.2	0.0
IMD	0.2	0.3	0.0	0.3
NEWS2	0.2	0.1	0.2	0.3
ACVPU	0.2	0.1	0.2	0.3
RR	0.2	0.1	0.2	0.3
BP	0.2	0.1	0.0	0.3
HR	0.1	0.1	0.2	0.0
Temperature	0.1	0.1	0.2	0.0
Creatinine	0.1	0.1	0.0	0.0
Oxygen saturations	0.1	0.0	0.0	0.2
Urea	0.0	0.0	0.0	0.0
Age	0.0	0.0	0.0	0.0
Sex	0.0	0.0	0.0	0.0
Ethnicity	0.0	0.0	0.0	0.0
Microbiology data	0.0	0.0	0.0	0.0
Prescriptions data	0.0	0.0	0.0	0.0

Missing data listed in descending frequency. CFS: clinical frailty scale, GCS: Glasgow coma scale, CRP: c-reactive protein, WCC: white cell count, IMD: index of multiple deprivation, NEWS2: national early warning score 2, RR: respiratory rate, BP: blood pressure, HR: heart rate.

2.7. Discussion

This study demonstrates a significant fall in CAP hospitalisation at QEHB during the pandemic. The decline in admissions was associated with significantly increased short- and long-term mortality, with a fall in 30-day readmissions. CAP admissions during the

pandemic had greater acuity, more severe CAP and higher incidence of sepsis, but processes of hospital care were unchanged.

Demographics were comparable, except for increased CCI in the later cohorts reflecting a greater burden of comorbidity. Significantly fewer participants were admitted from RH or NH during the pandemic. During the pandemic participants had greater physiological derangement demonstrated by increased RR and reduced consciousness with increased NEWS2 score. Blood urea and lactate were significantly higher, but CRP was lower during the pandemic. All assessed severity indices were higher during the pandemic. Together these data suggest that during the pandemic people with CAP were presenting to hospital later and with more severe disease.

This study did not consider the role of primary care contacts. Primary care contacts fell during the pandemic, and this was not fully replaced by virtual consultations (Bodilsen et al., 2021; Mansfield et al., 2021). Primary care prescribing rates for chronic diseases and antimicrobials also fell during the pandemic (Dale et al., 2023; Yang et al., 2023). Multiple studies have reported fear and avoidance of seeking healthcare during the pandemic (Taylor et al., 2020; Gogoi et al., 2023; Smolić, Fabijančić and Blaževski, 2023). These combined factors may have delayed or impacted people's ability to seek healthcare.

Given the likelihood of future pandemic events these findings indicate that delivery of non-pandemic healthcare should be altered to prevent this excess mortality. Public health campaigns in the event of a future pandemic should focus on encouraging people to continue seeking healthcare in the event of serious medical problems. The cardinal symptoms of CAP significantly overlap those of COVID-19, therefore rapid access to

PCR based testing will facilitate streaming of people to pandemic and non-pandemic healthcare locations.

Work by Tashiro *et al* suggests that moderate unenforced restrictions such as mask wearing and self-isolation if unwell significantly reduces incidence of respiratory tract infections without the huge socio-economic implications of lockdown (Tashiro et al., 2023). This could be of relevance to people with chronic conditions which confer high risk of poor outcome in CAP.

This data reflects multicentre studies which demonstrate the decline in admission with associated increase in mortality, however, is the first to present granular data which can be used to understand the factors influencing increased mortality.

2.7.1. Demographic factors

Age, sex, and comorbidities are frequently cited as risk factors for poor outcome in CAP (Cillóniz et al., 2013). In this study age, sex and prevalence of individual co-morbidities was unchanged, however CCI was significantly higher in the pandemic cohorts, demonstrating that although rates of individual comorbidities were not altered the burden of comorbidities was increased. While CCI was developed to predict long-term mortality in the community, it has been widely used to quantify comorbidity in studies of hospitalised participants. CCI has been demonstrated to be a significant predictor of in-hospital mortality in CAP (Nguyen, Saito and Wagatsuma, 2019).

Rates of do not attempt resuscitation (DNAR) and or treatment limitation decisions did not alter over time, other studies of hospitalised patients have demonstrated increased

rates of DNARs during the pandemic which may have influenced patient outcomes (Bows and Herring, 2022).

CFS was not routinely recorded with high rates of missing data, CFS is a metric which may reflect overall complexity and burden of morbidity summed as frailty. It may be that participants in the later cohorts with increased mortality were frailer. There are no validated scores for assessment of frailty using routinely recorded data in secondary care, development of tools to assess frailty using these data sources would be of benefit.

The age, sex and common comorbid conditions are similar to those presented in the 2019 BTS CAP audit data (W. S. Lim and Lawrence, 2019), suggesting this is a representative cohort.

2.7.2. Admission related factors

Timing of admission to hospital for unplanned episodes has been linked with increased adverse outcomes termed the weekend effect. The weekend effect is thought to be due to increased severity of illness rather than altered delivery of care (Bion et al., 2021).This study tested whether the change in mortality was associated with changed patterns of admission. There was no shift weekday versus weekend or time of day (in or out of usual working hours) across the cohorts. There was a significant reduction in admission from NH/RH, given that RH/NH residents are typically very frail with multiple comorbidities, it would be expected that reduced admissions would equate to reduced mortality.

2.7.3. CAP severity

Participants in the later cohorts had more severe pneumonia, with higher rates of sepsis and more physiological derangement. A Japanese study demonstrated a similar shift to more severe cases (Nagano et al., 2021). There were no differences in admission to ICU which would be expected given the increased illness severity. There are several plausible explanations for this:

1. There was no increased need for ICU admission despite statistical differences demonstrated here, or the sicker participants were deemed not appropriate for ICU admission.
2. During the pandemic delivery of acute respiratory care altered substantially with respiratory support units (RSUs). These typically provided level 2 care with non-invasive ventilatory therapy (Messer et al., 2021), therefore avoiding ICU admission.
3. Less people were admitted to ICU despite clinical need due to rationing of ICU beds related to pandemic pressure.

CRP was clinically and statistically significantly lower in cohort 2. CRP is an acute phase reactant which typically peaks 2-3 days after onset of inflammation (Markanday, 2015). The reduced CRP combined with more deranged physiology and increased mortality suggests that during the pandemic patients were presenting to hospital later, and therefore CRP had started to fall, however this study does not have serial CRP measurements to confirm this. Elevated CRP has been associated with increased risk of mortality in CAP, although change in serial CRP has been demonstrated to have a greater prediction for 30-day mortality (Chalmers, Singanayagam and Hill, 2008).

Urea has long been associated with risk of mortality in CAP and is used in both CURB65 (Lim et al., 2003) and pneumonia severity index (PSI) to predict mortality risk (Fine et al., 1997). The absolute difference in median urea is small and the rates of AKI and median creatinine were not different between cohorts. Both urea and creatinine reflect glomerular filtration rate, but urea is a less accurate measure as it can be influenced by factors other than glomerular filtration, hence creatinine is used in diagnosis of AKI (Kellum et al., 2012). Elevated urea with normal creatinine has been associated with dehydration, catabolic states, and pre-renal causes of AKI (Kellum et al., 2012), perhaps reflecting that renal dysfunction in CAP at point of admission is most likely due to pre-renal causes.

Serum lactate was significantly higher in cohort 2 than 1 or 3, again this absolute difference was small, but reflects a general shift to increased severity evidenced by both physiological and laboratory indices. Serum lactate is an independent predictor of mortality from both CAP (Chen, Wang and Guo, 2016; Frenzen et al., 2018) and sepsis regardless of infection source (Mikkelsen et al., 2009).

2.7.4. Quality indicators

This study has demonstrated that the increased mortality during the pandemic is associated with increased comorbidities, and more severe disease, this study also sought to understand whether provision of early in-hospital care was altered using quality standards defined by both CAP guidelines and acute medical guidelines.

Over the three cohorts there were no significant differences in the rates of adherence to BTS quality markers. Rates of adherence to these quality standards were broadly lower

than national BTS audit data from 2019 where 85% had a CXR within four hours, 74% received antibiotics within four hours, however rates of oxygen prescription were much higher in UHB data (83-86%) compared to 53% in national data (W. Lim and Lawrence, 2019). Implementation of the BTS care bundle has been associated with improved outcomes (Lim et al., 2016; Morrow et al., 2021). Receiving appropriate antibiotic therapy and CXR within four hours has also been associated with reduced mortality and length of stay (Chalmers et al., 2011; Priya Daniel et al., 2016; van den Bosch et al., 2016).

Adherence to the senior review indicators was significantly higher in the pandemic than in cohort 1. Senior medical review within the prescribed window in cohort 1 was much lower than SAM 2019 clinical audit (Society for Acute Medicine, 2019), but the later cohorts had much better adherence to these targets. It is important to note that SAM audit took place on a single day in June, data presented here are for winter seasons which typically have increased rates of admissions and therefore may have more delays associated in care. The reduced time for senior review would typically be expected to reduce mortality, however the converse is true in this study. This may reflect either increased staffing in AMU during the pandemic as many planned healthcare contacts were cancelled, reduced frequency of admissions therefore quicker time to review, or it could be that these patients were triaged as more unwell and therefore reviewed more rapidly.

2.7.5. Microbiology

Microbiological testing strategy is guided by CAP severity in BTS guidelines, where increased severity indicates increased testing (Lim et al., 2009). Therefore, it would be anticipated that testing rates would increase in cohort 2 and 3. However, significantly fewer sputum sample were sent in cohort 2 despite increased rates of severity. There were no changes in frequency of blood culture sampling. Compared to national CAP practice in 2019 there were fewer sputum and blood cultures samples sent across all timepoints. Analysis of pleural fluid microbiology results was not possible due to limited number of pleural fluid samples. The rates of positive blood culture samples were unchanged across time, however there was a significant increase in positive sputum samples in cohort 3. Given the more severe disease identified during the pandemic it would be expected that more positive microbiology samples would be identified (Haessler et al., 2022). It is possible that in the later cohorts more patients had pre-hospital antimicrobials which delayed presentation but would also reduce rates of positive microbiology (Ewig et al., 2002).

Nosocomial COVID-19 infection has been reported to increase risk of mortality(Ponsford et al., 2021), but nosocomial COVID-19 infection was not associated with increased risk of mortality in this study.

Other studies have examined patterns in microbiology associated with the pandemic. Amarsy *et al*. showed a significant drop in *S. pneumoniae* bacteriaemia during the pandemic, although testing rates remained similar (Amarsy et al., 2022). Other studies have shown that more cases had an aetiological agent identified, again despite similar levels of testing (Bodilsen et al., 2021). The inability to analyse results of microbiological

testing in this study limits its usefulness, although there were differences in rates of sputum positivity across the cohorts, this may not represent a clinically significant change (Ewig et al., 2002). Microbiology reports are free text and therefore uninterpretable during this study. The practicalities of sputum sampling are challenging, in real-world studies only 36% of people hospitalised with CAP can produce a sputum sample, and the probability of sputum sampling falls with increasing age (Ewig et al., 2002), therefore reduced sampling could reflect a sicker cohort.

COVID-19 accelerated use of PCR based testing for respiratory pathogens. Use of oropharyngeal swabs in a PCR pneumonia panel including bacterial causes has been shown to be equivalent to pathogen detection in induced sputum samples, this would be of particular use where sputum expectoration is unlikely (Serigstad et al., 2023).

2.7.6. Outcomes

Mortality in the first cohort broadly reflected national audit data in 2019 (BTS audit: 13.6% 30-day mortality, 15.1% at UHB), further Birmingham has higher CAP mortality than the national average (Public Health England, 2019). 30-day mortality was 56% higher during cohort 2 and 47% higher during cohort 3 compared to cohort 1. The increased mortality remained significantly different up to 360-days, however the percent change in mortality was reduced at later timepoints, particularly in cohort 3 where 360-day mortality was only 1% higher in cohort 3 than 1 (Table 2-9). This could suggest that the increased mortality associated with CAP during the pandemic was shifted to an earlier time, that many of those who died within 30-days pandemic cohorts may have died within a year at other times too.

Increased short-term CAP mortality of 40% was shown by Bodilsen *et al* (Bodilsen et al., 2021), but this is the first study to assess longer-term mortality and demonstrate that although remaining elevated at 360-days the mortality rates were less elevated than at 30 days.

There was a significant reduction in 30-day readmission to UHB during the pandemic This could be linked to the increased short-term mortality; where reduced short-term mortality is associated with increased rates of readmission, whereas increased short-term mortality reduces readmission. Increasing readmission following CAP combined with falling 30-day mortality have been observed, but the reason for the increased readmissions is not clear (Lawrence, McKeever and Lim, 2023). Only readmissions to UHB were examined, this could underestimate readmissions as participants may have attended other hospitals. The readmission rate in cohort 1 is similar to the national rate in the BTS CAP audit (Lim and Lawrence, 2019b), and UHB represents four hospitals with a bed capacity of 2700 across Birmingham, so readmission to a different trust is thought to be unlikely.

2.7.7. Logistic regression

Factors associated with poor outcomes in CAP are well established and discussed in greater detail in Chapter 1.4. As knowledge of these risk factors will have be used to guide clinical decision making, therefore impact outcomes, this study sought to understand whether there differing patterns of risk factors in the later cohorts which may explain the increased rates of mortality. It is important to note that due to the convenience sampling used in this study, it was not powered to detect differences in

mortality, further the altered sample sizes across groups combined with significantly different event rate (30-day mortality) means that power is likely to be different across the three cohorts.

Baseline characteristics

In the overall cohort, increasing age, presence of solid malignancy and male sex were associated with increased mortality, replicating published data (Cillóniz et al., 2013) however COPD which has previously been associated with increased risk of adverse outcome (Welte and Kohnlein, 2009) was associated with reduced mortality risk in this data. In the context of the pandemic people with COPD were considered extremely clinically vulnerable and asked to shield. People with COPD often have rescue packs at home containing antibiotics therefore reducing time to antimicrobials and given their vulnerability may have been prioritised at triage by primary or secondary care therefore influencing their outcome from CAP. Age and malignancy were also significant predictors of outcome in cohort 1, but not 2 or 3. Male sex was only predictive of increased mortality risk in cohort 3.

Physiological factors at admission:

In the overall dataset creatinine was associated with a modest reduction in 30-day mortality (OR 0.99 for each unit increase in creatinine), but presence of AKI and elevated urea were associated with increased risk of adverse outcome. Creatinine is significantly influenced by muscle mass (Nankivell et al., 2020), therefore people with sarcopenia (common in hospitalised patients (Pérez-Zepeda, Sgaravatti and Dent, 2017)) would be expected to have lower creatinine. Sarcopenia in hospitalised patients is associated with increased risk of adverse outcomes (Martone et al., 2017), therefore

having increased muscle mass; ergo, less frail might influence CAP outcomes. AKI is defined as a change in creatinine which may explain the difference in risk of mortality between the two factors. Alternatively, creatinine being associated with reduced risk may be a false positive finding due to this study not being powered to detect a difference. Other factors such as severity scores and serum lactate were associated with increased risk of adverse outcome as published previously. qSOFA was associated with reduced risk of mortality in cohort 2 (OR 0.45), given that qSOFA is predictive of sepsis this is unexpected and not previously demonstrated in the literature. The OR represents a substantial risk reduction, it could be that as sepsis can be seen as a treatable trait it confers protection, rather than for example malignancy or age which have no direct treatment, or that because qSOFA was elevated they received more appropriate treatment quickly, and therefore had better outcomes. Finally, qSOFA is known to not be an accurate predictor of 30-day mortality in CAP (Kolditz et al., 2016; Muller et al., 2017; Jiang et al., 2018; Grudzinska, Aldridge, et al., 2019) so this not a valid tool to use in mortality prediction in CAP and these data reflect that.

Processes of care

Microbiological testing was associated with altered risk of mortality. Blood culture sampling was associated with increased risk of mortality, while sputum sampling was associated with reduced risk. Blood cultures are indicated in CURB65 ≥2 according to BTS guidelines (Lim et al., 2009), or if there is suspicion of sepsis (Evans et al., 2021). Therefore, the relationship of increased mortality risk likely reflects high baseline mortality in these groups. The finding of sputum sampling being associated with reduced mortality is interesting, given that sputum sampling is indicated in the same CURB65

groups as blood culture. However, sputum expectoration is challenging, and increasing age reduces chance of a sputum sample being sent for analysis (Ewig et al., 2002). Therefore, the participants who did have a sputum sample sent may be younger and have other factors which reduce mortality. Conversely a sputum sample being sent could be an indicator of good quality care, which has been associated with improved outcomes (Lim et al., 2016). Antibiotic therapy ≤4 hours and senior review ≤14 hours were associated with increased risk of mortality; these factors have typically been associated with improved outcomes- but given the established evidence showing these participants with adverse features (high mortality risk) are likely to have been triaged more rapidly to having these interventions. These variables were largely only observed in the whole cohort, this suggests that the sample size played a role in their identification as significant variables. Blood and sputum sampling was significant in some cohorts and not others.

This study provides granular detail regarding CAP admissions before and during the pandemic. This work confirms other studies showing a decline in admissions and increased mortality, but extends the analysis by considering demographic, physiological and process of care factors which can impact CAP outcome.

2.7.8. General weaknesses

The main limitation of this work is that it is a retrospective single centre study. Although the key findings of reduced admissions and increased mortality are replicated by published international studies. There is a possibility that the findings identified here of a

more severe CAP phenotype with greater co-morbidity during the pandemic is a QEHB specific finding.

Birmingham had very high rates of COVID-19 and QEHB housed the most ventilated COVID-19 patients in a single unit across the UK (Atkin et al., 2021), Birmingham has previously been identified as an outlier in CAP care, with higher rates of hospitalisation and mortality (Public Health England, 2019). QEHB was under high strain during the study periods, other studies have reported more significant falls in hospital admissions in regions under high strain compared to low strain, but there are no reported differences in mortality dependent on strain. These findings should be replicated in wider cohorts across a spectrum of locations to reflect high and low COVID-19 strain. This data could be accessed through linkage of existing datasets such as BTS CAP audit, hospital episode statistics, primary care data and other NHS data flows. NHS data is largely held electronically, however there are significant operational challenges to integrating these data streams (Zhang et al., 2023).

This study focused on factors at admission, but most deaths in hospitalised CAP occur after four days of hospitalisation (Pessoa et al., 2020), early interventions were chosen as rapid implementation of treatment has been shown to improve outcomes in CAP and sepsis (W. S. Lim et al., 2016; Morrow et al., 2021; Singer et al., 2016). Further analysis of free text data such as that recorded during ward rounds was not possible in this study. It is possible that treatment decisions made later in the episode impacted mortality but are not measured in this study. This study does not contain cause of death data, it would be helpful to understand whether cause of death was altered during the pandemic.

The inability to assess free text data contained in the EPR limited some analyses, for example it would be useful to understand whether appropriate antimicrobials were used based on guidelines, allergies, and microbiology results. Being able to extract the days of symptoms, and or prior medical contacts before admission would facilitate understanding of whether the severity of CAP was increased by delayed presentation, or barriers to seeking healthcare. Incorporating natural language processing techniques into future studies would develop the findings identified here (Wu et al., 2022).

Missing data was a significant barrier to comparing the cohorts with regards to clinical frailty index, this would have enabled global comparison of functional status across groups. Although some of the measures reported can be a surrogate of frailty- for example DNAR and treatment limitations, but clearly these binary variables lack sensitivity compared to validated models.

Linking this data with primary care records would facilitate comparison of pre and post CAP episode healthcare contacts which would inform whether access to primary care was altered during the pandemic for people with CAP. CAP has specific importance in this situation as the cardinal symptoms overlap substantially with COVID-19 therefore people may have been asked not to attend primary care centres thus been less likely to have face to face consultation to enable physical assessment, therefore influencing outcome.

ICD coding combined with thoracic imaging within 24 hours of admission was used to define CAP, this has been shown have high rates of inaccuracy (W. Lim and Lawrence, 2019), where failure to identify new radiographic features consistent with CAP was the main reason for lack of diagnosis. However, this rate of inaccuracy is likely to remain

consistent over time so therefore the cohorts are comparable, but due to the inability to assess free text radiology reports to verify radiological changes, it is impossible to mitigate for as radiographs are not accessible through PIONEER. In time validation of AI generated CXR reports will enable analysis of large CAP datasets, or development of natural language processing models will enable free text CXR reports to be interpreted for statistical analysis. Inaccuracy in coding of comorbidities and ethnicity is well established in healthcare data (Scobie et al., 2021), meaning that the data presented here is likely to reflect those inaccuracies. Further coding of co-morbidities is binary, with non-severe and severe disease groups coded as one despite there being large discrepancies in the associated risk of disease and adverse outcomes. To maintain confidentiality PIONEER suppresses any observations that are recorded less than ten times in the dataset, for example any individual older than 100 years is assigned an age of ≥100 years. This limited some analyses such as that for pleural infection, where the small numbers prevented detailed analyses of rates of pleural infection across the cohorts.

This study shows a significant reduction in CAP admissions to hospital but does not provide data to support changes in incidence of CAP, or respiratory tract infection. It could be that due to healthcare pressures people with milder infections decided against admission, or they were seen in emergency department and due to risk of COVID-19 clinicians risk assessment for ambulatory management of CAP during the pandemic was altered, therefore only severe cases were admitted. Therefore, the reduced rate of admission may reflect that fewer people with more severe disease are attending hospital. UK surveillance data suggests that primary care consultations for pneumonia

were lower throughout 2020/21 and 2021/2022 than 2019/20, however in the winters prior to this there had also been significant variation in consultation rates (UK Health Security Agency, 2023b).

Determination of sample size and selection of variables used in logistic regression models is subject to controversy (van Smeden et al., 2019). Power calculations can be performed using a single variable such as age, but due to different effect sizes an appropriately sized study to detect a difference in age may not detect differences in all modelled variables. Other studies have suggested ten events (occurrence of 30-day mortality) per variable inputted into the model, so a dataset with 100 deaths could have 10 variables modelled (van Smeden et al., 2019). There were 356 deaths overall in this dataset, therefore the three models tested in the whole group had appropriate power, but these models would not be expected have appropriate power in the individual cohorts. Further the method used for variable selection is debated, classically univariate tests were performed on variables of interest and only those with statistical significance used in logistic regression, other methods include forwards and backwards selection of all variables with non-contributory variables removed in a stepwise fashion, while variables of known significance can be “forced” in (Ranganathan, Pramesh and Aggarwal, 2017). This study used variable selection based on knowledge of factors known to influence outcomes in CAP, as including all variables was not feasible due to low numbers in groups for stepwise selection and given the weaknesses in use of univariate statistics it was felt that biological plausibility was the most robust method. Due to the differing power in each cohort, it is possible that the observed differences in

factors associated with mortality are artefactual due to sample size. To strengthen this data, increasing the sample size would enable more robust assessment using statistical modelling.

2.8. Summary

This study shows reduced rates of hospitalisation for CAP during the pandemic, which was associated with 47-56% increases in mortality on a background of high CAP mortality. During the pandemic participants had a greater burden of comorbidity and more severe CAP with a greater incidence of sepsis. Rates of ICU admission was not different across the cohorts, length of stay was also unchanged. Readmission rates were significantly reduced during the pandemic. At 360-days following admission the difference in mortality rates compared to the baseline cohort was smaller than at early timepoints, suggesting that the increased early mortality may have represented a shift in the time of death- rather than increased death overall. This study is limited to a single centre experience, although with corroboration of key findings at a national and international level. Lack of verification of radiological findings in CAP also limits the generalisability, highlighting an issue common to CAP research using large datasets. This study suggests that in the event of a future pandemic there should be public health campaigns to encourage people to seek healthcare for urgent issues, and streaming of pandemic and non-pandemic illnesses using rapid PCR testing should be adopted.

3. UTILITY OF SEVERITY ASSESSMENT TOOLS IN COMMUNITY ACQUIRED PNEUMONIA

This chapter contains published work **"Early identification of severe community-acquired pneumonia: a retrospective observational study",** BMJ Open Respiratory Research, 2019 (Grudzinska et al., 2019a).

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Author contributions: FG conceived and designed the study, sought regulatory approvals, performed data collection, data analysis and wrote the initial manuscript. KA & SH performed data collection and reviewed the manuscript. PN provided support with statistical analysis. DP, MB, RD, JP, ES, DT and DD: designed and oversaw the study and provided critical feedback on manuscript.

The data regarding derivation and validation of the novel score is unpublished.

3.1. Abstract

Community acquired pneumonia is a leading cause of hospitalisation with high mortality and morbidity. Severity assessment is a cornerstone of CAP care, used to guide microbiological testing, antimicrobial treatment and inform risk of adverse outcomes. Established severity assessment tools lack implementation studies to demonstrate clinical utility; and most were established over twenty years ago, accuracy of predictive tools has been shown to wane over time.

This Chapter sought to test the diagnostic accuracy of established tools in a contemporary cohort of CAP cases and derive a novel tool with increased predictive accuracy for 30-day mortality.

This study is a retrospective observational cohort study with data collection between 2014-2016 at a single centre in Birmingham, UK. Utility of established pneumonia specific (CURB65) and generic (qSOFA and NEWS) tools were tested. A novel tool was developed in the derivation dataset and further tested in a validation cohort.

In the derivation dataset 1545 validated cases of CAP were identified. In this data CURB65 was more accurate at predicting 30-day mortality than qSOFA and NEWS. Logistic regression was performed to derive a novel score using nine routinely recorded variables. Birmingham Community Acquired Pneumonia Score (BCAPS) had greater accuracy at predicting 30, 90 and 365-day mortality in CAP compared to CURB65 in both the derivation and validation cohorts. External validation and implementation studies are required to endorse the utility of BCAPS, but this work demonstrates that refinement of severity assessment is possible and given the computational capacity at our fingertips severity assessment tools need not be simple anymore.

3.2. Introduction

Prognostication is a core skill in healthcare; however, physicians over-estimate survival using clinical assessment alone (Glare et al., 2003). Therefore, risk stratification or severity assessment tools are widely used.

Prognosis research focuses on prediction of a future event using mathematical modelling to predict an individual's probability of an outcome of interest using population level data. Prognostication has important implications in guiding treatment decisions, intensity of care needed, whether ambulatory management can be considered, guiding family and patient discussions and to accurately stratify participants in clinical research (Lim et al., 2009; NICE, 2014; Martin-Loeches et al., 2023). Use of severity index adjusted mortality can also be used to identify issues with quality of care. Risk stratification tools are designed to be used in combination with clinical gestalt. Severity assessment considers factors known to be predictive of mortality at a population level, individuals may have comorbid conditions, or other factors which make implementation of a risk assessment tool infeasible on an individual basis.

Use of severity assessment tools in CAP is advocated by international guidelines (BTS, ERS, ATS/IDSA and NICE (Lim et al., 2009; NICE, 2014; Metlay et al., 2019; Martin-Loeches et al., 2023)). They recommend that severity assessment tools are used to stratify microbiological testing, anti-microbial choice and to guide decision making around appropriate place of care. The two tools recommended by Respiratory Societies are the pneumonia severity index (PSI) and CURB65. PSI is widely used in United States of America and advocated by ATS/IDSA guidelines. CURB65 was developed in

2003 and has subsequently been adopted as the severity assessment tool of choice in the UK.

3.2.1. CURB65

CURB65 is used as a measure of care quality in CAP by the BTS audit and NHS Commissioning for Care Quality and Innovation framework (Lim et al., 2009; NHS England, 2022). CURB65 criteria are in Table 3-1.

Table 3-1: CURB65 score indices.

C: Confusion
U: Blood urea >7mmol/L
R: Respiratory rate >30 breaths/minute
B: Systolic blood pressure <90mmHg or diastolic blood pressure <=60mmHg
65: Age ≥65 years

Confusion defined as Mental Test score of <=8, or new disorientation in person, place or time.

CURB65 can be used to predict mortality and was originally developed to identify patients who could be considered for ambulatory management. In the derivation and validation study participants were divided into three risk categories: low risk (CURB65=0-1) with 1.5% 30-day mortality, intermediate risk (CURB65=2) 30-day mortality 9.2% and high risk (CURB65≥3) with 22% 30-day mortality (Lim et al., 2003). This study was performed in three multi-national cohorts demonstrating that a CURB65 score ≥2 had a sensitivity 75% and specificity 65% to predict 30-day mortality. However, there are important caveats to this study- they excluded participants who were admitted

from nursing homes or had common co-morbidities which influence mortality risk in CAP such as malignancy and bronchiectasis. Following development, external validation studies have demonstrated significant variation in performance of CURB65. Table 3-2 demonstrates the wide range of reported 30-day mortality rates according to CURB65 score.

Table 3-2: 30-day mortality by CURB65 score

CURB65 score	**30-day mortality (%)**	
	Derivation study	External studies
0	0.7	0-4.2
1	2.1	4.0-11.5
2	9.2	7.3-31.6
3	14.5	16.4-38.1
4	40	26.4-55
5	14	33-38.9

30-day mortality reported as percent. Data taken from(Lim et al., 2003; Ronan et al., 2010; Nüllmann et al., 2014; Wesemann et al., 2015; Grudzinska, Aldridge, et al., 2019; Baker et al., 2022)

The performance characteristics of CURB65 demonstrate significant variation dependent on the population. In a 2010 meta-analysis of severity assessment tools in CAP CURB65 was assessed in 17 studies with 15,596 CAP cases, demonstrating moderate score performance with poor calibration compared to derivation probabilities, especially in low-risk categories (Chalmers et al., 2010).

Table 3-3: Performance characteristics of CURB65

	Sensitivity	Specificity
≥1	98.6-100.0	11.4-26.5
≥2	85.1-96.0	40.1-52.2
≥3	56.9-71.0	46.0-80.8
≥4	23.0-37.0	94.4-95.3
≥5	3.0-3.4	99.0-99.5

Performance characteristics of CURB65 taken from (Yan Man et al., 2007; Chalmers et al., 2010; Ronan et al., 2010; Grudzinska, Aldridge, et al., 2019)

Studies have attempted to use CURB65 to predict other adverse outcomes such as ICU admission, complications, or length of stay without success. These outcomes are significantly influenced by healthcare systems and external pressures; therefore, pneumonia severity may play less of a role in determining outcome.

The validity of CURB65 in adults over the age of 65 years has been questioned. This is important as median age of hospitalised CAP in the UK is 75 years (W. Lim and Lawrence, 2019). Several studies have demonstrated variable performance of CURB65 with increasing age (Parsonage et al., 2009; Chen et al., 2010). The reasons underlying this are likely to be explained by changes in presentation features in older adults who present with less physiological derangement but increased cognitive impairment and other presentations such as falls (Guo et al., 2023; Chen et al., 2010; Metlay et al., 1997). Further, increasing age is associated with increasing risk of mortality, but a binary cut-off of 65 years does not incorporate the increasing risk at extremes of age (Cillóniz et al., 2013). In addition, CURB65 does not consider some of the factors most known to influence outcome such as presence of co-morbidities and composite metrics of frailty or multimorbidity. Severity assessment tools typically only predict short-term mortality,

readmission following CAP is increasing (Lawrence, McKeever and Lim, 2019), and studies investigating patient priorities demonstrate that mortality is rarely the most important outcome for patients (Sapey et al., 2019).

Real world use of CURB65 appears to be very variable with audit data suggesting that between 5-48% cases have a CURB65 score recorded at admission (Karmakar and Wilsher, 2010; Morrow et al., 2021). Implementation of CURB65 scoring does not clearly impact outcomes in observational data. There is limited data examining the direct impact of CURB65 on clinical decision making or patient outcomes. The best evidence for implementation of CURB65 is in reduction of broad spectrum anti-microbials without adverse impact on outcomes (Chalmers et al., 2011). Inadequate anti-microbial therapy is associated with increased risk of adverse outcomes in CAP (Priya Daniel et al., 2016), but inappropriate use of broad spectrum anti-microbials may contribute to development of anti-microbial resistance and morbidity (Llor and Bjerrum, 2014). Guidelines recommend use of CURB65 to rationalise anti-microbial therapy (Lim et al., 2009; NICE, 2014).

There are observational data suggesting increased mortality if a CURB65 score is not recorded at admission (Morrow et al., 2021) but this study does not control for important confounders such as age, disease severity or co-morbidity which also influence outcome. Use of CURB65 in preference to PSI was associated with lower mortality in a Dutch multi-centre study but this is not risk adjusted (Kaal et al., 2023). The data examining implementation of CAP bundles (which include CURB65 scoring) is stronger; implementation of the BTS CAP bundle improved proportion of patient's receiving antibiotics within four hours and reduced 30-day mortality (Lim et al., 2016), and BTS

CAP audit which uses CURB65 as a care quality measure has demonstrated a significant fall in mortality over the past decade (W. Lim and Lawrence, 2019), but this cannot be attributed to use of CURB65 alone.

3.2.2. Pneumonia Severity Index

PSI is a five-tier classification system based on twenty factors which include pre-morbid conditions and physiological assessment at time of hospitalisation. PSI was derived and validated in data from >50,000 episodes of CAP. PSI places greater emphasis on pre-morbid conditions than CURB65 (Fine et al., 1997).

Table 3-4: Pneumonia severity index

Demographics and co-morbidities	Physiological indices
Age	Altered mental status.
Sex	Respiratory rate ≥30 breaths/minute
Nursing home resident	Systolic blood pressure <90mmHg
Neoplastic disease	Temperature <35°C or >39.9°C
Liver disease	Heart rate ≥125 beats/minute
Heart failure	pH <7.35
Cerebrovascular disease	Blood urea ≥11mmol/L
Renal disease	Sodium <130mmol/L
	Glucose ≥14mmol/L
	Haematocrit <30%
	Partial pressure O2 <8kPa
	Pleural effusion on CXR

mmHg: milimetres of mercury, kPa: kilopascal, CXR: chest radiograph

Similarly, to CURB65 PSI demonstrates best performance in prediction of low-risk CAP to identify patients for ambulatory management. PSI demonstrates less variability than

CURB65 when performance characteristics are compared between the derivation and subsequent validation studies Table 3-5. Importantly PSI has been demonstrated to safely increase proportion of CAP patients treated as outpatients (Atlas et al., 1998; Yealy et al., 2005).

Table 3-5: 30-day mortality by PSI class

PSI Class	Derivation study	External validation studies	Sensitivity (%)	Specificity (%)
I	0.4	0-0.3	-	-
II	0.7	0.4-0.8	100	0
III	2.8	3.8-5.0	91.4-97.7	25.8-38.8
IV	8.5	8.1-9.3	83.9-91.4	49.5-50.2
V	31.5	22.1-24	46.0-63.2	83.6-84.8

Taken from(Fine et al., 1997; Yan Man et al., 2007; Aujesky and Fine, 2008; Chalmers et al., 2010)

PSI and CURB65 are both well validated, and while PSI has greater data to support implementation and clinical decision making it contains more variables and requires laboratory results which are not routinely recorded in CAP care such as PaO2. PSI and CURB65 are both more accurate at predicting low risk patients than high risk. Overall comparisons between PSI and CURB65 suggest similar performance suggesting that score choice should be driven by local factors. CURB65 is quicker to calculate and contains fewer variables, thus despite the lack of impact studies CURB65 is the tool recommended by UK guidelines (Lim et al., 2009; NICE, 2014).

3.2.3. Generic early warning scores

Sepsis guidelines have suggested use of quick sequential organ failure assessment (qSOFA) to identify patients at high risk of poor outcome from sepsis (Evans et al.,

2021). However, it has been demonstrated that pneumonia specific tools outperform generic sepsis or acute illness severity scores in prediction of mortality (Kolditz et al., 2016; Muller et al., 2017; Jiang et al., 2018). However, qSOFA is more accurate than CURB65 in predicting ICU admission during CAP (Muller et al., 2017).

National early warning score (NEWS) was developed by the Royal College of Physicians in 2012 to provide a consensus early warning score to be implemented across acute care. It is designed to be used as track and trigger method to identify deteriorating patients using serial measurements. It uses defined triggers to recommend clinical review. It was not derived to predict any specific outcome, however, has been widely validated to predict mortality against other early warning scores in both pre-hospital and admitted participants (Lindskou et al., 2023; Guan et al., 2022). NEWS was updated in 2017 to NEWS2, which reflected changes in oxygen saturation scales, confusion parameters and triggers for clinical review. NEWS was widely used across acute care in the NHS during the period of data collection.

CURB65 is twenty years old, and PSI is approaching it's twenty fifth anniversary. In the past twenty years 30-day mortality has significantly improved, and the demographics of people hospitalised with pneumonia have changed (Lawrence, McKeever and Lim, 2022).This means that prognostic accuracy of established models may wane due to changes in diagnosis and treatment (Steyerberg et al., 2013). Further hospitals are increasingly paper free enabling easy access to computational power to generate more complex tools which would not have been feasible in the past, increasingly point of care testing is used which could also be incorporated into severity assessment tools.

Severity assessment tools should not be used alone to influence treatment strategies. Each person with CAP may have physiological, social or demographic features which make management of their CAP challenging. But development of accurate tools which are developed and validated in a pragmatic cohort of patients will allow for stratification to improve CAP outcomes, enable clear discussions with patients and their families and accurately identify specific groups who may benefit from interventions such as steroids. Tools which provide accurate discrimination will also support implementation of CAP research by identifying at risk populations and allowing for baseline comparison. Prognostic models are abundant in the literature, yet impact or implementation studies to demonstrate clinical utility is lacking. Therefore, any future studies should include implementation to demonstrate clinical change.

CAP severity assessment is poorly utilised and has limited evidence for impact and clinical utility. The current gold standard performs well in low-risk patients, but not in high-risk groups. Further CURB65 underperforms in older adults. Given the aging population it is important to have well validated tool which accurately stratifies older adults.

3.3. Hypothesis

Development of a novel or updated CAP assessment tool will provide better prediction of 30-day mortality in a pragmatic cohort of hospitalised CAP.

3.3.1. Aims

1. Develop a cohort of physician validated CAP cases.
2. Examine utility of established scores: CURB65, qSOFA and NEWS

3. Derive a novel score to predict 30-day mortality in CAP.
4. Perform internal validation of the novel score.

3.4. Methods

3.4.1. Ethical approval

This was a retrospective observational study based at Queen Elizabeth Hospital, Birmingham. Ethical approval for the derivation study was not sought following Health Research Authority guidance, local institutional approval was granted (CARMS-14851). Ethical approval was granted for validation cohort (23/LO/0139) by Queens Square Ethics Committee. Due to the limited nature of data collection, high mortality and subsequent morbidity including cognitive impairment permission was granted to not approach individuals for consent, however any individuals who had opted out of use of data by NHS digital were excluded. Data were consecutive CAP cases admitted to QEHB between July 2014- January 2016 (derivation) and February 2016-Febuary 2017 (validation).

3.4.2. Data collection

Data were collected from episodes between 2014 and 2017 using the following International Classification of Disease codes: J12-18. Only adults aged ≥16 years were included. Transfers from other hospitals, or readmissions within 14 days were excluded. Cases were then hand reviewed to ensure they met both clinical and radiological definitions of CAP to generate a cohort of physician validated CAP cases. Data collection variables were defined by factors included in existing scores, plus factors known to significantly impact outcomes in CAP.

Table 3-6: Data variables collected in derivation study.

Physiological observations	Laboratory investigations	Demographics and co-morbidities	Outcomes and complications
GCS New onset confusion Respiratory rate Blood pressure Oxygen saturation Fi02 Temperature NEWS	Glucose Albumin & total protein Urea & Creatinine Bilirubin Haemoglobin, white cell count, neutrophil count and lymphocyte count Blood pH, lactate & base excess Microbiological investigations	Ethnicity Age Co-morbidities Medication history	Mortality Readmission Intensive care admission

GCS: Glasgow coma scale, FiO2: fraction inspired oxygen, NEWS: national early warning score.

For serially recorded observations the first documented observation on admission to hospital (either ED or AMU) was recorded. To ascertain new onset confusion the notes were reviewed at point of admission for evidence of reduced GCS, altered confusion scale within early warning score (AVPU), or any documentation which indicated new or worsening confusion.

3.4.3. Calculation of existing severity assessment tools

NEWS, qSOFA and CURB65 were calculated as per their validation studies (Lim et al., 2003; Singer et al., 2016; Royal College of Physicians, 2017).

3.4.4. Derivation of novel score

Continuous variables were dichotomised using a combination of normal values, cut-offs used in existing scores and optimal binning with 30-day mortality as the reference. Optimal binning is a statistical process used to discretise a continuous variable with reference to a nominal variable, where the cut-points are selected to optimise the relationship with the nominal variable(Dougherty et al., 1995).

30-day mortality was selected to enable comparison to CURB65 the reference standard. To test which cut-offs were most predictive of 30-day mortality in continuous variables forward, stepwise logistic regression was performed generating models for each variable with the different cut-offs.

These cut-offs were then used to perform both univariable and multivariable analysis to identify factors associated with 30-day mortality using Pearson X^2 test. Significance was accepted if $p<0.05$. Variables were tested for co-linearity. Odds ratios were generated for each variable and combined to generate a novel score. The novel score was then compared to CURB65 to compare accuracy in predicting 30-day mortality.

3.4.5. Power calculation for validation cohort

The sample size was estimated using the data from which the novel score was derived, which comprised n=1270 episodes with complete data, with a 30-day mortality rate of 19.0%. In this cohort, BCAPS returned an AUC of 0.811 (95% CI: 0.789-0.844) for the prediction of 30-day mortality, compared to an AUC of 0.690 (95% CI: 0.643-0.717) for the CURB65 score. However, the AUC for BCAPS was calculated based on data from which it was derived; overfitting of the model to the data will have resulted in the AUC being inflated, relative to what would be expected when applied to a validation cohort

(van Smeden et al., 2019). Since the degree of this AUC inflation is difficult to estimate without external validation, a lower AUC for BCAPS of 0.78 was arbitrarily assumed for the sample size calculation. Considering the above, the sample size calculation was performed using easyROC v1.3.1 based on AUCs of 0.78 vs. 0.69 for BCAPS vs. CURB65 and a 30-day mortality rate of 19.0%. Based on these values, a total sample size of n=635 would be required to detect a difference between the AUCs of BCAPS vs. CURB65 with 90% power and 5% alpha. The previous study found that approximately 20% of episodes had missing data prevented calculation of BCAPS, and so needed to be excluded. As such, the required sample size was increased to N=762, to account for missing data.

3.4.6. Statistical package

Data were analysed using Stata version 17.0 (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC.)

3.5. Results

3.5.1. Derivation cohort demographics and outcomes

For the derivation dataset 2895 cased identified by ICD codes were screened to generate 1545 cases of physician validated CAP (Figure 3-1). The cohort included mainly older adults, with median age 77, there was a high prevalence of multi-morbidity (defined as ≥2 chronic diseases). The most common co-morbidities were cardiac disease (53.5%), pulmonary disease (38.1%) and type 2 diabetes mellitus (20.0%).

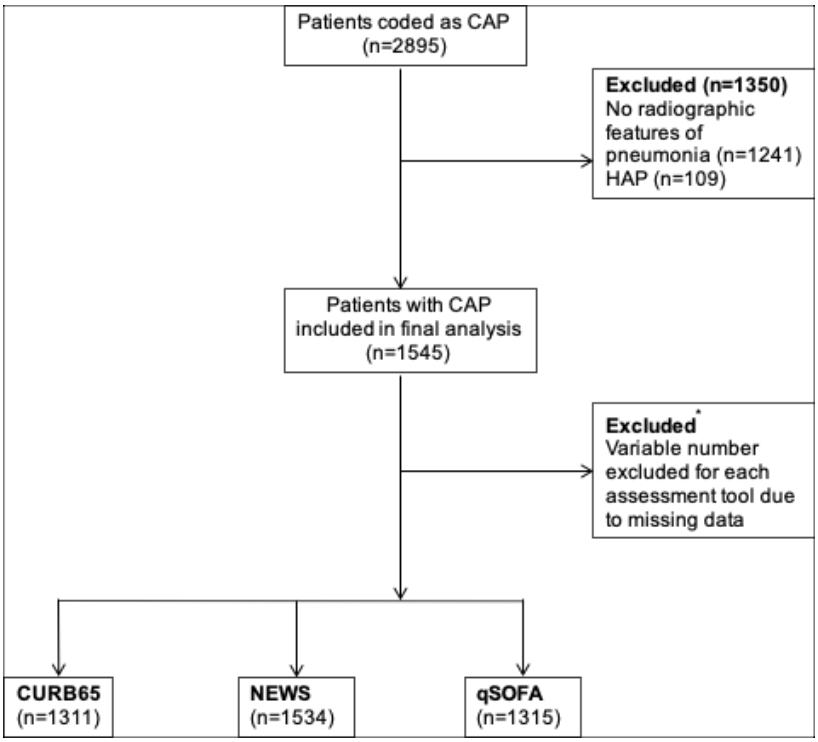

Figure 3-1: Modified consort diagram

*Reasons for exclusion of patients for each severity assessment tool (No. of cases excluded). NB Some cases were excluded due to more than one missing data point: CURB65: confusion (230), urea (5), respiratory rate (4), blood pressure (4), age >65 years (0). Lac-CURB: As for CURB65 plus lactate (227). qSOFA: mentation (230), respiratory rate (4), blood pressure (4). NEWS: temperature (9), oxygen saturations (5), level of consciousness (4), respiratory rate (4), blood pressure (4), heart rate (4). CAP= community acquired pneumonia, HAP= hospital acquired pneumonia, NEWS= National Early Warning Score, qSOFA= quick Sequential (Sepsis-related) Organ Failure Assessment.

Table 3-7: Summary Characteristics of Derivation Cohort

Variable	Derivation Cohort
Number of cases	1545
Median Age (IQR)	77 (63-86)
Female Sex (%)	760 (49.2)
White British Ethnicity (%)	1302 (84.3)
Multimorbidity (%)	1441 (91.3)
Comorbidities:	
Pulmonary disease (%)	588 (38.1)
Metabolic disease (%) (obesity/dyslipidaemia)	238 (15.4)
Solid malignancy (%)	204 (13.2)
Cerebrovascular disease (%)	161 (10.4)
Chronic kidney disease (%)	89 (5.8)
Dementia (%)	170 (11.0)
Diabetes Mellitus (%)	309 (20.0)
Cardiac disease (%)	827 (53.5)

IQR: interquartile range

Inpatient mortality was 15.4% rising to 19.0 % at 30-days following admission. ICU admission was required in 6.4% of cases. Median length of stay was 7 days. One year mortality was 33.1% in Table 3-8.

Table 3-8: Outcomes in Derivation Cohort

Outcome	Count (%)
Inpatient death	238 (15.4)
30-day mortality	293 (19.0)
90-day mortality	390 (25.2)
365-day mortality	511 (33.1)
30-day readmission	102 (6.6)
ICU admission	99 (6.4)
Median length of stay (IQR)	7 (4-14)

ICU: intensive care unit, IQR: interquartile range.

Missing data is shown in Table 3-14. Variables used to calculate CURB65, qSOFA and NEWS were missing in <1%, except for confusion which was missing in 15% of cases.

3.5.2. Utility of established severity tools to predict 30-day mortality.

CURB65 outperformed both qSOFA and NEWS in prediction of 30-day mortality in CAP in the derivation cohort. AUC was significantly greater for CURB65 (0.692) compared to qSOFA (0.632), p <0.0001, however CURB65 was not significantly better at predicting 30-day mortality compared to NEWS (AUC 0.668), p= 0.174.

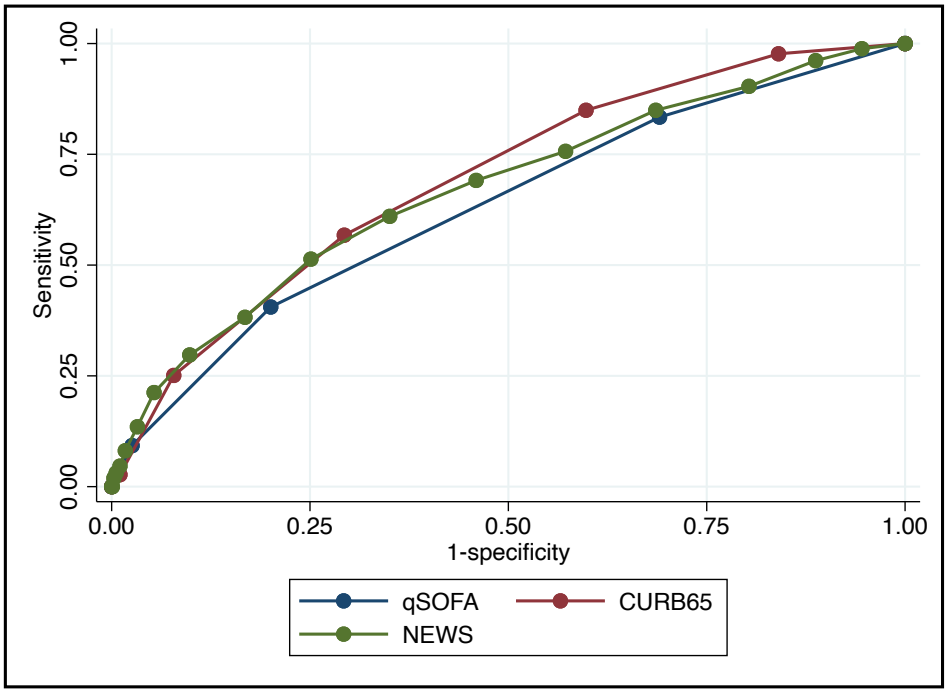

Figure 3-2: ROC assessing performance of CURB65, NEWS and qSOFA.

Area under curve (Standard error) 0.693(0.017), 0.670(0.192) and 0.6321(0.018) for CURB65, NEWS and qSOFA respectively. qSOFA= quick sequential organ failure assessment, NEWS= national early warning score.

Performance characteristics were calculated for prediction of 30-day mortality. For all tested scores sensitivity was greatest at low values. For CURB65 using a cut-off ≥2 resulted in 84.6% sensitivity and 42.9% specificity with a positive likelihood (+LR) of 1.5. For qSOFA a cut-off ≥2 resulted in 40.3% sensitivity with specificity of 79.8% and +LR

2.0. For NEWS a score between 5-6 resulted in sensitivity of 78.9% and specificity 39.9% with +LR 1.3.

Table 3-9: Performance Characteristics for CURB65

Cut-point	Sensitivity (%)	Specificity (%)	+LR	-LR
≥ 1	97.9	16.5	1.2	0.1
≥ 2	84.6	42.9	1.5	0.4
≥ 3	53.6	73.8	2.0	0.6
≥ 4	22.5	93.4	3.4	0.8
≥ 5	2.4	99.1	2.7	0.9

+LR: positive likelihood ratio, -LR: negative likelihood ratio.

Table 3-10: Performance Characteristics for qSOFA

Cut-point	Sensitivity (%)	Specificity (%)	+LR	-LR
≥ 1	83.6	30.9	1.2	0.5
≥ 2	40.3	79.9	2.0	0.7
≥ 3	9.1	97.3	3.4	0.9

+LR: positive likelihood ratio, -LR: negative likelihood ratio.

Table 3-11: Performance Characteristics of NEWS

Cut-point	Sensitivity	Specificity	+LR	+LR
≥medium	78.9	39.8	1.3	0.5
≥high	57.9	68.5	1.8	0.6

Low risk= NEWS <5, Medium risk=5-6, High risk ≥7. +LR: positive likelihood ratio, -LR: negative likelihood ratio.

3.5.3. Utility of existing severity scores to predict outcome in sub-groups.

Older adults versus younger adults

Ability of CURB65 to predict 30-day mortality was not significantly different across 4 age categories (<64, 65-74, 75-84 and >85 years), p=0.5688 by DeLongs test.

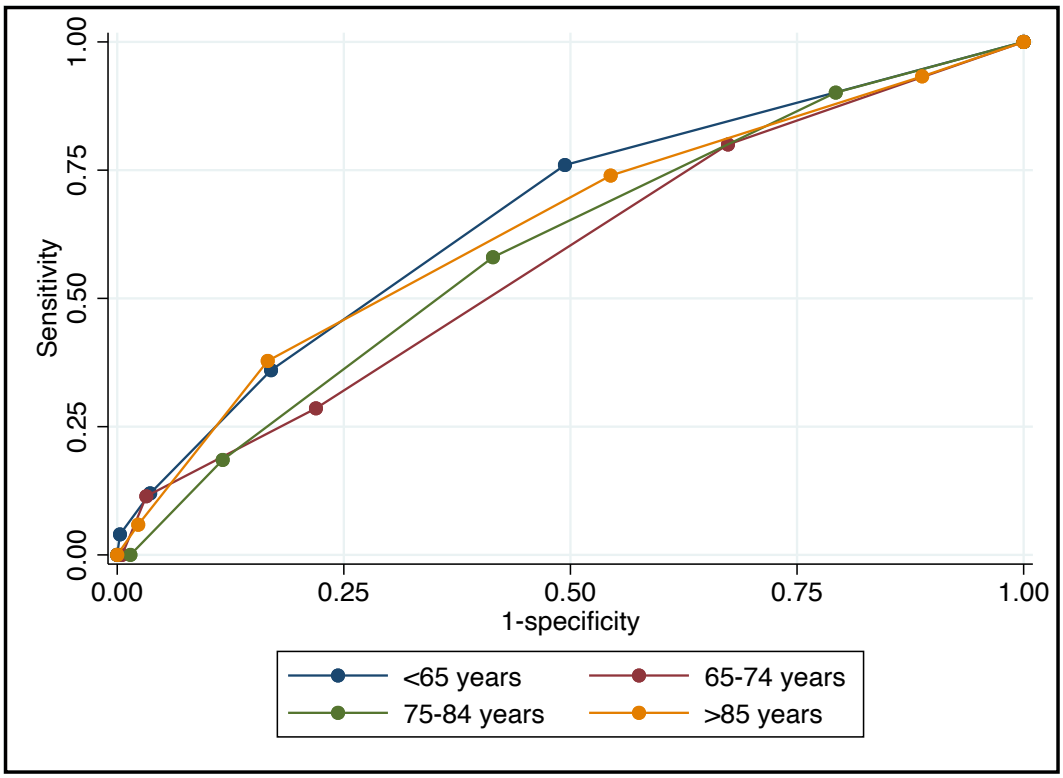

Figure 3-3: ROC assessing performance of CURB65 in increasing age.

AUC <64: 0.6616, 65-74: 0.5793, 75-84: 0.6007 and >85 years: 0.6389, *p*-value: 0.5688 by DeLongs. AUC: area under curve.

CURB65 in people with multimorbidity

CURB65 was significantly better at predicting 30-day mortality in people without multimorbidity compared to those with multi-morbidity (Figure 3-4), p <0.0001 by DeLongs test.

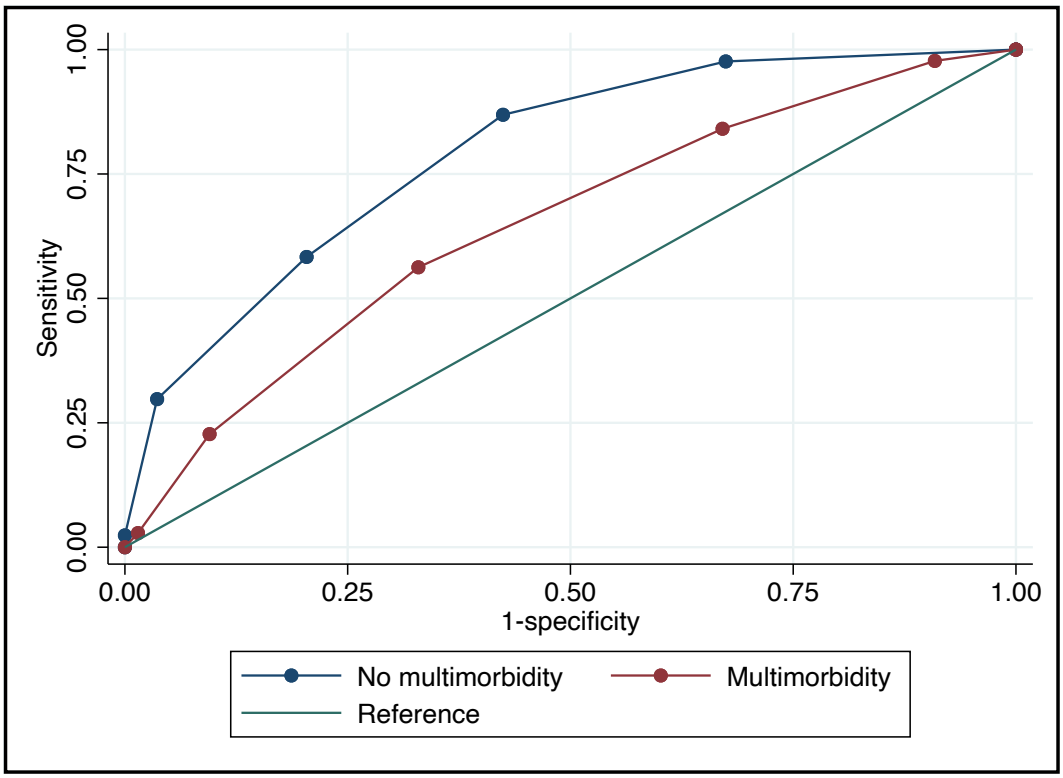

Figure 3-4: CURB65 to predict 30-day mortality in participants with multi-morbidity.

AUC without multimorbidity: 0.7922, with multimorbidity: 0.6491, *p*-value: <0.0001 by DeLongs test. AUC: area under curve

This effect was not seen when CURB65 was tested in specific co-morbidities rather than in multimorbidity. Presence of cardiac disease, diabetes mellitus, dementia, chronic pulmonary disease, or metabolic disease alone did not significantly affect ability of CURB65 to predict 30-day mortality.

3.5.4. Use of Existing tools to predict outcomes other than mortality.

ICU admission occurred in 99 (6.4%) cases. NEWS had the greatest accuracy to predict ICU admission compared to CURB65 and qSOFA (*p* <0.0001) but with only moderate accuracy (AUC 0.655).

Readmission within 30-days occurred in 6.6% cases. None of CURB65, qSOFA or NEWS were able to predict 30-day readmission, all had AUC <0.5.

Table 3-12: Accuracy of scores to predict adverse outcomes other than death.

	Number of cases	AUC (S.E)	*p*-value
ICU admission			
CURB65	83/1306	0.476 (0.03)	
qSOFA	83/1306	0.564 (0.03)	<0.0001
NEWS	83/1306	0.655 (0.03)	
30-day readmission			
CURB65	86/1306	0.495 (0.03)	
qSOFA	86/1306	0.485 (0.03)	0.8062
NEWS	86/1306	0.469 (0.03)	

AUC: area under curve, S.E: standard error, qSOFA: quick sequential organ failure assessment, NEWS: national early warning score.

3.5.5. Birmingham Community Acquired Pneumonia Score Derivation

Table 3-13 shows the various categories tested to dichotomise continuous variables as described in 3.4.4. To determine which cut-offs were most associated with 30-day mortality, forward, stepwise, logistic regression analysis was performed, creating separate models for each individual variable including the different cut-offs.

Table 3-13: Dichotomisation strategies used for continuous variables.

Variable	Cut-offs tested
Age	<61, 61-88, 89+years
Respiratory rate	>24 breaths/minute
Systolic BP	<80 mmHg
Diastolic BP	<60 mmHg
Temperature	<35.4 °C or >37.4 °C
Heart rate	>120 bpm
Urea	>9 mmol/L
Creatinine	>130 µmol/L
WCC	> 11 10^9/L or <4 10^9/L
Total Protein	<65 g/L
Albumin	<32, 32-41, 42+ g/L
Bilirubin	>30 µmol/L
Haemoglobin	<110 g/L
Platelets	<150 or >450 109/L
Neutrophils	>7.5 109/L
Lymphocytes	>4.0 or <0.5 109/L
Neutrophil/lymphocyte ratio	>29.39
CRP	>29 mg/L
pH	<7.31
Lactate	>3.7

BP= blood pressure, mmHg= millimetres of mercury, bpm=beats per minute.

Univariate analysis

Univariate association between 30-day mortality and each potential predictor variable was analysed using a Pearson X^2 test. Missing data was typically less than 1% with confusion, total protein, and blood gas indices demonstrating missing data >10%.

Significant associations were demonstrated in 28 predictor variables as shown in Table 3-14.

Table 3-14: Univariate analysis to predict 30-day mortality.

Clinical/Biochemical Feature	Number of cases included (%)	Odds Ratio (95% CI)	*p* value
Age <61, 61-88, ≥89	**1545 (100)**		**<0.001**
Sex	1545 (100)	0.887 (0.687 – 1.144)	0.355
RR>24	**1541 (99.7)**	**2.129 (1.629 – 2.783)**	**<0.001**
SBP<80	**1541 (99.7)**	**4.296 (2.262 – 8.159)**	**<0.001**
DBP<60	**1541 (99.7)**	**1.934 (1.453 – 2.575)**	**<0.001**
Required O2 on admission	**1544 (99.9)**	**2.419 (1.837 – 3.183)**	**<0.001**
Temp <35.4	**1536 (99.4)**	**4.087 (2.674 – 6.246)**	**<0.001**
Temp >37.4	**1536 (99.4)**	**0.602 (0.441 – 0.823)**	**0.001**
HR >120	**1541 (99.7)**	**1.452 (1.066 – 1.976)**	**0.017**
Confused	**1315 (85.1)**	**2.543 (1.862 – 3.231)**	**<0.001**
Urea >9	**1540 (99.7)**	**3.003 (2.311 – 3.901)**	**<0.001**
Creatinine >130	**1540 (99.7)**	**1.816 (1.365 – 2.418)**	**<0.001**
White cell count >11.0	1541 (99.7)	1.280 (0.979 – 1.673)	0.070
Total Protein <65	**1252 (81.0)**	**2.235 (1.680 – 2.975)**	**<0.001**
Albumin <32, 32-41, ≥42	**1511 (97.8)**		**<0.001**
Bilirubin >30	1508 (97.6)	1.666 (0.979 – 2.834)	0.057
Haemoglobin <110	**1541 (99.7)**	**1.598 (1.215 – 2.101)**	**0.001**
Platelet <150	1541 (99.7)	1.375 (0.994 – 1.901)	0.053
Neutrophil count >7.5	**1541 (99.7)**	**1.490 (1.107 – 2.005)**	**0.008**
Lymphocyte count >4.0	**1541 (99.7)**	**2.554 (0.997 – 6.545)**	**0.043**
Lymphocyte count <0.5	**1541 (99.7)**	**1.872 (1.403 – 2.499)**	**<0.001**
Neutrophil: lymphocyte ratio >29.39	**1541 (99.7)**	**2.552 (1.853 – 3.514)**	**<0.001**
C-reactive protein >29	**1520 (98.4)**	**1.740 (1.194 – 2.534)**	**0.004**
pH <7.31	**1340 (86.7)**	**3.945 (2.823 – 5.513)**	**<0.001**

Lactate >3.7	**1318 (85.3)**	**3.516 (2.577 – 4.797)**	**<0.001**
Cardiac disease	1545 (100)	1.252 (0.968 – 1.619)	0.087
Diabetes Mellitus	1545 (100)	0.744 (0.530 – 1.043)	0.085
Dementia	**1545 (100)**	**1.579 (1.091 – 2.285)**	**0.015**
Chronic kidney disease	1545 (100)	1.256 (0.751 – 2.102)	0.385
Cerebrovascular disease	**1545 (100)**	**1.478 (1.008 – 2.167)**	**0.044**
Solid malignancy	**1545 (100)**	**2.039 (1.464 – 2.840)**	**<0.001**
Haematological malignancy	**1545 (100)**	**0.548 (0.309 – 0.972)**	**0.037**
Metabolic disease	**1545 (100)**	**0.573 (0.382 – 0.859)**	**0.007**
Neurological Disease	1545 (100)	0.853 (0.518 – 1.404)	0.532
Endocrinopathy*	1545 (100)	1.024 (0.631 – 1.660)	0.925
Chronic pulmonary disease	1545 (100)	0.811 (0.621 – 1.059)	0.124
Rheumatological disease	1545 (100)	0.732 (0.472 – 1.134)	0.161
Psychiatric disease	**1545 (100)**	**0.615 (0.385 – 0.982)**	**0.040**
Gastrointestinal disease (excluding liver)	1545 (100)	0.724 (0.417 – 1.113)	0.139
Chronic liver disease	1545 (100)	0.914 (0.375 – 2.228)	0.843
Other co-morbidity	**1545 (100)**	**1.818 (1.049 – 3.152)**	**0.031**
Solid organ transplant	**1545 (100)**	**0.155 (0.021 – 1.148)**	**0.036**
Benign prostatic hypertrophy	1545 (100)	0.851 (0.394 – 1.837)	0.680
Peripheral vascular disease	1545 (100)	1.070 (0.433 – 2.641)	0.884
Leg ulcers	1545 (100)	0.265 (0.035 – 2.003)	0.166
No co-morbidity	1545 (100)	0.826 (0.513 – 1.330)	0.431
Statin	1545 (100)	0.912 (0.693 – 1.202)	0.515
Systemic steroid	1545 (100)	0.932 (0.708 – 1.226)	0.614
Inhaled corticosteroid	**1545 (100)**	**0.692 (0.510 – 0.939)**	**0.017**
Aspirin	1545 (100)	1.166 (0.874 – 1.557)	0.296

*Endocrinopathy excluding diabetes mellitus. Significant features shown in bold text.

Multivariate analysis

A stepwise, forward, logistic regression analysis was performed with 30-day mortality as the dependent variable using predictor variables which reached significance in the univariate analysis. 956 cases were included in the analysis. Table 3-15 shows which variables that were identified as significantly associated with 30-day mortality in the forward stepwise model.

Table 3-15: Stepwise, forward logistic regression analysis

Feature	Odds Ratio (95% CI)	*p* value
Age (61-88 compared to <61)	**5.628 (2.608 – 12.143)**	**<0.001**
Age (>88 compared to <61)	**8.510 (3.693 – 19.612)**	**<0.001**
Respiratory rate>24		0.059
Systolic BP<80		0.716
Diastolic BP<60		0.489
Required O2 on admission	**1.659 (1.118 – 2.462)**	**0.012**
Temp <35.4	**2.487 (1.360 – 4.549)**	**0.003**
Temp >37.4		0.832
Heartrate >120		0.349
Confused	**1.544 (1.052 – 2.265)**	**0.026**
Urea >9		0.060
Creatinine >130		0.288
Total Protein <65		0.734
Albumin Overall (<32, 32-41, ≥42)		**<0.001**
Albumin (<32 compared to ≥42)	**11.261 (5.042 – 25.151)**	**<0.001**
Albumin (32-41 compared to ≥42)	**3.240 (1.535 – 6.842)**	**0.002**
Haemoglobin <110		0.991
Neutrophils >7.5		0.908
Lymphocytes >4.0		0.779

Lymphocytes< 0.5		0.877
Neutrophil: lymphocyte ratio >29.39	**2.385 (1.537 – 3.702)**	**<0.001**
C-reactive protein >29		0.451
pH <7.31	2.372 (1.483 – 3.794)	<0.001
Lactate >3.7	2.858 (1.868 – 4.371)	<0.001
Dementia		0.243
Cerebrovascular disease		0.278
Solid malignancy	1.871 (1.178 – 2.972)	0.008
Haematological malignancy		0.273
Metabolic disease	0.558 (0.316 – 0.986)	0.044
Psychiatric disease		0.153
Other co-morbidity		0.713
Solid organ transplant		0.200
Inhaled corticosteroid		0.286

To maximise the number of cases included in the analysis, total protein was removed from the model as it was a frequent cause of missing data. This increased the number of cases included in the model to 1168, and number of deaths included increased to 244 from 206. A stepwise, forward, logistic regression analysis was performed, and the table below shows which variables were identified as significantly associated with 30-day mortality and so included in the model. Factors reaching significance are in bold text.

Table 3-16: Stepwise, forward logistic regression analysis with variables forced out.

Clinical/Biochemical Feature	Odds Ratio (95% CI)	*P* value
Age overall (<61, 61-88, ≥89)		**<0.001**
Age (61-88 compared to <61)	**4.635 (2.331 – 9.219)**	**<0.001**
Age (>88 compared to <61)	**7.668 (3.657 – 16.077)**	**<0.001**
Respiratory Rate >24	**1.560 (1.100 – 2.212)**	**0.013**
Systolic BP <80		0.277
Diastolic BP <60		0.559
Required O2 on admission	**1.517 (1.059 – 2.174)**	**0.023**
Temperature <35.4	**2.403 (1.389 – 4.155)**	**0.002**
Temperature >37.4		0.506
Heart rate >120		0.691
Confused		0.080
Urea >9	**1.732 (1.235 – 2.429)**	**0.001**
Creatinine >130		0.077
Albumin Overall (<32, 32-41, ≥42)		**<0.001**
Albumin (<32 compared to ≥42)	**10.034 (4.899 – 20.550)**	**<0.001**
Albumin (32-41 compared to ≥42)	**3.309 (1.699 – 6.443)**	**<0.001**
Haemoglobin <110		0.610
Neutrophils >7.5		0.898
Lymphocytes >4.0		0.295
Lymphocytes <0.5		0.826
Neutrophil: lymphocyte ratio >29.39	**2.146 (1.432 – 3.214)**	**<0.001**
C-Reactive Protein >29		0.430
pH <7.31	**2.304 (1.494 – 3.553)**	**<0.001**
Lactate >3.7	**2.052 (1.380 – 3.053)**	**<0.001**
Dementia		0.726
Cerebrovascular disease		0.340

Solid malignancy	**1.992 (1.302 – 3.050)**	**<0.001**
Haematological malignancy		0.230
Metabolic disease	**0.583 (0.347 – 0.980)**	**0.042**
Psychiatric disease		0.276
Other co-morbidity		0.894
Solid organ transplant		0.125
Inhaled corticosteroid		0.237

This analysis was repeated using a backward regression method which confirmed that the same variables were found to be significantly associated with 30-day mortality, except for metabolic disease which did not meet significance in the backward regression model (p=0.051).

Having identified these factors as independently associated with 30-day mortality, these features were assessed for co-linearity by calculating the variance inflation factor (VIF). There was no evidence of co-linearity between the factors in Table 3-16 (all VIFs <2.0).

Calculation of Novel Score

To weight factors appropriately predictive factors were assigned a score value related to their OR as seen in Table 3-17. Solid malignancy was forced out of the model to reduce complexity of calculation as it has not been demonstrated to be associated in other models and had a low OR relative to other predictor variables.

Table 3-17: Score Generation

Clinical/Biochemical Feature	Odds Ratio (95% CI)	Score value
Age (61-88 compared to <61)	4.635 (2.331 – 9.219)	5
Age (>88 compared to <61)	7.668 (3.657 – 16.077)	8
Respiratory Rate>24	1.560 (1.100 – 2.212)	2
Required O2 on admission	1.517 (1.059 – 2.174)	2
Temperature <35.4	2.403 (1.389 – 4.155)	2
Urea>9	1.732 (1.235 – 2.429)	2
Albumin (<32 compared to 42+)	10.034 (4.899 – 20.550)	10
Albumin (32-41 compared to 42+)	3.309 (1.699 – 6.443)	3
Neutrophil: lymphocyte ratio>29.39	2.146 (1.432 – 3.214)	2
pH<7.31	2.304 (1.494 – 3.553)	2
Lactate>3.7	2.52 (0.380 – 3.053)	2

3.5.6. Missing data

Missing data was initially handled by complete case analysis as most variables had missing data in <1% cases, however key variables (pH, lactate, confusion and albumin) had missing data exceeding 10% (Table 3-14). Multiple imputation was carried out using mi package in Stata. Univariate & multivariate analyses were repeated in the multiple imputation datasets and there were no significant differences noted. Therefore, complete case analysis was used for all analyses presented.

3.5.7. Performance of Birmingham Community Acquired Pneumonia score (BCAPs) in derivation cohort.

BCAPS was calculated in 1156 (75%) of derivation cohort cases, cases were excluded if missing data were present. Leading causes of missing data were lactate, pH and confusion (Table 3-14).

BCAPS was able to predict 30-day mortality more accurately than CURB65 (AUC BCAPS: 0.811 vs 0.6799, p <0.0001 by DeLongs test).

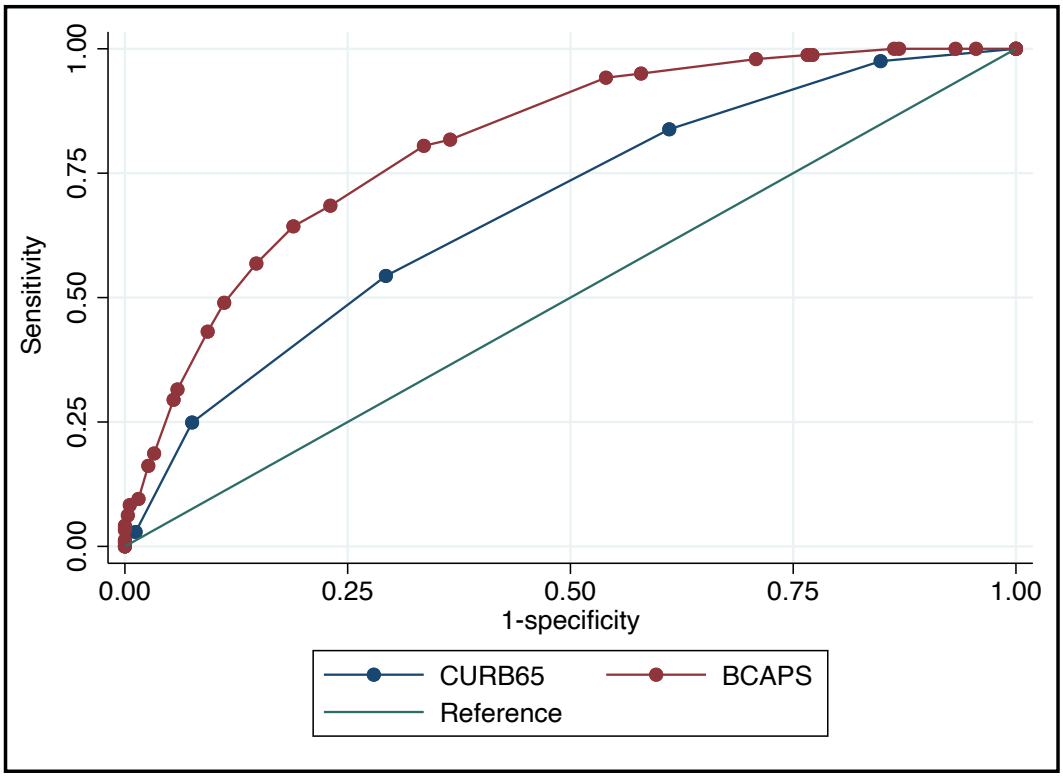

Figure 3-5: Comparison of BCAPs and CURB65 to predict 30-day mortality

Receiver operator curve analysis was carried out on 1156 cases with complete data to compare BCAPS and CURB65. AUC was BCAPS:0.811 vs 0.6799, p-value <0.0001 by DeLongs test. BCAPs: Birmingham Community Acquired Pneumonia score. AUC: area under curve.

Distribution of BCAPs was significantly altered in people who died within 30 days of admission (Figure 3-6). BCAPS was significantly higher in people who died within 30-days of admission (15 [IQR7]) vs. 10 [IQR 5] p=0.0001 by Kruskal-Wallis)

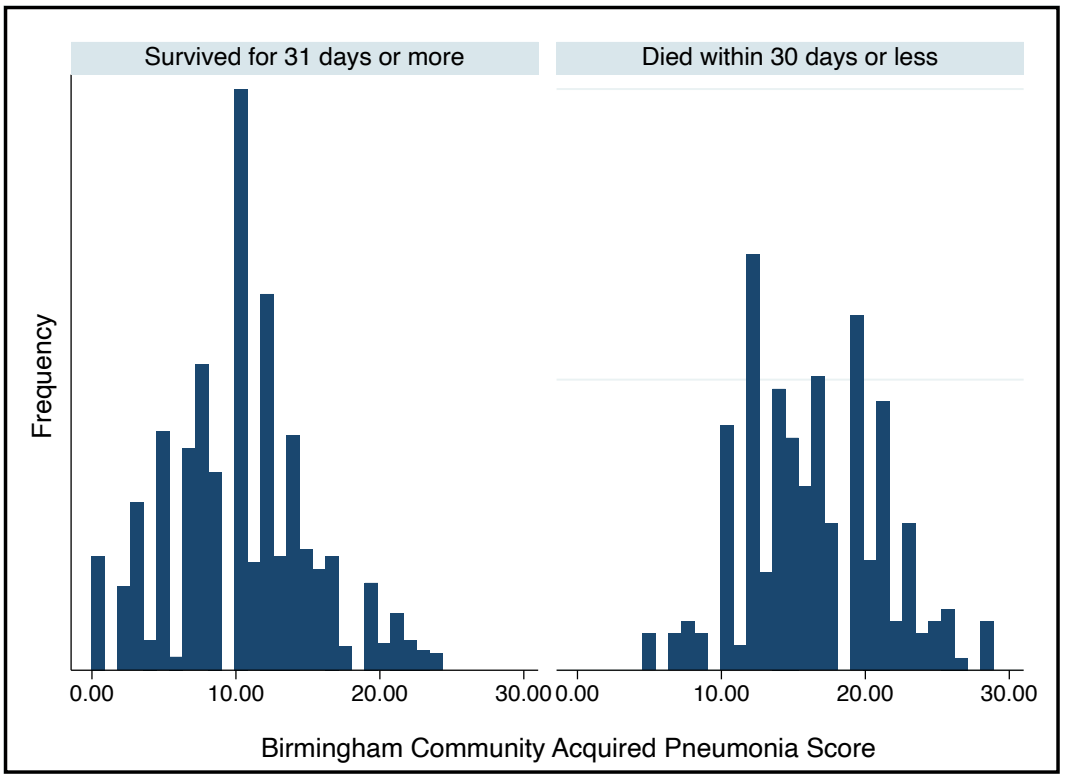

Figure 3-6: Distribution of BCAPs dependent on 30-day mortality

Histogram demonstrating distribution of BCAPS score according to 30-day mortality. BCAPS was significantly higher in people who died within 30-days of admission. (15 [IQR7]) vs. 10 [IQR 5] *p*-value: 0.0001 by Kruskal-Wallis.

To further examine the utility of BCAPS, BCAPS was discretised into optimal bins with 30-day mortality as the reference which resulted in three risk categories in Table 3-18.

Table 3-18: Development of risk categories for BCAPS

	BCAPS score	Survived ≥30 days	Died <30 days (%)
Low	0-10	628	50 (7)
Moderate	10-14	218	60 (21)
High	>14	147	150 (50)

Low score:<10, moderate score: 10-14, high score:>14.

BCAPS performance characteristics are defined in Table 3-19. BCAPS ≥moderate had sensitivity 80.7% and specificity 63.2% with +LR 2.2 and low +LR 0.3 to predict 30-day mortality.

Table 3-19: Performance Characteristics of BCAPS

Cut-point	Sensitivity	Specificity	+LR	-LR
≥Moderate	80.7	63.2	2.2	0.3
≥High	57.7	85.2	3.9	0.5

Moderate score: 10-14, high score:>14. +LR: positive likelihood ratio, -LR: negative likelihood ratio.

BCAPS to predict long-term mortality.

Ability of BCAPS to predict 90 and 365-day mortality was tested with CURB65 as the gold standard. BCAPS was significantly better at predicting 90 and 365-day mortality than CURB65.

Table 3-20: Ability of BCAPS to predict long-term mortality.

	Area under curve	*P* value
90-day mortality		
CURB65	0.680	<0.0001
BCAPS	0.780	
365-day mortality		
CURB65	0.674	<0.0001
BCAPS	0.761	

BCAPS: Birmingham Community Acquired Pneumonia Score

BCAPS in different age groups

BCAPS demonstrated reduced accuracy to predict 30-day mortality as age increased, with best predictive accuracy in people <64 years of age (p= 0.0223)

Table 3-21: ROC analysis of BCAPS in age groups

Age Group	Event rate (%)	Area under curve (S.E)	*p*-value
<64 years	28/428 (7)	0.8449 (0.03)	0.0223
65-74 years	42/232 (15)	0.8427 (0.03)	
75-84 years	39/175 (22)	0.7403 (0.03)	
>85 years	129/432 (29)	0.7569 (0.03)	

S.E: standard error.

BCAPS by multi-morbidity

BCAPS performed significantly better in people without multi-morbidity (AUC: 0.8856) than in people with multi-morbidity (AUC: 0.7698), p <0.001 by DeLongs test.

3.5.8. Validation of BCAPS in external dataset

A further cohort of physician validated CAP cases from 2016 were sought from QEHB. 1515 cases were reviewed to generate a cohort of 824 cases (Figure 3-7). The derivation and validation cohorts were well matched for age, gender and outcome measures. Presence of multi-morbidity was significantly higher in the validation cohort (91.3% vs 93.6%, p<0.001). Distribution of CURB65 and qSOFA were also different (Figure 3-8).

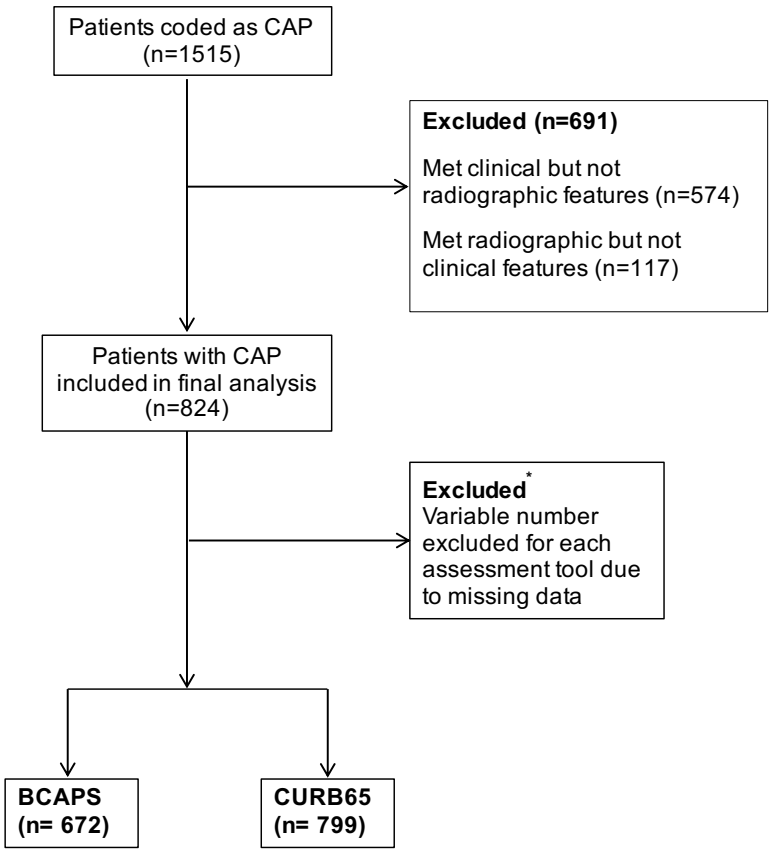

Figure 3-7: Modified consort diagram for Validation cohort.

*Reasons for exclusion of patients for each severity assessment tool (No. of cases excluded). NB Some cases were excluded due to more than one missing data point: CURB65: confusion (8), urea (10), respiratory rate (12), blood pressure (13), age (0). BCAPS: Age (0), respiratory rate (12), Oxygen on admission (12), Temperature (12), urea (10), Albumin (24), Neutrophil: lymphocyte ratio (13), lactate (94) and pH (82). CAP: community acquired pneumonia, BCAPS: Birmingham community acquired pneumonia score.

Table 3-22: Summary Characteristics of Derivation and Validation Datasets

	Derivation	Validation	*P* value
Number of cases	1545	824	
Age	77 (63-86)	75 (61-84)	0.060
Female Sex	760 (49.2)	410 (49.8)	0.740
Presence of co-morbidity	**1441 (91.3)**	**771 (93.6)**	**<0.001**
CURB65 score	2 (1-3)	2 (1-3)	0.027
qSOFA score	1 (0-1)	1 (0-1)	0.010
ICU admission	99 (6.4)	64 (7.8)	0.183
Inpatient death	238 (15.4)	112 (13.6)	0.380
30-day mortality	293 (19.0)	139 (16.9)	0.347
90-day mortality	390 (25.2)	189 (22.9)	0.353
360-day mortality	511 (33.1)	295 (35.8)	0.315

qSOFA: quick sequential organ failure assessment, ICU: intensive care unit. Chi-square for categorical variables and Mann-Whitney for continuous variables.

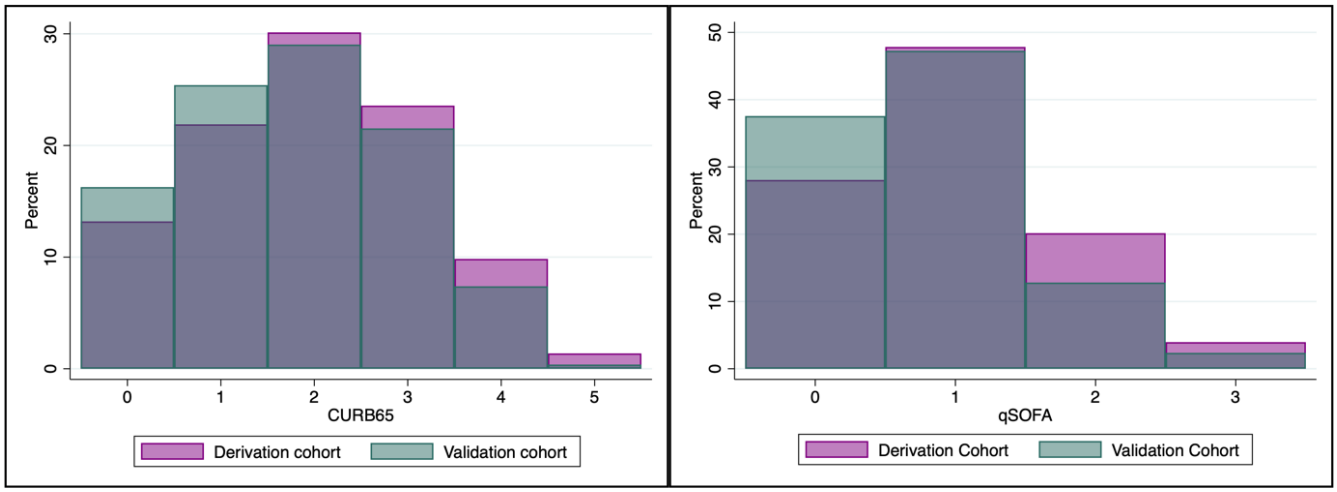

Figure 3-8: Distribution of CURB65 and qSOFA.

Histogram showing distribution of CURB65 and qSOFA across derivation and validation cohorts. In both scores there is a shift to lower scores in the validation cohort, *p*-value:0.027 for CURB65 and 0.01 for qSOFA.

Missing data in validation cohort

Scores were calculated in 799 (97%) cases for CURB65 and 672 (81%) cases for BCAPS. Missing data values are in Table 3-23. Overall missing data was low, however blood gas indices led to higher missing data values. However, there were still sufficient cases to meet the power calculation (n=635).

Table 3-23: Missing data in validation cohort.

Variable	Missing data (%)
Confusion	8 (1.0)
Temperature	12 (1.4)
Blood Pressure	13 (1.5)
Heartrate	7 (0.8)
Respiratory rate	12 (1.4)
Oxygen requirement	12 (1.4)
Albumin	24 (2.9)
pH	82 (9.9)
Lactate	94 (11.3)
NLR	10 (1.2)
Urea	10 (1.2)

NLR: neutrophil: lymphocyte ratio

Utility of BCAPS to predict 30-day mortality in validation cohort.

BCAPS was more accurate in predicting 30-day mortality than CURB65 (AUC:0.80 vs. 0.72, p=0.0009 by DeLongs test.

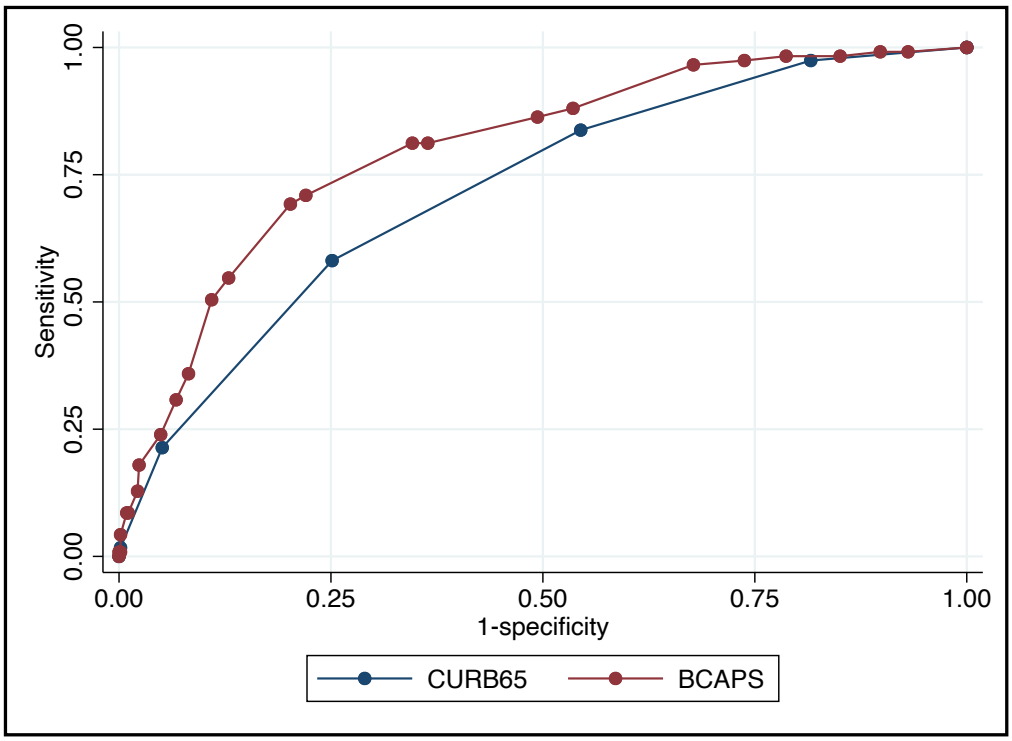

Figure 3-9: BCAPS compared to CURB65 to predict 30-day mortality

Receiver operator curve analysis was carried out on 672 cases with complete data to compare BCAPS to CURB65. AUC: CURB65 0.720 vs. 0.800 in BCAPS, *p*-value:0.0009 by DeLongs test. BCAPs: Birmingham Community Acquired Pneumonia score. AUC: area under curve.

Performance characteristics in validation cohort

Using a moderate cut-off for both BCAPS and CURB65, BCAPS demonstrated lower sensitivity (80.7% vs. 83.8%) whereas BCAPS demonstrated better specificity (65.7% vs. 46.9%). Overall BCAPS demonstrated higher positive likelihood ratios for 30-day mortality.

Table 3-24: Performance characteristics of BCAPS in Validation Cohort

Cut-point	Sensitivity (%)	Specificity (%)	+LR	-LR
≥Moderate	80.7	65.7	2.3	0.3
≥High	50.4	89.2	4.6	0.6

Moderate score: 10-14, high score:>14. +LR: positive likelihood ratio, -LR: negative likelihood ratio.

Table 3-25: Performance Characteristics of CURB65 in Validation Cohort

Cut-point	Sensitivity (%)	Specificity (%)	+LR	-LR
≥1	97.8	19.1	1.2	0.1
≥2	83.8	46.9	1.6	0.3
≥3	55.8	76.2	2.3	0.6
≥4	20.6	94.9	4.0	0.8
≥5	1.5	99.9	9.7	1.0

+LR: positive likelihood ratio, -LR: negative likelihood ratio.

Validation BCAPS in varying age groups

In the validation cohort BCAPS performed equally in all age groups (p=0.7842).

Table 3-26: Performance of BCAPS across age groups in validation cohort

Age Group	Event rate (%)	Area under curve (S.E)	*p*-value
<64 years	18/225 (8)	0.8185 (0.06)	0.7842
65-74 years	23/125 (18)	0.7624 (0.06)	
75-84 years	39/175 (20)	0.7537 (0.04)	
>85 years	59/147 (40)	0.7371 (0.04)	

Validation of BCAPS in participants with multi-morbidity

BCAPs was more accurate in people without multi-morbidity than people with multi-morbidity (AUC 0.893 vs. 0.777), p= 0.0037 by DeLongs.

3.6. Discussion

CURB65 is the recommended tool of choice to assess CAP severity and guide clinical decision making in the UK. However, real-world implementation CURB65 is variable, it is unclear why CURB65 is not used more widely. It could reflect the lack of studies demonstrating clinical utility and impact on outcomes. This study demonstrates that of existing scores CURB65 provides greatest accuracy in prediction of 30-day mortality but is less accurate in the presence of multimorbidity. This data was used to generate a novel severity assessment tool for use in CAP: BCAPS, BCAPS was more accurate than CURB65 at predicting mortality, but demonstrated similar limitations in older people and presence of co-morbidity.

3.6.1. CURB65

CURB65 predicts 30-day mortality with greater accuracy than generic sepsis or early warning scores in a large cohort of physician validated CAP with real world co-morbidities. In this study, age did not influence the accuracy of 30-day mortality prediction as seen in other studies (Parsonage et al., 2009; Chen et al., 2010). However, presence of multimorbidity did significantly decrease the accuracy of CURB65 to predict mortality, but presence of individual common co-morbidities alone did not influence prediction. This is the first demonstration of impact of multimorbidity in CURB65. Further, this study confirms that CURB65 has very limited ability to predict adverse outcomes other than death. CURB65 performs better at low values and is therefore optimised to identify low risk cases rather than those at high risk, in this study CURB65 score of 0 indicates a negative likelihood ratio 0.1 suggesting 30-day mortality is very unlikely in that group so CURB65. In this study and others CURB65 has been demonstrated to

predict mortality more accurately than generic tools, this suggests that pneumonia specific tools should be used to guide care even in the presence of sepsis. qSOFA performed particularly poorly compared to CURB65 and NEWS. It is important to note that at the time of data collection both NEWS and CURB65 were in common clinical use, however qSOFA was not published until February 2016 so was therefore unlikely to have been in common clinical use. Therefore, NEWS and CURB65 may have been used to guide management and therefore outcomes which may reflect some of their increased accuracy compared to qSOFA. Further qSOFA was designed to predict in-patient mortality in suspected infection rather than 30-day mortality in CAP. This study reflects others that have demonstrated qSOFA is a poor predictor of mortality in CAP (Kolditz et al., 2016; Muller et al., 2017; Jiang et al., 2018).

3.6.2. Birmingham Community Acquired Pneumonia Score

A novel score was derived, aiming to improve precision in predicting 30-day mortality compared to the gold standard of CURB65. BCAPS is a composite score consisting of nine variables which are commonly measured in clinical practice. It was derived in a pragmatic cohort without excluding any co-morbidity or social factors.

BCAPS demonstrates greater accuracy than CURB65 in prediction of 30-day mortality. BCAPS is also more accurate at predicting longer term mortality at 90- and 365-days following admission. However, accuracy of BCAPS is impacted by increasing age, and presence of multi-morbidity in the derivation cohort only.

BCAPS has been validated in cohort separated by time, but not hospital site. In this validation cohort BCAPS demonstrated increased accuracy to predict 30-day mortality

compared to CURB65. In the validation cohort the ability of BCAPS to predict mortality was not significantly impacted by age, although this data should be interpreted with caution as the numbers are much smaller and the power calculation for overall comparison was 635 participants whereas each age group only compared 120-209 participants. Further mortality rates in participants >85 years was much lower in the validation cohort than derivation cohort. This difference shows the importance of validation in external datasets.

BCAPS was impacted by presence of multi-morbidity; in participants without multi-morbidity mortality prediction was significantly more accurate. This was also true for CURB65 and given the prevalence of multimorbidity suggests that any future tools should consider these composite assessments.

Other studies have shown that ability of CURB65 to predict mortality is influenced by increasing age. This is logical as CURB65 performs best lower scores, and age ≥65 means a minimum score of 1, so older adults will always score higher. None the less this analysis should be interpreted with caution as there are relatively few participants in each group. Given the published variance in CURB65 in older adults BCAPS was also assessed in the same way, in the derivation cohort BCAPS had better accuracy at lower age groups, but in the validation data BCAPS performed equivalently across all age groups. Performance of CAP tools at increased age is crucial. Median age of hospitalised CAP is 75-77 years in this study, and similar to national audit data (W. Lim and Lawrence, 2019). Here, 30-day mortality increases 5-fold in >85 years compared to <64 years suggesting that a binary cut-off of 65 years may not be identifying the same risk population as identified in the 2003 derivation and validation studies. BCAPS uses

two risk categories (61-88 and >88 years) with weighted scores to indicate increasing risk with increasing mortality, but even this change to allow for increasing risk suggests that dichotomising continuous variables impacts sensitivity and therefore performance. Sensitivity analyses to test score performance in presence of co-morbidities demonstrated that presence of individual common co-morbidities (CVS disease, pulmonary disease, diabetes mellitus and dementia) did not impact predictive capacity of CURB65 or BCAPS. However, presence of multi-morbidity (defined as ≥2 long-term conditions) significantly impacted accuracy with greater accuracy in people without multi-morbidity. This is an important observation as between 91-93% of participants in this study had multi-morbidity, and this reflects estimates from other studies of CAP (Cillóniz et al., 2013). Multimorbidity is expected to rise over the coming decade (Kingston, Robinson, Booth, Knapp and Jagger, 2018), therefore any severity assessment tool needs to perform well in this population. It is interesting to note that presence of single pathologies alone did not influence predictive ability. This may reflect the complexity of managing multiple co-morbidities- e.g., balancing cardiac failure with chronic renal failure and potential use of nephrotoxic drugs. Co-morbidities are predictive of complications in-hospital (Corrales-Medina et al., 2011), mortality and re-admissions (Cillóniz et al., 2013). Hospitalisation with CAP increases rates of exacerbations of cardiovascular and respiratory disease in the following year (Bornheimer et al., 2017; Wagenvoort et al., 2017). This data suggests that rather than considering single co-morbidities, severity assessment tools should consider composite measures such as multi-morbidity. This study used a pragmatic and broad definition as used in NICE guidance of ≥2 long-term health conditions. This definition has been criticised as clearly

some long-term health conditions confer greater risk than others but is an easily applied variable to existing datasets. Another composite measure which would be interesting to explore would be frailty. Frailty is common in older adults and has significant overlap with multi-morbidity. Again, frailty influences risk of developing CAP, risk of complications and mortality (Iwai-Saito et al., 2021), therefore including these composite variables may improve predictive accuracy.

Mortality from CAP has fallen by 50% in the past decade, but re-admissions are increasing (W. S. Lim and Lawrence, 2019). Unplanned re-admission is frequently used as a surrogate of health care quality, in some healthcare systems reduction of unplanned readmissions is used as a financial incentive. The inevitability of readmission following CAP has been explored, where following expert case note review only in 14% of cases was re-admission deemed to have been avoidable (Boussat et al., 2022). People who are readmitted are more complex with greater numbers of co-morbid conditions, higher CCI and higher levels of socio-economic deprivation (Andruska et al., 2015; Lawrence, McKeever and Lim, 2023). The mantra of reducing mortality in CAP has significantly benefitted many, but this may not be the priority of people with CAP. CAP is predominantly a disease of older adults. Studies investigating healthcare priorities in older adults with chronic health conditions have consistently demonstrated that maintaining independence is the highest priority followed by survival (Stegmann et al., 2019). Healthcare priorities amongst older adults in CAP specifically have been investigated in small sub-study of an RCT in CAP, Sapey *et al* showed in 80% of participants avoiding readmission was the highest priority, mortality was only the highest

priority in 7% (Sapey et al., 2019). Individual patient preferences clearly demonstrate maintenance of independence is key, but from a health economic perspective- maintained independence and avoidance of readmission would be beneficial. Further score development should consider important patient preferences for outcome. Being able to accurately identify people at high risk of readmission or other adverse outcomes would enable stratification and facilitate research in re-admissions.

The validation study presented here confirms that BCAPS is an accurate predictor of 30-day mortality in CAP. The validation dataset was collected at a different time to the derivation data and therefore will be liable to variation in case mix, treating physicians and changing guidance which increases confidence in BCAPS. However, the validation data is from the same hospital site.

To further develop this tool BCAPS requires validation in external datasets, and ideally these would include implementation studies which demonstrate the utility of BCAPS compared to CURB65 the gold standard. Implementation studies in CAP are also limited by lack of directed therapies. Other than antibiotics the evidence for management in CAP in areas such as fluid therapy, nutrition, mobilisation is scarce and usually relies on extrapolation or expert opinion. Therefore, use of more discriminatory tools which can predict other adverse outcomes may be better placed in research where they can provide baseline stratification on basis of severity, but also identify participants who are at high risk of a specific outcome to give targeted therapy whether that be drug targets or mobilisation for example. The recent Cape Cod trial gives a good rationale for this. They identified that steroids improved outcomes in people with severe CAP defined by need for ventilatory support but without septic shock, they also showed compared to

other trials that early intervention is likely to be key (Dequin et al., 2023). Therefore, a specific tool which predicts likelihood of severe CAP requiring IMV at point of admission would enable early steroid treatment without the risk of steroids in people who are unlikely to benefit.

This study developed a physician validated cohort of CAP to derive and validate BCAPS. Typically, large studies have used ICD coding to identify cases, but coding is inaccurate in approximately 40% of cases (Lindenauer et al., 2012; P Daniel et al., 2016) this results in a dataset which is inaccurate and contains primary diagnoses other than CAP. The derivation study collected data on over forty variables. The choice of variables was based on existing literature which included the established severity assessment tools but also more recent evidence for poor outcomes. To generate a clinical decision tool continuous variables were dichotomised, but to standardise this approach and identify cut-offs in a methodologically sound way a combination of factors known from clinical expertise and statistical methods were used, multiple cut-offs were tested to identify the cut-offs most predictive of 30-day mortality. These multiple cut-offs were then weighted according to odds ratio.

The sample size was appropriate for validation study, with sample size calculation generated from derivation data with an estimate of model over-fitting accounted for. This dataset contains minimal missing data <1% for most variables, and multiple imputation was completed to check for impact of missing data.

3.6.3. Limitations

There are important limitations to this study. It is a UK focussed study which did not consider PSI, as the accepted gold standard tool in the UK is CURB65. Comparison of PSI was not considered as it requires PaO2 which is not routinely measured in CAP. The derivation dataset was not collected based on a power calculation; it was a pragmatic approach to collecting consecutive CAP admissions with physician validated diagnoses over two years. Using pmsampsize package in Stata the suggested sample size to use the predictor variables (43) collected in this study with an event rate (30-day mortality) of 19% would indicate a sample size of 5161 cases. Given the rate of missing data (~20%) and inaccuracy of ICD coding (~40%) this would mean screening ~8000 CAP cases to find ~5000 validated cases with complete data. This could be reduced by considering fewer predictor variables in initial model development. Reducing candidate predictor variables to 10 reduces sample size to 1201 cases. The major barrier to use of large datasets in CAP is diagnostic inaccuracy, typically cases that are coded as CAP are removed from datasets due to lack of new radiological changes. In the validation dataset 46% ICD coded cases were excluded, of these excluded cases 83% were due to lack of radiological features of CAP. Manual case note review is labour intensive, but recent developments in AI suggest that AI could be used to interpret chest radiographs in CAP therefore increasing diagnostic accuracy of ICD coded CAP (Becker et al., 2022).

This study dichotomised continuous variables for ease and accessibility of score calculations, despite having tested multiple cut-offs for best accuracy this approach has been its limitations. Continuous variables are rarely linear predictors and modelling as

such can impact power and confounding in subsequent use of the tool. The recommended approach is to transform continuous variables using fractional polynomials or similar, however this is not commonly implemented in clinical research tools due to the perceived complexity. But given the increasing computational power of statistical programmes this should be considered for future development.

This study requires external validation, preferably in a multicentre cohort to ensure that geographical variation in case mix and CAP management does not impact accuracy of mortality prediction. Further if established to be accurate BCAPS would need to undergo implementation studies to demonstrate clinical utility and safety. A typical implementation study would compare BCAPS to the gold standard of risk stratification (CURB65) when used to guide ambulatory care. Implementation studies of a novel score may be challenging due to the widespread use of CURB65 in national audit programmes which may influence clinical equipoise, however there is very limited data to support clinical utility of CURB65 and this data demonstrates that its accuracy appears to have waned over the past twenty years. However, the poor uptake of CURB65 in clinical practice may suggest that the limited clinical utility is recognised and therefore they rely on clinical gestalt.

Identification of patient priorities in CAP, especially in older adults with multimorbidity will benefit CAP research as a whole. But specifically in prognostic research it may help refine score development for specific research priorities such as readmission or long-term mortality.

3.7. Summary

Predictive ability of CURB65 appears to have waned over the past twenty years, this may reflect changing demographics with increasing age and prevalence of multi-morbidity. CURB65 underperforms in older adults and people with multi-morbidity- this population represents the cohort most at risk of CURB65. Further CURB65 has very limited data to support its clinical utility despite widespread use in national audit programmes. The lack of clinical utility may be the driver for poor uptake in clinical practice. In a cohort of validated CAP cases a novel score was derived which shows increased accuracy in prediction of 30-, 90- and 365-day mortality compared to CURB65. The novel score BCAPS shows similar limitations to CURB65 in older adults and people with multimorbidity, but overall accuracy is significantly greater. BCAPS has undergone internal validation demonstrating its utility but requires further external validation and implementation studies. A severity assessment tool which demonstrates clinical utility will be helpful in clinical practice but also in identifying specific cohorts of patients for research who may have increased mortality, or other adverse outcomes.

4. REAL-TIME ASSESSMENT OF NEUTROPHIL METABOLISM AND OXIDATIVE BURST USING EXTRACELLULAR FLUX ANALYSIS

This chapter is a published manuscript validating use of Extracellular Flux (XF) in isolated human neutrophils to establish metabolic rates *ex-vivo*. This work was required to generate the data in Chapter 5. Typically, XF technology has been used in adherent cell lines, and given the propensity of neutrophils to be activated and undergo apoptosis thorough validation was required.

This was a collaborative piece of work conducted by FG and Alice Jasper. This work is published in Frontiers in Immunology titled **"Real-time assessment of neutrophil metabolism and oxidative burst using extracellular flux analysis"** (Grudzinska et al., 2023b).

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Authors contribution: FG conceived and designed the study, recruited participants and undertook all experimental assays (data in Figures 1-3 and 6-7 were completed jointly with AJ. Data in Figures 4-5 were completed solely by FG), performed data analysis and wrote the initial manuscript. AJ conceived and designed the study and undertook experimental assays (data in Figures 1-3 and 6-7), performed data analysis and reviewed the initial manuscript. ES, DT, MC, AS and JB designed and planned the study and revised the manuscript and approved final version.

4.1. Abstract

Neutrophil responses are critical during inflammatory and infective events, and neutrophil dysregulation has been associated with poor patient outcomes. Immunometabolism is a rapidly growing field that has provided insights into cellular functions in health and disease. Neutrophils are highly glycolytic when activated, with inhibition of glycolysis associated with functional deficits. There is currently very limited data available assessing metabolism in neutrophils.

Extracellular flux (XF) analysis assesses real time oxygen consumption and the rate of proton efflux in cells. This technology allows for the automated addition of inhibitors and stimulants to visualise the effect on metabolism.

We describe optimised protocols for an XFe96 XF Analyser to (i) probe glycolysis in neutrophils under basal and stimulated conditions, (ii) probe phorbol 12-myristate 13-acetate induced oxidative burst, and (iii) highlight challenges of using XF technology to examine mitochondrial function in neutrophils. We provide an overview of how to analyse XF data and identify pitfalls of probing neutrophil metabolism with XF analysis.

In summary we describe robust methods for assessing glycolysis and oxidative burst in human neutrophils and discuss the challenges around using this technique to assess mitochondrial respiration. XF technology is a powerful platform with a user-friendly interface and data analysis templates, however we suggest caution when assessing neutrophil mitochondrial respiration.

4.2. Introduction

Neutrophils are vital in the host response to infection and inflammation. Neutrophil dysfunction is described at the extremes of age, in overwhelming infection, in chronic diseases and is associated with poor outcomes for patients (Grudzinska, Brodlie, et al., 2019; Giam, Shoemark and Chalmers, 2021). In sepsis and other disease states there is broad dysfunction with all neutrophil functions reduced (Alves-Filho, Spiller and Cunha, 2010; Sapey et al., 2017). Ineffective neutrophil responses are harmful during the acute response where dysfunction may drive increased mortality and morbidity (Winkelstein et al., 2000; Patel et al., 2018). Then in the resolution phase persistent neutrophilic responses potentiates tissue damage as seen in acute respiratory distress syndrome (ARDS) (Matthay, Ware and Zimmerman, 2012) and unopposed neutrophil action in alpha-1 anti-trypsin deficiency (McCarthy, Reeves and McElvaney, 2016). In patients with ARDS or sepsis, studies of neutrophil extracellular traps (NETs) demonstrate that there is an optimal level of *ex-vivo* function with poor outcomes for those with the highest or lowest levels of NETosis (Patel et al., 2018; Lefrancais et al., 2018). While alterations to a single cell function suggests involvement of a single molecular pathway, broad cellular dysfunction implicates a central driver such as altered metabolism. Immunometabolism is defined as the role of cellular metabolism in the regulation of immune cell function (Bird, 2019).

Murine work by Sadiku and colleagues has demonstrated that regulation of neutrophil function is linked to metabolism (Sadiku et al., 2017). Neutrophils are highly glycolytic (Borregaard and Herlin, 1982) and inhibition of glycolysis using 2-deoxyglucose (2-DG)

significantly reduces migration, phagocytosis, NETosis and oxidative burst. Neutrophils possess a functional network of mitochondria; however, mitochondrial oxidative phosphorylation does not appear to be required for adenosine triphosphate (ATP) synthesis to meet the energetic demand of neutrophils (Fossati et al., 2003; van Raam et al., 2008; Maianski et al., 2004). The bulk of ATP is synthesised by glycolysis (Bao et al., 2014; Sadiku et al., 2021). Sadiku *et al* demonstrated that neutrophils from patients with chronic obstructive pulmonary disease had lower baseline ATP levels and exhibited reduced glycolysis in response to a pathogen compared to age matched controls (Sadiku et al., 2021). Moreover, the glycolytic capacity of neutrophils can be impacted by treatment with lipopolysaccharide (LPS) (Pan et al., 2022). Developing methods to understand neutrophil metabolism may provide insights into mechanisms underpinning disease and could offer a new therapeutic paradigm, improving outcomes for patients with acute and chronic disease.

Extracellular Flux (XF) analysis assesses real-time measurement of oxygen consumption rate (OCR) and proton efflux rate (PER) of cells. XF technology allows automated addition of different stimuli or inhibitors with flexibility over timing to visualise the effect of stimulation and inhibitory factors on cellular metabolism. XF technology requires low cell numbers relative to other forms of respirometry and allows for simultaneous measurement of PER and OCR as indicators for glycolysis and mitochondrial function, respectively. The streamlined workflow combined with user-friendly interface and analysis templates have seen a worldwide boom in the use of these instruments for probing cellular metabolism. In this manuscript we describe

optimised protocols for using an XFe96 XF Analyser to probe (i) glycolysis in neutrophils under basal and stimulated conditions and (ii) phorbol 12-myristate 130-acetate (PMA)-induced oxidative burst. We highlight limitations of using XF technology to examine mitochondrial function in neutrophils. Finally, we provide an overview of how to analyse XF data and identify pitfalls of probing neutrophil metabolism with XF analysis.

4.3. Materials, Equipment and Methods

4.3.1. Study subjects

11 otherwise healthy volunteers aged 22-49 years old were recruited from staff members in the Queen Elizabeth Hospital Birmingham. Written informed consent was given by all participants in accordance with the University of Birmingham Research Ethics Committee (ERN 12-1185R2).

4.3.2. Isolation of blood neutrophils

Neutrophils are notoriously challenging cells to work with due to their short lifespan and propensity for activation. The upmost care must be taken during cell isolation and assays should be commenced immediately following isolation to reduce risk of apoptosis.

Peripheral blood neutrophils were isolated from lithium-heparin anticoagulated (BD Bioscience, New Jersey, USA) whole venous blood as previously described (M. J. Hughes et al., 2020). Briefly, whole blood was mixed 6:1 with 2% dextran (Merck Life Sciences, UK) (w/v in 0.154 M saline) for 30 minutes to allow erythrocyte sedimentation. Percoll (GE Healthcare, New York, USA) was made using 9:1 v/v Percoll to sterile saline (1.54 M) which was further diluted to 56% and 80% v/v solutions in 0.154 M saline. The buffy coat was layered on Percoll discontinuous gradients and centrifuged at 470 x *g* for 20 minutes with no brake or acceleration. Neutrophils were removed and washed in phosphate-buffered saline (Merck Life Sciences, UK) (PBS) (250 x *g*, 10 minutes, full brake and acceleration) before being resuspended at 4 x 10^6/mL in phenol red and sodium bicarbonate free, filter sterilized Roswell Park Memorial Institute-1640 (RPMI) (Merck Life Sciences, UK, R8755), supplemented with 2mM L-glutamine, 1mM sodium pyruvate,

25mM glucose and 5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Merck Life Sciences, UK), pH 7.4, which will be referred to as XF media. Neutrophil purity was >99% for all assays, except in Figure 7 where neutrophil purity is detailed.

4.3.3. Extracellular Flux Assays

All assays were carried out using XFe96 V3 PS Cell Culture microplates (Agilent Technologies, California, USA) coated with 22.4 µg/mL Corning® Cell-Tak™ (Corning Inc., New York, USA) solution and stored overnight at 4°C. On the day of the assay pre-coated microplates were bought to room temperature and seeded with 2 x 10^5 cells/well (glycolysis and mitochondrial respiration) or 5 x 10^4 cells/well (oxidative burst) in 50µL XF media. Wells A1, A12, H1 and H12 contained only 50 µL/well XF media to account for background correction wells. The XFe96 microplate was centrifuged at 200 x *g* for 1 minute with maximum acceleration but no brake at room temperature before incubation in a non-CO_2, 37°C (PO_2 of 141.4 mmHg) incubator for 25-30 minutes, all incubation steps were carried out under the same conditions. Wells were observed under a light microscope to ensure cell adherence before gently adding 130 µL/well pre-warmed XF media, after addition of media the plate was incubated for a further 15 minutes in a non-CO_2, 37°C incubator. 25 µL of each inhibitor/stimulant was added to injection ports A-C of the sensor cartridge (Agilent Technologies, California, USA) as follows, with stated concentrations referring to final well concentrations: *Glycolysis*- port A: 160 nM PMA (Merck Life Sciences, UK), port B: 2 µg/ml oligomycin (Barlow, Solomon and Affourtit, 2018)(Merck Life Sciences, UK), port C: 50 mM 2-DG (Technologies, 2019)(Merck Life Sciences, UK). *Mitochondrial respiration* - port A: 2 µg/ml oligomycin, port B: 3 µM BAM-

15(Barlow, Solomon and Affourtit, 2018) (Bio-techne, Minneapolis, USA) and port C: 2 µM rotenone/antimycin A (Barlow, Solomon and Affourtit, 2018) (Merck Life Sciences, UK). *Oxidative burst* - port A: XF media, port B: 2 µM rotenone/antimycin A and port C: 160 nM PMA. Or port A: 2 µM rotenone/antimycin A, port B: 10ng/ml tumour necrosis factor α (TNFα) (Merck Life Sciences, UK) or XF media, port C: compound of interest. *N*-Formyl methionyl-leucyl-phenylalanine (fMLP) (Merck Life Sciences, UK). Lipopolysaccharide (LPS) ultrapure from Escherichia coli E011:B4 (Sigma-Aldrich). Heat killed *Streptococcus pneumoniae* (SP) and non-typeable *Haemophillus influenzae* (NTHI) were donated by Dr Kylie Belchamber (Belchamber et al., 2019) Mitochondrial damage associated molecular patterns (mtDAMPS) were donated by Dr Jon Hazeldine (Hazeldine et al., 2015). All compounds were prepared in XF media.

4.3.4. Data analysis

OCR and extracellular acidification rate (ECAR data) were exported from WAVE to GraphPad Prism and Microsoft Excel for downstream analysis. Seahorse analytics (https://seahorseanalytics.agilent.com) was used to convert ECAR to PER using the buffer factor 2.8 as calculated by the manufacturer's protocol (https://www.agilent.com/cs/library/usermanuals/public/usermanual-xf-buffer-factor-protocol-cell-analysis-S7888-10010en-agilent.pdf) for RPMI supplemented with 25 mM glucose. Glycolytic parameters were calculated as follows: Basal glycolysis - PER value prior to the first injection, PMA-induced glycolysis - maximum PER reached over the course of the assay, and glycolytic response to PMA – PMA-induced glycolysis minus basal glycolysis. Area under the curve analysis was performed on GraphPad Prism to

generate oxidative burst parameters. Mitochondrial respiration parameters were calculated by importing OCR data into pre-formatted analysis templates in Microsoft Excel to calculate basal respiration, ATP-linked respiration, proton leak respiration, maximal respiration, and non-mitochondrial respiration. Mean and standard error of the mean (SEM) are presented for data. Paired t-test was performed for Figure 4-4. Linear regression analysis was performed in GraphPad Prism for mitochondrial data Figure 4-8.

4.4. Representative results

4.4.1. Achieving a confluent monolater of neutrophils in XFe96 microwell plates

To establish optimal cell seeding concentrations, 1 x 10^4 to 5 x 10^5 neutrophils per well were seeded into Cell-Tak™ coated XFe96 microwell cell culture plates. Cell confluence was assessed by eye using an inverted optical light microscope. The manufacturer recommends cell confluence between 50-90% while avoiding cell clusters. Figure 4-1 demonstrates 50-90% confluence for 5 x 10^4 and 2 x 10^5 neutrophils/well with a clear monolayer and no clustering of cells. Higher seeding densities resulted in cell clusters and disrupted the assessment of metabolism. Thus, a range of cell densities between 5 x 10^4 and 2 x 10^5 cells/well were examined for each XF assay to identify optimal cell densities.

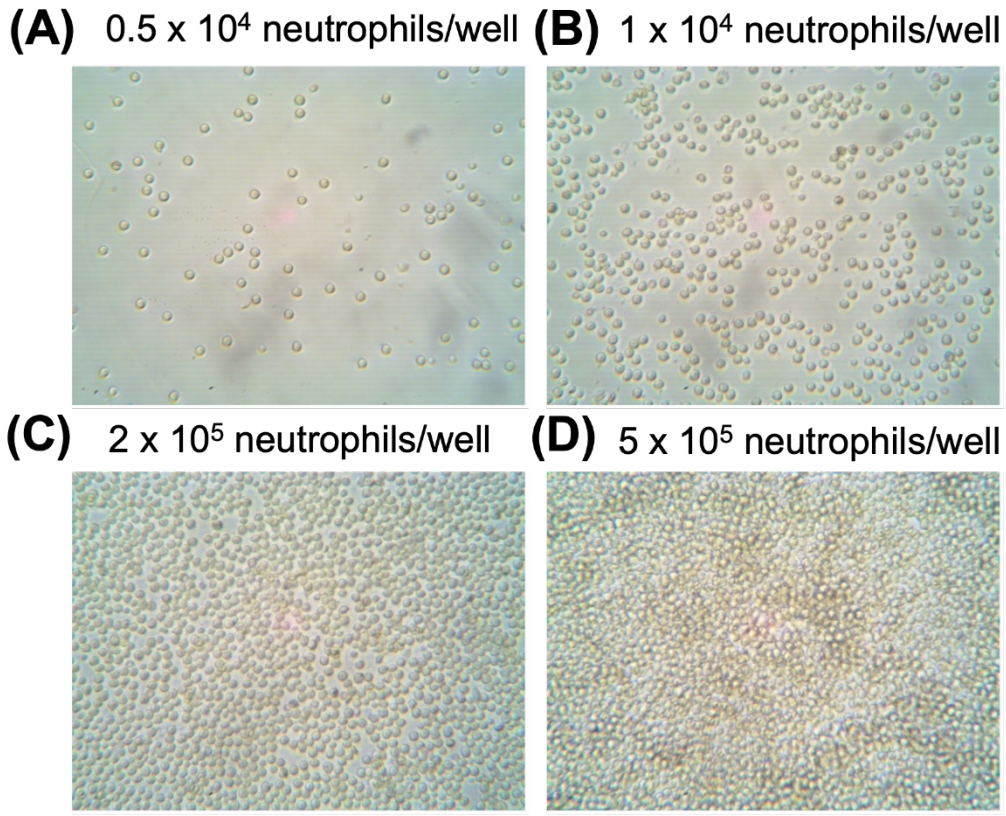

Figure 4-1: Seeding densities of neutrophils in XFe96 coated microplates.

Human peripheral blood neutrophils were isolated from whole blood of donors before seeding into Cell-Tak™ coated XFe96 cell culture plates at (A) 1 x 10^4, (B) 5 x 10^4, (C) 2 x 10^5 or (D) 5 x 10^5/well. XFe96 cell culture microplates containing neutrophils were imaged under an inverted light microscope (45x magnification) and examined by eye for confluence percentage. Confluence assessments were combined with experimental data to achieve the optimal number of neutrophils per well for each test which achieved the correct confluence and consistent data outputs.

4.4.2. Oxidative Burst

Oxygen consumption rate (OCR) measured by XF technology can be used to quantify oxidative burst response in mononuclear cells such as neutrophils. We highlight this in the representative oxidative burst trace in Figure 4-2A using an acute injection of PMA to activate neutrophils seeded in XFe96 microplates. Consistent with an oxidative burst, PMA leads to a typical transient bell-shaped increase in oxygen consumption.

Importantly, when rotenone plus antimycin A is added before PMA, mitochondrial complexes I and III are inhibited and the subsequent PMA-induced increase in OCR is independent of mitochondrial oxygen consumption. To quantify the oxidative burst, we assessed a) time to maximal oxygen uptake, b) maximum oxygen consumption as demonstrated by the arrow and c) total oxygen consumption after PMA injection (shaded area under the curve, Figure 4-2A). These metrics give a reliable quantitative comparison of oxidative burst between groups where both speed and magnitude of oxidative burst may be impacted.

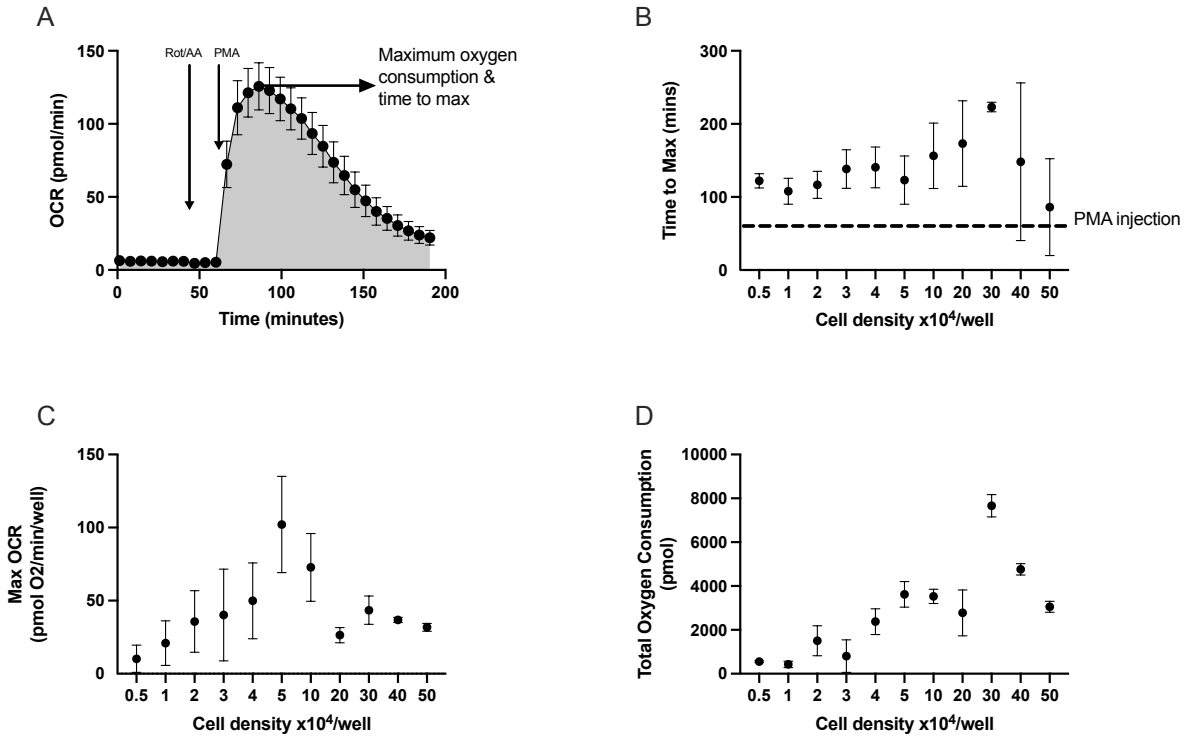

Figure 4-2: Representative trace and optimal cell seeding density to assess neutrophil oxidative burst.

Neutrophils seeded at 0.5 – 5 x 10^4/well in XFe96 cell culture microplates were sequentially injected with 1) XF media (injection 1), 2 µM rotenone/antimycin A (injection 2) and 160 nM phorbol 12-myristate 13-acetate (PMA) (injection 3). (A) is a representative oxygen consumption rate (OCR) trace (n = 1). Area under the curve analysis was performed, to calculate (B) time to maximum OCR, (C) maximum OCR reached and (D) total oxygen consumption over the duration of the test for each cell density (as shown in figure 2). Graphs B-D represent the mean ± standard error of the mean (SEM) from 3 distinct donors.

4.4.3. Cell density and PMA titration

Given that both cell density and/or PMA concentration will affect the metrics used to quantify an oxidative burst using XF technology, we explored to what extent these factors effect neutrophil oxidative burst by assessing neutrophil cell density and PMA concentration. Initially, between 0.5 x 10^4 and 5 x 10^5 neutrophils/well were seeded onto XFe96 cell culture microplates as described in methods. Baseline OCR was measured over a period of 4 measurement cycles (2 min 30 sec mix, 30 sec wait, 3 min measure)

prior to sequential injections of 2 μM rotenone plus antimycin A and 160 nM PMA to establish oxidative burst test parameters (Figure 4-2A). To quantify the oxidative burst time to maximum OCR Figure 4-2B, maximal OCR (Figure 4-2C) and total oxygen consumption (Figure 4-2D) were calculated after injection of 160 nM PMA. Neutrophils seeded at 5 x 10^4/well showed the greatest OCR of 102.12 pmol O_2/min/well, while OCR decreased in cell densities greater than 5 x 10^4/well (Figure 3C). Combining assessment of OCR from oxidative burst activity (Figure 4-2) with confluence assessments (Figure 4-1), we show that neutrophils seeded between 0.5 x 10^4 and 5 x 10^4 per well are optimal for assessment of oxidative burst.

To establish the optimal dose of PMA required to induce an oxidative burst in 5 x 10^4/well neutrophils, a dose response of PMA was performed including 640, 320, 160, 80, 40, 20, 10, 5 and 2.5 nM. PMA concentrations below 160 nM yielded inconsistent peaks of oxygen consumption (dotted line, Figure 4-3A). Both maximum OCR (Figure 4-3B) and total oxygen consumption (Figure 4-3C) over the course of the assay were consistently increased from baseline at PMA concentrations above 160 nM. Based on our data in Figure 4-3, and in agreement with published literature (Swain, Romero and Dranka, 2018; Hickman, Herrera and Jaspers, 2019) we recommend using 160 nM PMA for assessing oxidative bursts from 5 x 10^4/well neutrophils when using the XFe96 analyser. Additionally, 160 nM PMA allows for detection of both increases and decreases between groups, whereas higher concentrations result in the maximal oxidative burst response for 5 x 10^4 neutrophils/well, so increases caused by disease states or pharmacological treatments would not be detected.

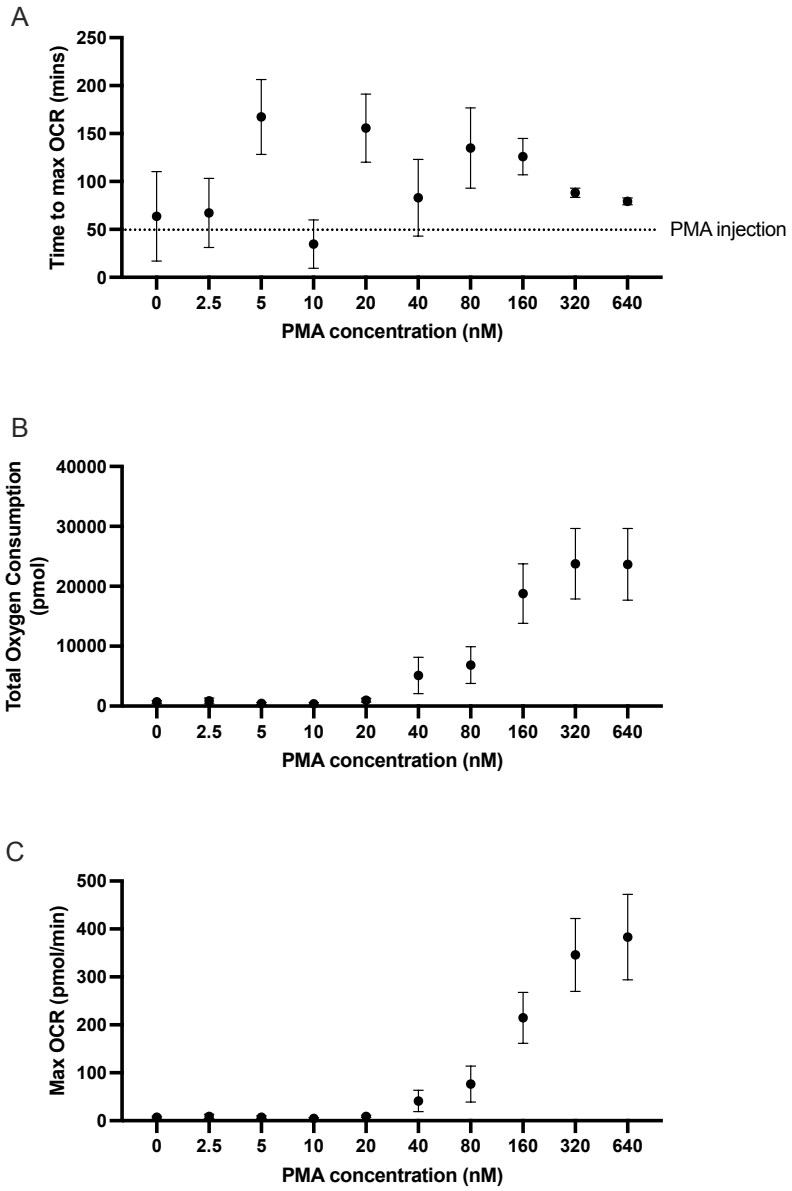

Figure 4-3: PMA-induced OCR from neutrophils

Human peripheral blood neutrophils were seeded at 5 x 10^4 cells/well onto Cell-Tak™ coated XF XFe96 microplates. Oxidative burst was probed by sequential injection of media, 2 µM rotenone plus 2 µM antimycin A and phorbol 12-myristate 13-acetate (PMA) (640, 320, 160, 80, 40, 20, 10, 5 or 2.5 nM). Oxygen consumption rate (OCR) was monitored over the duration of the test, and results were exported to GraphPad Prism for downstream analysis. Time to max OCR (A), maximum OCR (B) and total oxygen consumption (C) were calculated from area under the curve analysis. Graphs represent means ± standard error of the mean (SEM) from 5 distinct donors.

4.4.4. Assessment of oxidative burst induced by physiological stimuli.

Using neutrophils seeded at $5x10^5$ cells/ well we explored the effect of physiological stimuli confirmed to generate reactive oxygen species by other methods (McGovern et al., 2011; Swain, Romero and Dranka, 2018; Hazeldine et al., 2019; Britt et al., 2022). Experimental procedure was as described in 4.4.3, except injection strategy was A:2 μM rotenone plus antimycin A, B:10ng/ml TNFα or XF media, C: fMLP, LPS, heat killed bacteria or mtDAMPS at a range of concentrations. Unprimed neutrophils did not generate a detectable increase in oxygen consumption when challenged with heat killed *SP*, *NTHI* or LPS at a range of concentrations. fMLP at 0.01μM- 10μM generated a small and rapid peak in OCR (13.35-8.11 pmol O_2/min/well). mtDAMPs generated a peak oxygen consumption of 18.01 pmol O_2/min/well 130 minutes following injection without priming (Figure 4-4). Priming with TNFα results in significantly greater oxygen consumption when neutrophils are challenged with fMLP (p <0.001) and mtDAMPs (p= 0.002) compared to unprimed neutrophils. Peak oxygen consumption is rapid within three minutes of injection and overall oxygen consumption is lower than that induced by PMA. Peak oxygen consumption induced by fMLP was 38.12 pmol O_2/min/well fMLP and 29.7 pmol O_2/min/well by mtDAMPs (Figure 4-4A). Total oxygen consumption was greatest by mtDAMPs 4409 pmol compared to 4033pmol induced by 10μM fMLP. TNFα priming does not result in detectable oxygen consumption when neutrophils are challenged with heat killed bacteria or LPS compared to XF media alone (Figure 4-4A and B). Treatment with TNFα alone resulted in a small increase in total oxygen consumption 1533pmol compared to 443pmol in unstimulated neutrophils (Figure 4-4). Therefore, we recommend use of priming agents prior to testing compounds other than

PMA when investigating oxidative burst by XF analyser, and titrating cell density to achieve OCR well within the limits of detection.

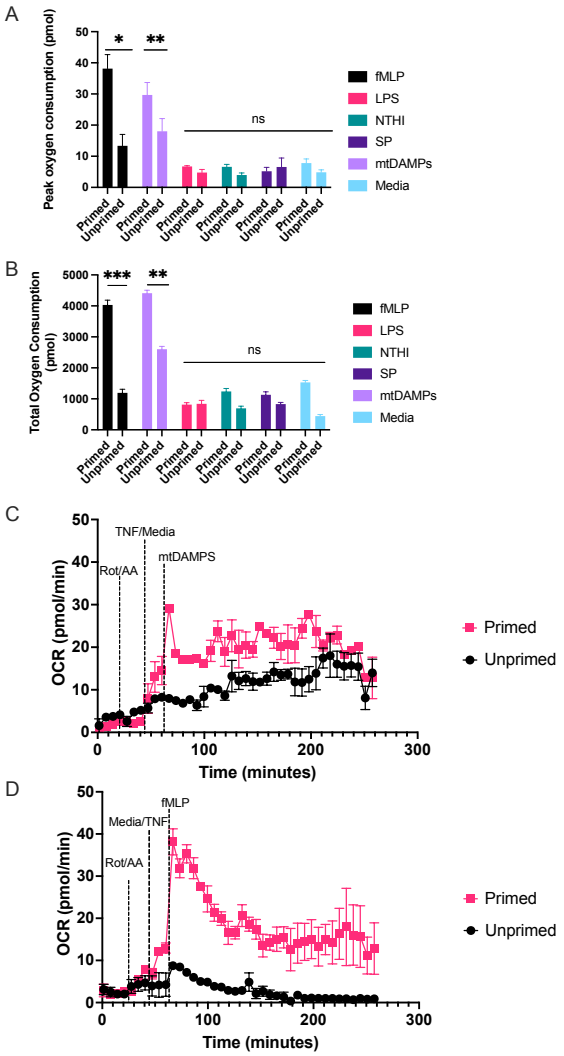

Figure 4-4: Induced OCR from activated neutrophils.

Human peripheral blood neutrophils were seeded at 5 x 10^4 cells/well onto Cell-Tak™ coated XF XFe96 microplates. Oxidative burst was probed by sequential injection of 2 µM rotenone plus 2 µM antimycin A, 10ng/ml TNFα (primed), or XF media (unprimed) followed by 10µM fMLP, or 10µm LPS, NTHI at 100 bacteria:1 neutrophil, SP at 100 bacteria: neutrophil or 100µg/ml mtDAMPS. Oxygen consumption rate (OCR) was monitored over the duration of the test, and results were exported to GraphPad Prism for downstream analysis. Peak OCR (A) and total oxygen consumption were calculated from area under the curve analysis. A-B represent means ± standard error of the mean (SEM) from 3 distinct donors. C-D are representative traces from two distinct donors. Data for 100ng/ml PMA not shown. A) fMLP primed vs. unprimed P= 0.0024, mtDAMPs p=0.043, others non-significant. B) fMLP p=<0.001, mtDAMPs p=0.002, others non-significant. fMLP: *N*-Formyl methionyl-leucyl-phenylalanine, LPS: lipopolysaccharide, NTHI: non typeable *Haemophillus influenzae, SP: Streptococcus pneumoniae*, mtDAMPS: Mitochondrial damage associated molecular patterns, OCR: oxygen consumption rate, TNFα: tumour necrosis factor.

4.4.5. Glycolytic Assessment

Extracellular acidification rate and proton efflux rate

XF analysis can also provide detailed information on cellular glycolytic flux. Until recently (Teslaa and Teitell, 2014), ECAR was commonly used as an indicator of cellular glycolytic rate, given that at neutral pH conversion of glucose to lactate releases protons and acidifies the medium. However, the relationship between extracellular acidification and glycolytic rate can be confounded by other acidification mechanisms. Specifically, in cells that have active mitochondrial oxidative phosphorylation, CO_2 generated in the tricarboxylic acid cycle can be spontaneously or enzymatically hydrated to carbonic acid (H_2CO_3), which dissociates to HCO_3^- + H^+ in media at physiological pH. Of significance, conversion of one glucose molecule to lactate yields 2 lactate + 2 H^+, whereas complete oxidation of one glucose to CO_2 yields 6 HCO_3^- + 6 H^+, therefore when extracellular acidification of glucose is oxidized to CO_2, extracellular acidification is three times higher than conversion to lactate. Moreover, XF analysis of ECAR from changes in media pH does not consider the buffering capacity of the media, which often contain low amounts of HEPES or other buffering agents. It is therefore also important to correct for buffering capacity of the XF assay media. This correction converts ECAR into proton efflux rate (PER), which is preferable when interpreting glycolytic flux.

Stimulant/activator

Glycolytic rate assessed using XF technology is obtained in cells exposed to exogenous glucose and mitochondrial complex inhibitors such as oligomycin or rotenone plus antimycin A. Typically, treatment of cells with mitochondrial respiratory complex inhibitors

such as oligomycin increases PER because of increased glycolytic flux to sustain cellular ATP supply to meet the energetic needs of the cell when mitochondrial ATP synthesis is abolished. However, in neutrophils, under basal conditions with surplus exogenous glucose, oligomycin has no effect on PER (Figure 4-5), suggesting a lack of mitochondrial contribution to ATP supply in blood derived neutrophils. To measure increased glycolytic rate in neutrophils a stimulation step is required to increase ATP hydrolysis rather than abolition of mitochondrial respiration. This is demonstrated in neutrophils treated ± PMA, whereby without PMA stimulation oligomycin has no impact on PER but when stimulated with PMA, PER increases more than 2-fold (Figure 4-5A). Oligomycin and PMA have no synergistic effect compared to PMA alone over 270 minutes (Figure 4-5B).

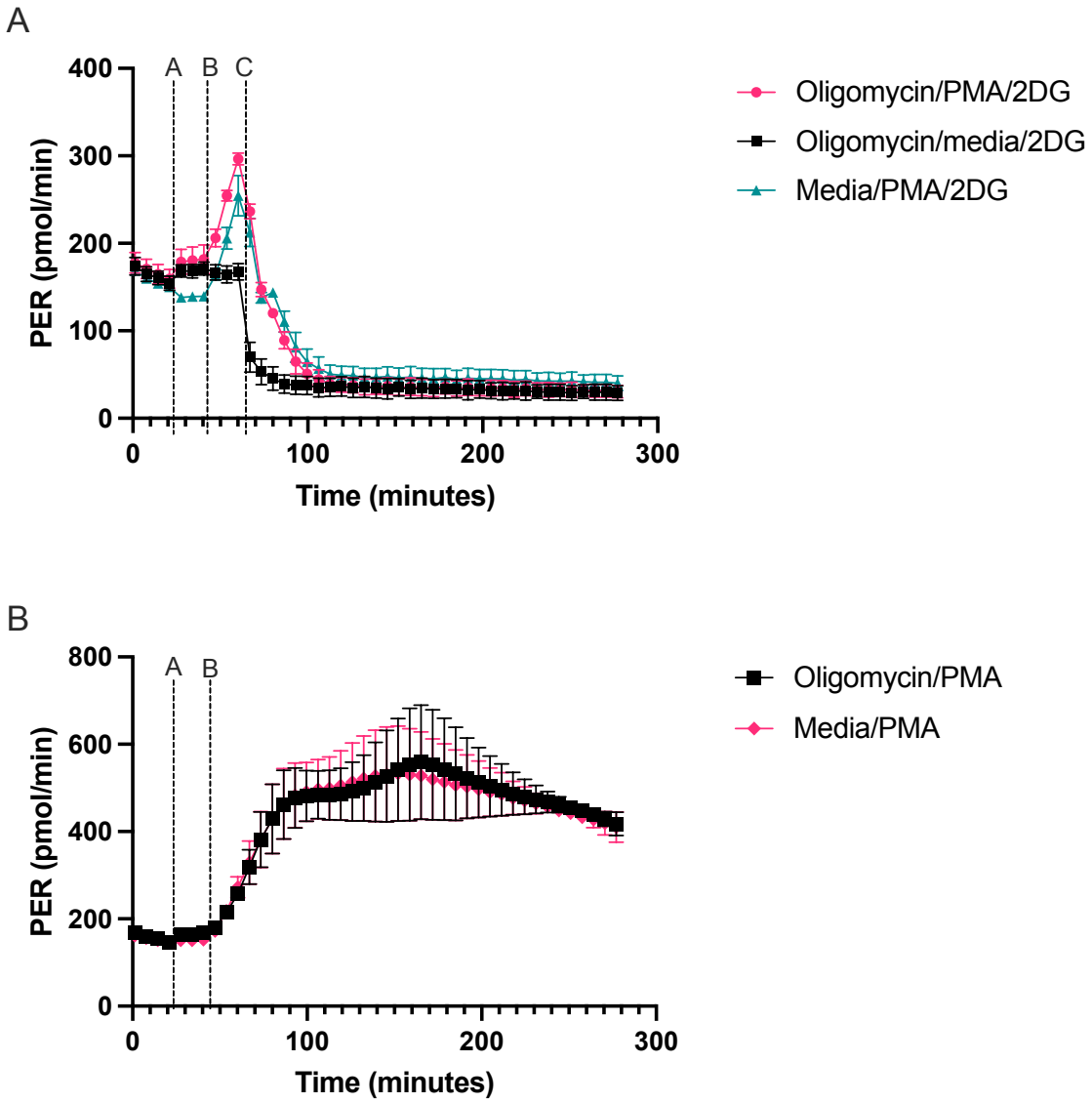

Figure 4-5: PER from neutrophils exposed to oligomycin or PMA.

Human peripheral blood neutrophils were seeded at a density of 2 x 10^5 cells/well onto a Cell-Tak™ coated XFe96 microplate. (A) 2 µM oligomycin or XF media was injected first, followed by sequential injections of 160nM PMA or XF media and finally 50 mM 2-Deoxy-D-Glucose (2-DG) at defined time points as shown on the figure. (B) 2 µM oligomycin or XF media was injected first, followed by 160nM PMA. Extracellular acidification rate (ECAR) was measured across the duration of the test. Following data export, ECAR readings were converted to proton efflux rate (PER) using Seahorse analytics by accounting for the buffer capacity of the media. Data was exported to GraphPad Prism and expressed as mean ± standard error of the mean (SEM) from 3 distinct donors.

Cell density

To establish what cell seeding density is required to obtain consistent and reliable glycolytic rates from neutrophils stimulated with PMA, PER was assessed in XFe96 microplates containing 0.5 – 5 x 10^5 isolated neutrophils per well. As indicated by increases in PER, basal glycolytic rate positively correlated with increased cell density (Figure 4-6A). However, PMA-induced fold change in PER was consistent only up to 2 x 10^5 cells/well (Figure 4-6B). For this reason, we suggest that 2 x 10^5 cells/well is an optimal cell density for assessing PER from neutrophils using the XFe96 analyser. This number of cells sits at the middle of the detectable baseline range and would allow for increases and decreases in glycolytic rate to be assessed under disease states. Importantly, 2 x 10^5 cells/well also coincides with our confluence assessment (Figure 4-1).

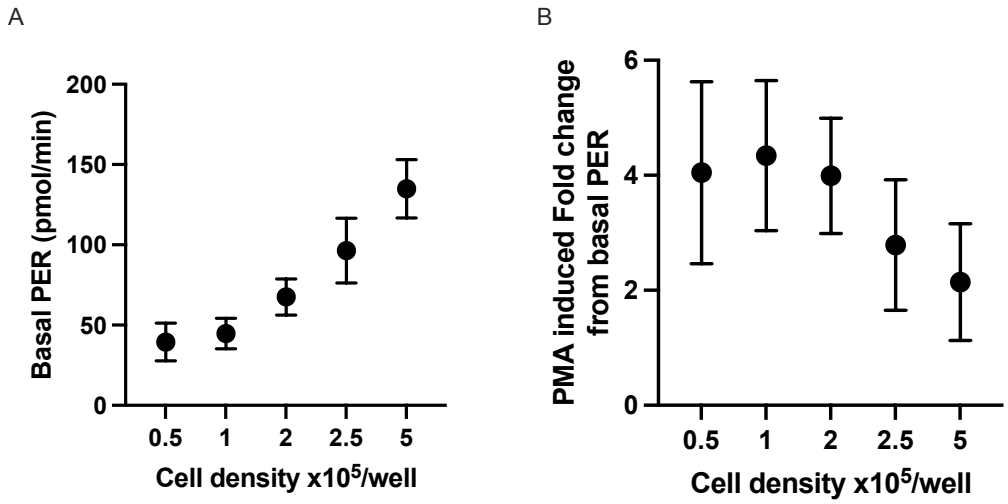

Figure 4-6: Glycolytic parameters from neutrophils seeded at different densities.

Glycolytic rates of isolated neutrophils seeded at 0.5, 1.0, 2.0, 2.5 and 5 x 10^5 cells/well were established from proton efflux rate (PER) data as described previously. Basal glycolysis (A) represents final baseline measurement of glycolysis prior to stimulation, (B) phorbol 12-myristate 13-acetate (PMA)-induced fold change from basal PER. Data represent means ± standard error of the mean (SEM) from 3 distinct donors.

4.4.6. Mitochondrial respiration

Cell density

To establish optimal neutrophil seeding density for assessment of mitochondrial respiration using XF analysis, a range of cell seeding densities were examined from 0.5 – 5 x 10^5 cells/well. At cell concentrations less than 2 x 10^5, basal OCR was less than 20 pmol O_2/min/well (Figure 4-7), below the recommended threshold for OCR measured with the XFe96 analyser. Only at 2.5 and 5 x 10^5 cells/well was OCR detectable above the 20 pmol O_2/min/well threshold (Figure 4-7). Of interest, the typical OCR curve for assessing mitochondrial function with XF technology (Brand and Nicholls, 2011) is not apparent at lower cell seeding densities (Figure 4-7). Notably, OCR from 2.5 and 5 x 10^5 cells/well do show sensitivity to the mitochondrial compounds oligomycin, BAM-15 and

rotenone plus antimycin A, however there is considerable variability at such high cell seeding densities (Figure 4-7). Together with confluence images (Figure 4-1), at appropriate cell seeding densities (< 2 x 10^5 cells/well), OCR is below the limit of detection for the XFe96 analyser and thus OCR obtained from neutrophils at these densities should be treated with caution when inferring mitochondrial respiratory activity.

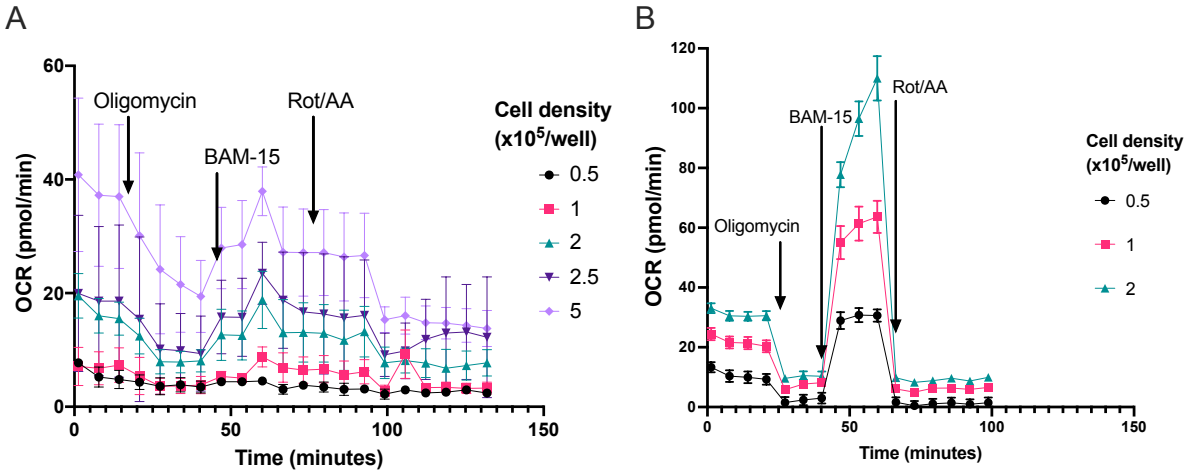

Figure 4-7: Probing OCR from neutrophils and PBMCs seeded at different cell densities.

(A) Mitochondrial respiratory activity of isolated neutrophils seeded between 0.5-5 x 10^5 cells/well were probed by sequential injection of 2 µM oligomycin (injection 1), 3 µM BAM-15 (injection 2) and 2 µM rotenone/antimycin A (Rot/AA) (injection 3), to establish rates of adenosine triphosphate (ATP)-coupled, maximal mitochondrial respiratory activity and non-mitochondrial oxygen consumption, respectively. Cell purity was assessed using H&E staining, mean neutrophil purity was 97% the contaminating cells were 2% monocytes and 1% lymphocytes. Data are means ± standard error of the mean (SEM) from 4 distinct donors (B) Mitochondrial respiration of isolated PBMCs seeded between 0.5-2x10^5 cells/well were probed by sequential injection of 2 µM oligomycin (injection 1), 3 µM BAM-15 (injection 2) and 2 µM rotenone/antimycin A (Rot/AA) (injection 3), to establish rates of adenosine triphosphate (ATP)-coupled, maximal mitochondrial respiratory activity and non-mitochondrial oxygen consumption, respectively. Data are means ± standard error of the mean (SEM) 4 technical replicates.

Cell purity

Given that lower cell seeding densities (<2x10^5 cells/well) make it difficult to obtain reliable OCR (Figure 4-7), it could be argued that increasing cell seeding would be beneficial for probing mitochondrial energy metabolism in neutrophils. That said, OCR measured during a mitochondrial stress test with higher neutrophil densities could be related to contamination from other mononuclear cells, which have higher mitochondrial activity. Typically, density gradient isolations are >97% pure, but contamination with other mononuclear cells such as monocytes or lymphocytes is a common issue when working with neutrophils.

To test this, we probed OCR in neutrophils contaminated with peripheral blood mononuclear cells (PBMCs) seeded at 2 x 10^5 cells/well. This was achieved by mixing neutrophils with defined concentrations of PBMCs to yield cell populations containing either 100%, 70% or 50% neutrophils. OCR were significantly higher in cell populations that contain PBMCs (Figure 4-8E and F). In 100% pure neutrophil isolations, basal OCR was 4.5 (SEM 2.0) pmol O_2/min/well compared to 26.72 (SEM 2.6) pmol O_2/min/well at 50% purity (Figure 4-8E and F). Basal OCR appeared to be dose-dependently higher than pure neutrophil populations with increasing proportion of PBMCs (18-fold higher for 30% contamination and 26-fold higher for 50% contamination, compared to pure neutrophils) (Figure 4-7E). Also, the sensitivity of OCR to mitochondrial compounds is much greater from cell suspensions containing PBMCs (Figure 4-8E). OCR traces from 100% pure neutrophils fall below the 20 pmol O_2/min/well threshold for the XFe96 analyser for the entirety of the mitochondrial assay, as shown in Figure 7E. We also

show that basal OCR is different in neutrophil isolations between 95-100% purity (Figure 4-8F). When corrected for non-mitochondrial respiration (calculated as OCR after rotenone and antimycin A injection), parameters of mitochondrial respiration including basal mitochondrial respiration (Figure 4-8C), maximal respiration (Figure 4-8B) and ATP production (Figure 4-8A) correlate negatively with neutrophil purity ($p < 0.001$). It is also worth noting that non-mitochondrial oxygen uptake did not significantly change ($p = 0.8252$) with increasing PBMC concentration (Figure 4-8D).

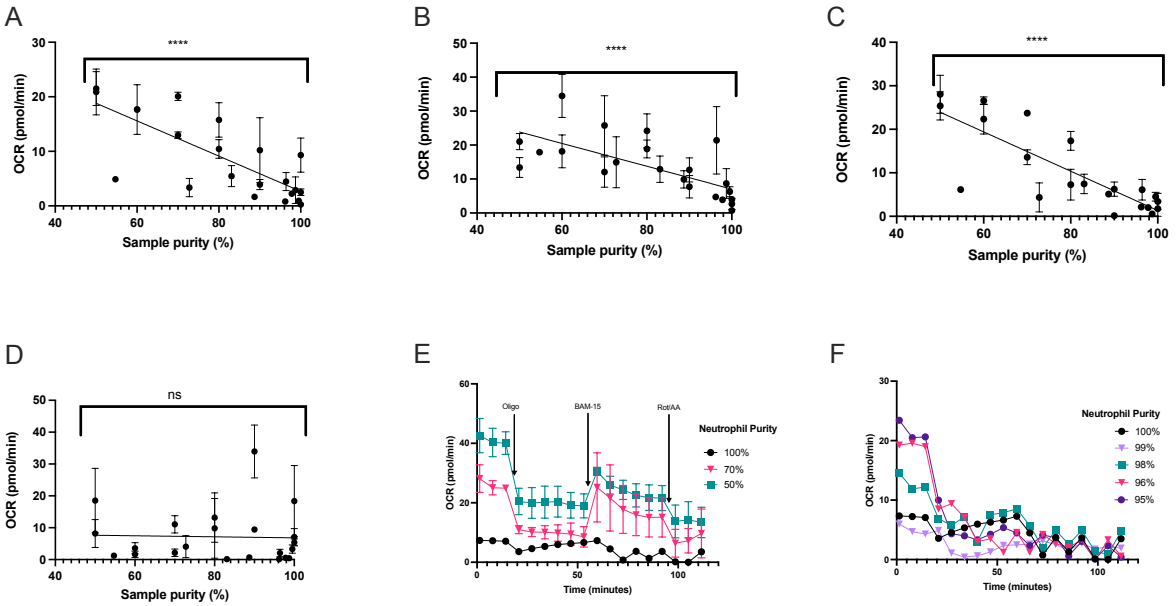

Figure 4-8: Mitochondrial respiration in varying neutrophil purities.

Pure neutrophil isolations were contaminated with up to 50% peripheral blood mononuclear cells (PBMCs) harvested from the same donor and cells seeded at 2 x 10^5/ well. Mitochondrial respiration was probed for in neutrophils by sequential injection of 2 µM oligomycin, 3 µM BAM-15 and 2 µM rotenone plus 2 µM antimycin A (Rot/AA). (A) adenosine triphosphate (ATP)-coupled mitochondrial respiration, (B) maximal mitochondrial respiration, (C) basal mitochondrial respiration, (D) non-mitochondrial oxygen consumption, (E) mean oxygen consumption rate (OCR) ± standard error of the mean (SEM) over the duration of the assay, representing 100%, 70% or 50% neutrophil purities from 2 distinct donors and (F) mean oxygen consumption rate (OCR) ± standard error of the mean (SEM) over the duration of the assay, representing 100%, 99%, 98%, 96% and 95% neutrophil purities from a single donor. Data in A-D consists of three unique donors, for each donor the cell preparation was intentionally contaminated to achieve 22 samples with varying neutrophil purity between 100% neutrophils and 50% neutrophils. Data points shown are mean ±SEM for four technical replicates for each donor.

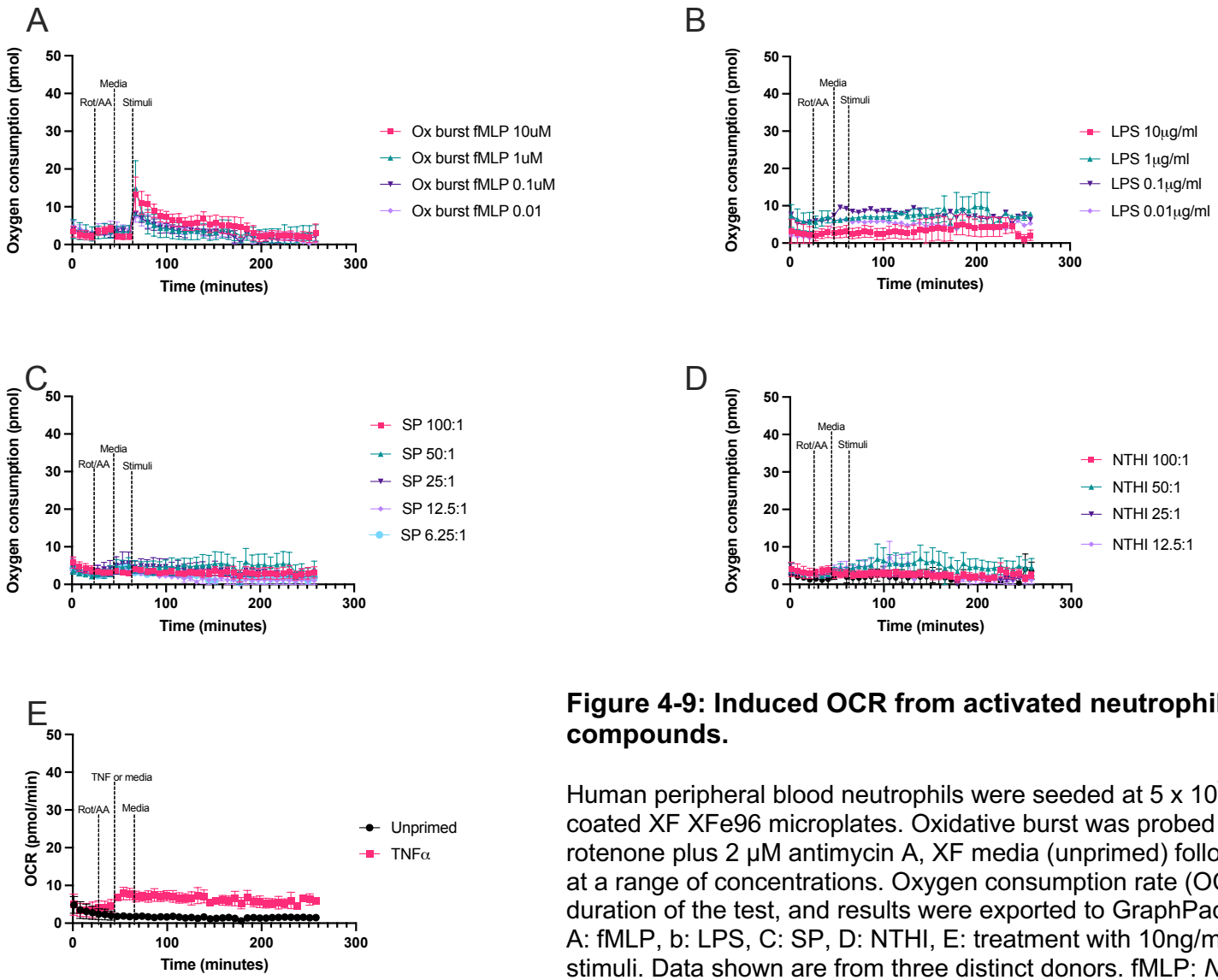

Figure 4-9: Induced OCR from activated neutrophils using a range of compounds.

Human peripheral blood neutrophils were seeded at 5 x 10^4 cells/well onto Cell-Tak™ coated XF XFe96 microplates. Oxidative burst was probed by sequential injection of 2 µM rotenone plus 2 µM antimycin A, XF media (unprimed) followed by fMLP, or LPS, NTHI, SP at a range of concentrations. Oxygen consumption rate (OCR) was monitored over the duration of the test, and results were exported to GraphPad Prism for downstream analysis. A: fMLP, b: LPS, C: SP, D: NTHI, E: treatment with 10ng/ml TNFα with no subsequent stimuli. Data shown are from three distinct donors. fMLP: *N*-Formyl methionyl-leucyl-phenylalanine, LPS: lipopolysaccharide, NTHI: non typeable *Haemophillus influenzae, SP: Streptococcus pneumoniae*, mtDAMPS: Mitochondrial damage associated molecular patterns, OCR: oxygen consumption rate, TNFα: tumour necrosis factor.

4.5. Discussion

Neutrophils are highly glycolytic cells (Borregaard and Herlin, 1982); inhibition of glycolysis significantly impacts neutrophil effector functions (Fossati et al., 2003). Neutrophils utilise glycogen stores to synthesise ATP in the absence of oxygen and are capable of gluconeogenesis to increase glycolytic capacity (Sadiku et al., 2017). The use of glycolytic inhibitors abolishes neutrophil ATP levels, while they remain unchanged after inhibition of mitochondrial respiratory complexes (Kumar and Dikshit, 2019).

Activated neutrophils rapidly consume oxygen as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase forms superoxide anions, reactive oxygen species and hydrogen peroxide, a process commonly referred to as oxidative burst. PMA is a protein kinase C activator and a robust activator of neutrophils, albeit non-physiological (Swain, Romero and Dranka, 2018). In cell seeding densities beyond 5 x 10^4 neutrophils/well, we saw a reduction in the size of the oxidative burst response. Typically, it would be anticipated that greater cell seeding densities would produce a larger oxidative burst response, however, due to cell overcrowding and/or reduced PMA stimulation relative to number of cells, this was not the case. We demonstrated that 5 x 10^4 neutrophils/well combined with an injection strategy incorporating 2 µM rotenone plus 2 µM antimycin A followed by 160 nM PMA, produces a substantial and consistent oxidative burst response, without maximising the neutrophil reactive oxygen species (ROS) response. This allows for detection of both elevated and suppressed oxidative burst responses caused by disease states or exogenous treatments. We show that PMA concentrations lower than 160 nM do not produce a consistent peak following PMA injection, and therefore area under the curve analysis

is unable to quantify a reliable oxidative burst response. Data in Figure 4-2 and Figure 4-3 demonstrate that when assessing oxidative burst in neutrophils a multi-parameter approach should be taken as using a single parameter may lead to false assumptions as seen in the variability of time to max oxygen consumption) which is due to detection of very small peaks at extremes of cell density and or PMA concentration, and are not likely to be a true difference in time to maximum oxygen consumption. PMA is a potent and non-physiological activator of neutrophils; therefore, we explored the utility of other stimuli in the oxidative burst assay. We demonstrated that priming with TNFα enhances detection of the increased oxygen consumption induced by fMLP and mtDAMPs. In unprimed cells there was a small increase in oxygen consumption following fMLP/mtDAMPs, but this was well below the recommended limits of detection for OCR. Heat killed *SP*, NT*HI*, or LPS did not induce a detectable increase in oxygen consumption with or without priming. Heat killed bacteria and LPS have been used by others to induce oxidative burst, although with different methods (Hazeldine et al., 2019; Britt et al., 2022).

More commonly used methods to measure oxidative burst detect generation of ROS by a range of fluorescent, chemiluminescent probes rather than detecting environmental oxygen consumption (Murphy et al., 2022a). While these methods can be highly specific this can also be considered a weakness if assay conditions are not carefully controlled. Different probes have varying selectivity for different species of ROS and vary in their ability to detect intra and extracellular ROS (Murphy et al., 2022a), this is frequently an issue in commercially available kits where the selectivity is not always clearly documented. In addition, chemiluminescent probes such as luminol and lucigenin are prone to errors related to redox recycling as the probes can

generate radicals that produce superoxide (Zielonka, Lambeth and Kalyanaraman, 2013). Using the XF analyser to assess oxidative burst allows for measurement of the total oxygen uptake from the environment which may better represent total ROS generation, especially when mitochondrial uptake of oxygen is abolished. This assay typically uses half the cell number of chemiluminescent assays and enables simultaneous indication of glycolysis through PER data.

Neutrophils rely on glycolysis for synthesis of ATP. In the traditional glycolytic rate test (Technologies, 2019) used in other cell types, injection of oligomycin (ATP synthase inhibitor) or rotenone and antimycin A pushes glycolysis to its capacity, as cells are unable to produce ATP via mitochondrial respiration and therefore upregulate glycolysis to meet the energetic demands of the cell. When testing this assay on human neutrophils, we observed no change in glycolysis after oligomycin injection. A recent publication has highlighted that by inhibiting mitochondrial respiration using mitochondrial respiratory complex inhibitors in resting state cells, cells only shift to glycolysis as a compensatory mechanism for basal ATP demand requirements, thus these agents may not actually shift cells to maximum glycolytic capacity as previously thought (Mookerjee, Nicholls and Brand, 2016). This confirms our findings, highlighting the need for a stimulant to increase ATP demand. Here we used 160 nM PMA, to increase glycolytic activity in neutrophils and assess downstream glycolytic parameters. Seeding more than 2 x 10^5 neutrophils per well to probe glycolytic activity resulted in inconsistent and lower glycolytic responses. Similarly, to the oxidative burst test, this is likely due to overcrowding of cells in the bottom of the well, which compromises the monolayer required to run these assays.

The manufacturer states a limit of detection of 20 pmol O_2/min/well for basal OCR from cells assessed with the XFe96 and XFp analysers. OCR below this threshold cannot be interpreted reliably and should be used with caution to infer mitochondrial function. Here, neutrophil mitochondrial metabolism is too low to detect using current XF analysers – OCR are below the limit of detection and do not change in response to mitochondrial stressors. Moreover, this was not overcome by increasing cell seeding density. In fact, our data showed that increases in cell seeding density yields amplified the low levels of contamination by mononuclear cells which appear to skew mitochondrial respiration tests using XF analysis. These data highlight the importance of using highly pure neutrophil preparations when performing metabolic assays and suggest caution to be taken when inferring mitochondrial respiration from OCR of neutrophil preparations.

Of interest, in two recent studies, a typical mitochondrial OCR trace using XFe96 technology was reported in neutrophils seeded between 0.5-1.5 x10^5 cells/well with isolation purities >95% (Porter et al., 2018; Pan et al., 2022). At similar cell seeding densities, in our study we were unable to achieve equivalent traces. Such responses were only apparent with much less pure (<70%) neutrophil preparations. We show that basal OCR are higher in preparations with greater PBMC contamination at levels typically deemed acceptable for neutrophil assays (Figure 4-7), however these data should be interpreted with caution given the lower limit of detection for XF analysers. Given that PBMCs have higher rates of mitochondrial respiration than neutrophils, our data demonstrated that contamination with PBMCs has significant implications when assessing mitochondrial respiratory parameters from isolated neutrophils. Notably, in both papers, basal OCR are below the optimal minimum detection

threshold for the XFe96 analyser. Additionally, the results as presented are problematic. For example, Porter *et al*. show higher OCR in neutrophils after rotenone and antimycin A injection than basal OCR, suggesting a negative basal rate of mitochondrial respiration (Porter et al., 2018). Unless non-mitochondrial oxygen consumption is stimulated in such experiments, for example by increased ROS, such finding is not theoretically possible. Nonetheless, if increased ROS are responsible for such increases in OCR this needs to be corrected to accurately calculate parameters of mitochondrial function.

Consistent with our findings, Chacko *et al*. show that PER is not responsive to oligomycin in neutrophils (Chacko et al., 2013). Moreover, neutrophil OCR is not sensitive to mitochondrial compounds, corroborating our findings and agreeing with the central dogma for a minimal role of mitochondrial oxidative phosphorylation in neutrophil energy generation. Our data, combined with existing evidence, suggests that mitochondrial ATP synthesis is below the limit of detection for XF analysers, and thus play an insignificant role in supplying ATP to meet basal energetic demands in mature neutrophils. Mitochondrial ATP synthesis does appear to be required for messenger purposes (Fossati et al., 2003; Bao et al., 2015) Further, mitochondrial respiration appears to be important in supporting oxidative burst in the absence of glucose by providing NADPH oxidase (Davies et al., 2019).

XF analysers account for the pH and oxygen content in background wells, which contain no cells. This enables background correction of the values to ensure readouts represent actual metabolism rather than changes in ambient O_2 concentration or pH fluctuations affecting media parameters. Actual oxygen

concentration of XF media was not assessed in each experiment, however the use of background wells should account for any variability in oxygen concentration, further the same experimental conditions were maintained across donors to limit any variability in oxygen concentration in media and therefore oxygen consumption. For adherent cell cultures, normalisation is required to account for well-well cell density differences across the plate. When using neutrophils, cells are plated from pre-diluted homogenous cell suspensions at defined cell seeding densities per well and therefore normalisation to cell number, protein content or DNA will have modest effects on OCR or ECAR. This is, of course, if even cell seeding across the plate is obtained by the experimenter. Reporting ECAR values (traces or extrapolated values) are no longer accepted as representative for glycolysis. Media often has a large buffering capacity, which by its nature can mask pH changes, and subsequent acidification rates. Therefore, by calculating and applying a buffering factor for each specific media to account for the buffering capacity, ECAR values can be converted to PER, a more representative readout for glycolytic tests (when corrected for any mitochondrial-linked PER).

XF analysers allow for high throughput data and multiple metabolic tests to be performed on a single assay plate, with a minimal demand for cell numbers. The dynamics of oxygen consumption and change in pH over the duration of the assay allows for the depiction of cellular metabolism and metabolic responses over time. XF analysers accurately predict lactate production compared to direct lactate measurement (Mookerjee et al., 2015). Other methods to assess glycolysis include direct measurement of glucose and or lactate in assay media, use of glucose analogs, measurement of rate limiting glycolytic enzymes, glucose tracing and

metabolite quantification. Each of these has strengths and limitations, we recommend a combined approach to interrogation of metabolism for robust data. These are discussed in detail in (Teslaa and Teitell, 2014). Alternative respirometers such as the Oroboros O2k analyser require 2mL of solution, with a recommended 2-4 million cells (based on HL-60 data) per chamber (Silva et al., 2018; Sunbalova LF; Calabria E; Volani C; Gnaiger E, 2020), more than 40x the requirement for a well of a XFe96 cell microplate. XF technology also incorporates a pH probe to allow the determination of the glycolytic pathway alongside oxygen consumption simultaneously, without the need for additional modules. As mentioned previously, neutrophils are notoriously easy to activate by mechanical, chemical, and biological stressors. Respirometers such as Oroboros allow for inhibitor/stimulant injections but operate using continuous stirring motions, which may activate neutrophils, skewing metabolic readouts. Adherent cells lend themselves naturally to XF assays, as they can be cultured in a monolayer directly in the cell plate. By incorporating a molecular adhesion step, cells in suspension can be manipulated into a monolayer to perform metabolic assays without exposing them to rigorous mixing. It has been demonstrated previously that plate adhesion using Cell-Tak™ does not affect neutrophil ROS production (Porter et al., 2018).

XF analysers are highly sensitive; however, care must be taken in ensuring an evenly distributed monolayer of cells is formed. If cells are not distributed directly under the probe, or if they begin to detach from the base of the well due to disruptions, probes can lack fine discrimination. Further, introduction of pharmaceutical treatments or other stimulants/inhibitors requires further assay validation to ensure they do not interact with the probes themselves, which are sensitive to pH. Despite these

limitations, we have demonstrated how XF technology can be harnessed for neutrophil bioenergetic analysis. XF analysers are costly to run compared to other respirometers, however the high throughput and automated nature of the system allows for efficient data collection.

In conclusion, we have presented optimised protocols for the assessment of neutrophil glycolysis and oxidative burst using XF technology. The metabolic tests outlined here can be applied to any project context by incorporating pharmacological treatments, disease state neutrophils or neutrophils from different developmental stages. We demonstrate robust methodology to achieve neutrophil monolayers to perform metabolic assays for assessing glycolytic rate and oxidative burst. We highlight the challenges of these assays and mitigating strategies when assessing mitochondrial respiration. Overall, XF technology represents a powerful platform with a user-friendly interface and data analysis templates which has the potential to revolutionise our understanding of neutrophil metabolism. The protocols described in our manuscript provide a template to enhance discovery and ensure a standardised approach to neutrophil measurements using XF analysis.

5. NEUTROPHIL FUNCTION AND METABOLISM IN OLDER ADULTS WITH COMMUNITY ACQUIRED PNEUMONIA AND SEPSIS

Parts of this chapter have been published two manuscripts:

1. "**Neutrophils in community-acquired pneumonia: parallels in dysfunction at the extremes of age**" Thorax, 2019. (Grudzinska et al., 2019b)

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Authors contribution: FG conceived and designed the review, undertook literature searches, and wrote the initial manuscript and completed revisions. MB, BS, TJ, AS, DT and ES designed and planned the review and revised the manuscript and approved final version.

2. "**Observational cohort study protocol: neutrophil function and energetics in adults with community acquired pneumonia and sepsis: Pneumonia Metabolism in Ageing (PUMA)"** BMJ Open Respiratory Research, 2023. (Grudzinska et al., 2023a)

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5.1. Abstract

Community acquired pneumonia (CAP) is a leading cause of hospitalisation in older adults and is associated with high rates of adverse outcomes. Given the ageing population and lack of therapeutic advances in CAP new strategies to manage the burden of this disease are needed.

Neutrophil dysfunction has been demonstrated in CAP and is associated with poor outcomes. To investigate the mechanism underlying this neutrophil dysfunction, older adults with CAP and age/co-morbidity matched controls were recruited to assess *ex-vivo* neutrophil function and metabolism.

This study demonstrates that neutrophils from older donors with CAP display a broad range of defects. *Ex-vivo* neutrophils from CAP donors migrate inaccurately to IL8, have an impaired respiratory burst in response to PMA, and demonstrate increased degranulation compared to age matched controls, but do not display altered metabolism assessed by extracellular flux and RNA-sequencing. However, rates of glycolysis are significantly altered in healthy young adults compared to CAP and older adult controls.

These data confirm published literature suggesting that in older adults neutrophil dysfunction may contribute to poor outcomes with increased risk of tissue damage leading to complications, but that glycolytic metabolism is not implicated in these changes in older adults with CAP.

5.2. Introduction

5.2.1. Neutrophils in older adults in health

Neutrophil numbers are maintained in the older adult, both in the periphery and as progenitors in the bone marrow (Fulop Jr. et al., 1985), however older adults are more prone to neutropenia during infection as recruitment in response to G-CSF is blunted (Chatta et al., 1993). Neutrophils from older adults display altered cytokine production with decreased pro-inflammatory and increased anti-inflammatory cytokines (Schroder et al., 2006; Dalboni et al., 2013). Toll like receptor signalling (implicated in neutrophil ROS generation, cytokine production and increased survival) is decreased in older adults (Volkova et al., 2012), and adhesion molecules (CD62L and CD50) are downregulated in healthy older adults (De Martinis, Modesti and Ginaldi, 2004). This may impair neutrophil migration into tissues in response to infection, resulting in impaired inflammatory responses. Multiple groups have shown that neutrophil chemotaxis is impaired in the healthy older adult (Antonaci et al., 1984; McLaughlin, O'Malley and Cotter, 1986; Polignano et al., 1994; Sapey et al., 2017). In addition, neutrophils from healthy older adults display a decreased capacity to phagocytose opsonised bacteria (Butcher et al., 2001; Weiskopf, Weinberger and Grubeck-Loebenstein, 2009; Simell et al., 2011), which is likely linked to reduced expression of the key receptor for antibody mediated phagocytosis CD16b or Fcgamma IIIb (Butcher et al., 2001). Phagocytosis of unopsonised bacteria appears to be preserved (Emanuelli et al., 1986), supporting this theory.

ROS generation and associated anti-microbial killing is impaired in neutrophils from older adults (Antonaci et al., 1984; Tortorella et al., 1993; Polignano et al., 1994; Wenisch et al., 2000; Peters et al., 2009; Hazeldine et al., 2014), although these defects are not uniform in that defective ROS generation has been shown with

Staphylococcus aureus (S.aureus) stimulation, but ROS generation was maintained when stimulated by *Escherichia coli* (Wenisch et al., 2000).

Generation of NETs has been shown to be impaired in healthy older adults (Hazeldine et al., 2014; Kruger et al., 2015), while others have demonstrated increased NETosis in older adults, but in the study demonstrating increased NETs, the NETs had lower anti-microbial capacity assessed by *S.aureus* killing suggesting that neutrophils from older donors are more sensitive to NETosis, but their NETs are ineffective risking tissue damage without the associated benefit of anti-microbial killing (Sabbatini et al., 2022).

These contrasting studies demonstrate the importance of considering experimental conditions and case mix where for example frailty can influence neutrophil function more than age (Wilson et al., 2020).

Neutrophils from older donors have increased susceptibility to spontaneous and induced apoptosis and reduced capacity to prolong their lifespan in response to survival signals (Fulop Jr. et al., 1997; Schroder et al., 2006; Tortorella et al., 2006; Wessels et al., 2010). This could mean that neutrophils recruited to the site of infection survive for less time, and as such are less functionally active than in younger people.

Older adults have increased neutrophil counts in induced sputum and bronchoalveolar lavage (BAL) samples compared to young adults in health (Pignatti et al., 2011; Meyer et al., 1996), the BAL neutrophils from older adults displayed significantly higher ROS in response to PMA and had greater levels of the pro-inflammatory cytokine IL6 (Meyer et al., 1996).

Importantly when considering interventions to improve immune cell function, age-related neutrophil dysfunction does not appear ubiquitous or permanent. A study of older cyclists showed reduced features of immunosenescence across several cell types and functions compared to non-physically active older adults (Duggal et al., 2018) and physical activity has been shown to reduce systemic inflammation in a prospective study of older adults (Taaffe et al., 2005). Further, frailty which is often considered synonymous with ageing is an independent predictor of neutrophil migratory accuracy (Wilson et al., 2020), and frailty is a potentially modifiable trait.

5.2.2. Neutrophil response to infection

During infection there is increased release of mature and immature neutrophils from the bone marrow mediated by G-CSF (Chatta et al., 1993). Inflammatory mediators and bacterial products cause neutrophil priming and induce neutrophil stiffening (Drost et al., 1999; Skoutelis et al., 2000). In sepsis, reduced neutrophil deformability is associated with poor outcomes (Drost et al., 1999), and increased numbers of primed neutrophils are seen in the periphery (Patel et al., 2018). Whilst this increased priming is associated with poor outcomes in sepsis (Whitmore et al., 2016), failure of neutrophil priming is also associated with increased risk of invasive infection (Elbim et al., 1999).

Animal models have been used to understand the role of neutrophils in pulmonary infections. In murine models of *S.pneumoniae* infection, neutrophil influx peaks at 18 hours following infection and pneumonia is typically resolved (either by clearance or death) within a week. If neutrophil recruitment is delayed or neutrophils are depleted bacterial burden and mortality are increased (Nieminen et al., 2008; Bou Ghanem et al., 2015). Conversely prolonged or excessive neutrophil recruitment increases risk of

S.pneumoniae bacteriaemia (Domon et al., 2016). However, when neutrophils are depleted after the peak of infiltration, bacterial burden and mortality are reduced, suggesting that the damage associated with neutrophils can outweigh their anti-microbial effects (Bou Ghanem et al., 2015).

CXCR1 and CXCR2 are key receptors in neutrophil signalling pathways, differential rates of down-regulation and duration of signalling of each has altered downstream effects on migration, priming and ROS generation (Baggiolini, 2001; Jones et al., 1996b; Sanz & Kubes, 2012; Weathington et al., 2006). Neutrophil CXCR1 surface expression is not altered in sepsis, as CXCR1 is rapidly restored after internalisation; but CXCR2 levels are supressed in sepsis and correlate with acute illness severity (Cummings et al., 1999; Chishti et al., 2004).

Endothelial adhesion molecules are up regulated in response to inflammation, which may aid the migration of neutrophils into the tissues. Serum E-selectin is upregulated in meningococcal disease compared to healthy controls and returns to the level of healthy controls after convalescence (Bruserud et al., 1995), while the adhesion molecules ICAM1 and VCAM1 correlate with organ dysfunction in sepsis (Gearing and Newman, 1993). Circulating serum levels of P and E selectin have been shown to be increased in both CAP and COVID compared to healthy controls (Loyer et al., 2022), this upregulation of adhesion markers occurs in a pathogen and environmental dependent manner (Moreland et al., 2004).

5.2.3. Neutrophils in older adults with pulmonary infection

Ex-vivo studies of neutrophil function in adults during infection have shown a stepwise change in migration associated with severity of infection (Sapey et al., 2017). Similar results have been demonstrated for phagocytosis where in moderate

CAP, neutrophil phagocytosis was increased compared to controls, but severe CAP had supressed phagocytosis (Reiné et al., 2020). Abnormal phagocytosis has also been linked with risk of developing nosocomial infection during critical illness (Morris et al., 2011a). The relationship between illness severity and effector function has also been shown in NETosis in adults with sepsis where *ex-vivo* generation of NETs was lower in participants with septic shock compared to sepsis (Patel et al., 2018). Neutrophil ROS is increased in sepsis and high levels of ROS were associated with higher sequential organ failure assessment (SOFA) score (Martins et al., 2008). Neutrophils from people with COVID-19 infection showed increased transmigration and NETosis with suppressed phagocytosis and ROS (Belchamber et al., 2022), increased degranulation has also been shown in COVID-19 infection (Borella et al., 2022; Loyer et al., 2022).

During pulmonary infection in older adults and aged mice there is increased pulmonary invasion of neutrophils (Menter et al., 2014; Miller and Linge, 2017). Early neutrophil invasion is a key step in controlling infection and inflammation (Bou Ghanem et al., 2015), however their prolonged neutrophilic inflammation seems associated with increased risk of lung injury, especially in older adults (Bruunsgaard et al., 1999). Severe disease outcomes in pulmonary infections such as ARDS frequently correlate with increased neutrophil influx to the lung, and this contributes to the belief that neutrophils play a key role in development of acute lung injury (Pechous, 2017).

Neutrophil migration has been demonstrated to be impaired in older adults with CAP and remains diminished up to six weeks following the episode of CAP (Sapey et al., 2017). Circuitous neutrophil migration would increase risk of tissue damage from

toxic proteases (Liou and Campbell, 1996; Cepinskas, Sandig and Kvietys, 1999). Impaired bacterial killing mechanisms increases risk of systemic dissemination of bacteria and prolongs duration of infection. Compromised phagocytosis leads to reduced bacterial killing, consequently frustrated phagocytosis where a neutrophil fails to engulf a pathogen and releases toxic phagolysosome contents into tissues further exacerbating tissue damage (Francis et al., 2022)

Reduced NETosis has been identified in older adults with CAP (Ebrahimi et al., 2018); however, patients with the highest levels of NETs had increased mortality, and high levels of NETs have been previously shown to be predictive of progression to ARDS and high mortality (Lefrancais et al., 2018). This data may suggest that impaired NETosis seen in older adults is protective; particularly as both *S.pneumoniae* and *H.influenzae* have developed mechanisms to evade NETs (Beiter et al., 2006; Wartha et al., 2007; Juneau et al., 2011) and NET formation in *S.pneumoniae* CAP (murine) has been shown to generate greater collateral damage than provide protection (Narayana Moorthy et al., 2013). Yet in animal models of impaired NETosis there is decreased lung injury but reduced bacterial clearance and increased levels of pro-inflammatory cytokines (Lefrancais et al., 2018). Suggesting that there are optimal levels of NETosis during infections, where either too much or too little is damaging.

Neutrophils are therefore a potential target for modulation to improve outcomes in CAP. Neutrophils from older adults with CAP demonstrate widespread dysfunction, and this phenotype correlates with poorer outcomes (Sapey et al., 2019).

5.2.4. Proposed mechanisms and trialled therapies to improve neutrophil function in CAP.

The data presented so far demonstrates that that excessive or inaccurate neutrophil activity can worsen outcomes for patients, yet underactivity is also problematic. However, the mechanism(s) underlying this dysfunction are not clearly understood. Of particular importance is the temporal relationship of acute infection with neutrophil dysfunction in older adults. There are no data assessing longitudinal changes in neutrophil function including periods of acute infection. People with CAP may represent the spectrum of immunosenescence where they have the poorest function and are therefore most likely to develop severe infection.

Phosphoinositol 3 kinase

The phosphoinositol 3 kinase (PI3K) signalling pathway has been implicated in neutrophil migration and in particular the ability of neutrophils to appropriately target infectious or inflammatory signals (Hawkins et al., 2010). Constitutive expression of PI3K on neutrophils from older adults is associated with reduced migratory accuracy but accuracy can be restored by *in-vitro* inhibition of PI3K (Sapey et al., 2014). *In-vitro* inhibition of PI3Kδ restored neutrophil phagocytosis and bacterial killing in donors with critical illness (Scott et al., 2021).

Downstream targeting of PI3K can be achieved using statins (Kinsella et al., 2011; Al-Ani, 2013; Silveira et al., 2013). Statins are among the most common drugs prescribed in the UK (THSC, 2014) and there is a range of observational evidence suggesting patients who are taking a statin prior to admission have better outcomes from infection and sepsis (Tleyjeh et al., 2009; Janda et al., 2010; Grudzinska et al., 2017). Pre-dosing with statins improves outcomes in murine sepsis models (Catron et al., 2004). Treatment with high dose simvastatin *in-vitro* improved neutrophil

chemotaxis in patients with pulmonary infections (Sapey et al., 2017) but not if sepsis was present. A RCT of high dose simvastatin in CAP demonstrated reduced NETosis and improved migratory accuracy to CXCIL8 compared to placebo. The treatment group also had more sustained falls in SOFA score, and increased hospitalisation free survival (Sapey et al., 2019).

Haemopoietic stimulating factors.

Granulocyte- colony stimulating factor (G-CSF) and granulocyte- macrophage colony stimulating factor (GM-CSF) are haemopoietic factors which stimulate proliferation and differentiation of myeloid cells. G-CSF is neutrophil specific, whereas GM-CSF has broader effects on innate immune cells (Bo et al., 2011). *In-vitro* treatment of neutrophils from patients with sepsis with GM-CSF improved phagocytosis (Morris et al., 2011b), and animal studies have demonstrated that pre-treatment with G-CSF or GM-CSF improves outcomes (Attalah et al., 2002; Steinwede et al., 2011; Balamayooran et al., 2012). However human clinical trials of GM-CSF or G-CSF in sepsis and or pneumonia have failed to replicate these results with no improvement in mortality (in the absence of neutropenia) (Cheng et al., 2004, 2007; Bo et al., 2011; Stephens et al., 2008) , however GM-CSF improved neutrophil phagocytosis during critical illness, but not migration or respiratory burst (Pinder et al., 2018). In addition use of these agents has the theoretical risk of increasing neutrophilic inflammation and the organ dysfunction seen due to this; some studies have demonstrated a small increase in adverse outcomes (Stephens et al., 2008) , however several large meta-analyses have not demonstrated a significant increase in adverse outcomes with either G-CSF or GM-CSF treatment (Cheng, Stephens and Currie, 2007; Bo et al., 2011).

Modulation of NETosis

Generation of NETs is impaired in older adults. Manipulation of NET generation in animal models has generated mixed results. In mice with impaired NETosis challenged with a sepsis model there was reduced lung injury, but poor bacterial clearance with elevated pro-inflammatory cytokines(Lefrancais et al., 2018), however in wild type mice given DNAse to degrade NETs following injury there was reduced lung injury and mortality (Lefrancais et al., 2018). Other studies have shown that DNAse treatment increased neutrophil accumulation in the lungs, increasing the risk of neutrophil mediated damage with no survival benefit (Meng et al., 2012). These early findings in animal models seem to suggest that modulation of NETosis may play a role in managing infections, however the role and timing of treatment needs further research.

Studies targeting neutrophils suggests timing is crucial, and further studies to identify this window for therapy are warranted. Indeed, further characterisation of patients who would benefit from this kind of stratified treatment by deeply phenotyping the abnormalities in neutrophil function, including neutrophil metabolism would improve our mechanistic understanding of this dysfunction.

It is important to consider the high burden of morbidity following pneumonia and consider whether ongoing therapy following acute infection to improve neutrophil function would be warranted. This requires long term follow up and analysis of neutrophil function in survivors.

Studies of aged human CAP have not assessed neutrophil function before the infective insult, so it is unclear whether the impaired effector functions seen in CAP

reflect a lower baseline preceding the event or a response to CAP. However, exposing neutrophils from young adults to pooled plasma from older patients with CAP and sepsis (CAP+S) replicates the functional deficits seen in sepsis (Sapey et al., 2017), which suggests that the CAP+S environment itself can alter cellular function.

These data demonstrate impaired neutrophil responses to infection in older adults across a range of functions, migration, phagocytosis, NETosis and ROS. Given the broad dysfunction seen in these cells, it is suggestive of dysfunction in a single core cellular pathway which would impact all measured effector functions. Impaired metabolism leading to the inability to generate ATP depresses migration, phagocytosis, ROS and NETosis in neutrophils.

5.2.5. Neutrophil Metabolism

Immunometabolism is a rapidly developing field which describes the interface between immunology and cellular metabolism. This interface can describe how nutrients, or the environment influences immune cell function, but also how immune cells can influence tissue homeostasis. Cellular metabolism is a central regulator of function and activation of immune cells (Bird, 2019).

Neutrophil metabolic pathways and impact on effector function are described further in Figure 5-1. A range of compounds can be used to pharmacologically alter cellular metabolism, and the commonly described compounds are in Table 5-1.

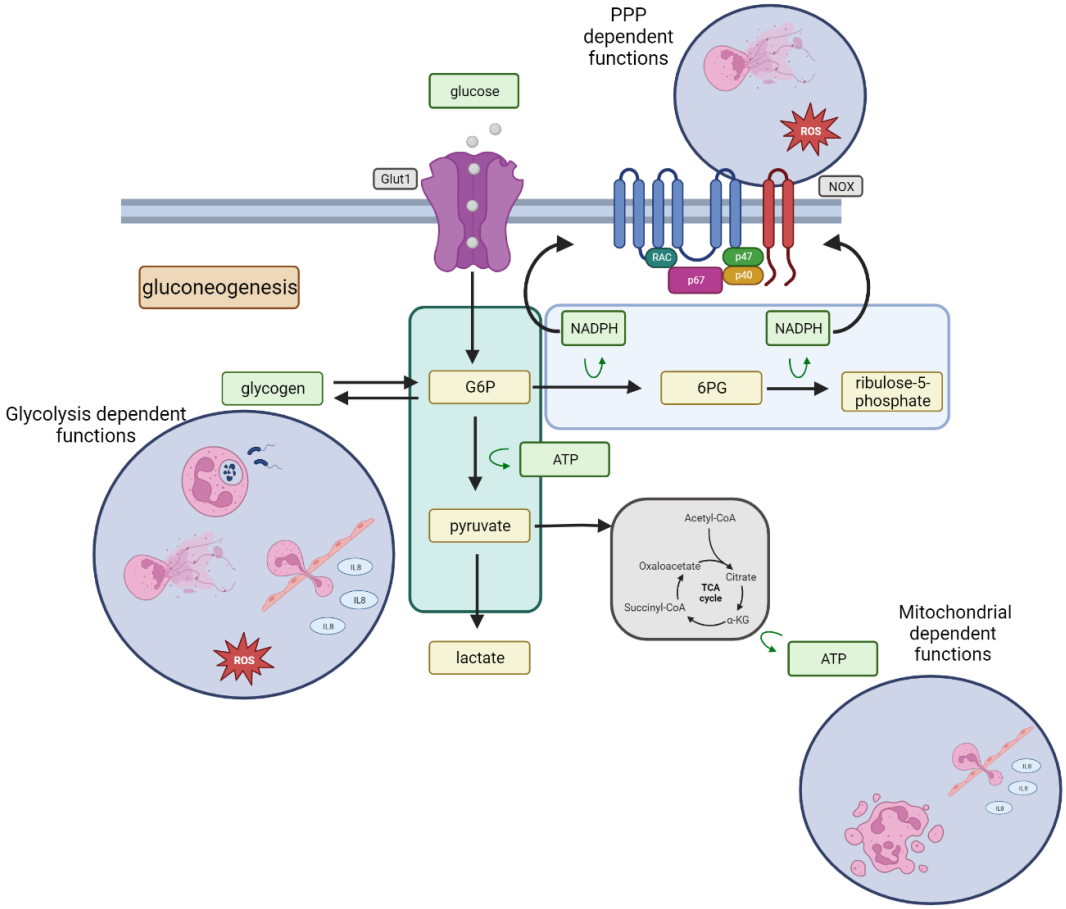

Figure 5-1: Metabolic pathways in neutrophils and their relevance to function

Neutrophils operate in a wide range of environmental conditions and therefore have a plastic approach to nutrients. In addition to glucose neutrophils can perform gluconeogenesis and use glycogen. Shunting through the pentose phosphate pathway (PPP) regenerates NAD and biosynthetic intermediates for protein synthesis impacting reactive oxygen species (ROS) generation and neutrophil extracellular traps (NETosis). The majority of ATP in neutrophils is generated through glycolysis. Inhibition of glycolysis prevents migration, phagocytosis, NETosis and ROS. Mitochondrial function is important during differentiation and for control of apoptosis. Purinergic signalling also appears to play a role in chemotaxis.

Table 5-1: Compounds commonly used in metabolism research.

Compound	Function
Anti-mycin A	Inhibitor of electron transfer at complex III
2-deoxyglucose	Non metabolisable glucose analogue in which the 2-hydroxyl group is replaced by hydrogen therefore it accumulates at step 2 of glycolysis
Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)	Increases mitochondrial membrane permeability to dissipate transmembrane potential
Metformin	Inhibition of mitochondrial complex I and activation of 5' adenosine monophosphate activated protein kinase (AMPK).
Oligomycin	ATPase synthase inhibitor

Neutrophil metabolism was first investigated in 1933 when it was noted that neutrophils undergoing phagocytosis had increased oxygen consumption (Baldridge and Gerard, 1932). This observation was furthered by Sbarra and colleagues in 1959 where they demonstrated neutrophils were capable of phagocytosis in both normoxia and hypoxia and that anti-mycin A did not affect phagocytosis. This was the first demonstration that neutrophil phagocytosis was not dependent on mitochondrial respiration (Sbarra and Karnovsky, 1959).

Neutrophils have relatively few mitochondria and do not appear to have a significant role in energy metabolism (Fossati et al., 2003). Instead, the energy required for neutrophil chemotaxis and functions is derived from glycolysis (Borregaard and Herlin, 1982). Neutrophil effector functions are metabolically demanding, ATP levels fall rapidly during phagocytosis (Stjernholm, 1967). Inhibition of glycolysis using 2-DG inhibits a range of neutrophil functions; degranulation (Smith, Iden and Bowman,

1984) phagocytosis (Boxer, Baehner and Davis, 1977; Fan et al., 2021) and migration. Mitochondrial inhibitors have no effect on ATP generation, phagocytosis, ROS or NETosis (Toller-Kawahisa and O'Neill, 2022). Treatment with 2-DG causes profound loss of ATP with <1% of level compared to untreated cells (Maianski et al., 2004), and mitochondrial inhibitors do not impact ATP levels in neutrophils. Sadiku *et al* investigated how inhibition of glycolysis, fatty acid oxidation or oxidative phosphorylation impacted overall neutrophil energy status assessed by ATP /Adenosine diphosphate (ADP) ratios. Only inhibition of glycolysis caused failure of energy status to be maintained (Sadiku et al., 2017).

Neutrophil mitochondrial metabolism appears to be important in murine granulocyte differentiation in bone marrow (Thomas Riffelmacher et al., 2017), but there are no human data regarding differentiation and mitochondrial function. CCCP inhibits neutrophil chemotaxis (Bao et al., 2015), but this appears to be independent of energy balance and act through purinergic signalling. Purinergic receptors include P2Y family of GPCR. P2Y2 and A2 are the most abundant receptors on neutrophils and are involved in regulation of chemotaxis (Y. Chen et al., 2006). It is thought that mitochondrial inhibitors disrupt chemotaxis by disrupting purinergic signalling rather than overall ATP balance. The role of purinergic signalling in chemotaxis was described by Kondo *et al* using microscopy to reveal spatiotemporal ATP release. The study demonstrated that LPS generated a broad uniform release of ATP rather than a localised response associated with pseudopod protrusion seen with fMLP. This suggested that LPS interfered with neutrophil polarity and therefore migration. Also, the study showed that priming with LPS enhanced the subsequent release of ATP following fMLP stimulation, and that removing the excess ATP with apyrase

improved neutrophil chemotaxis (Kondo et al., 2019). These signalling events take place at the cell surface and are therefore liable to influence from exogenous ATP, such as the high levels seen in sepsis (Cauwels et al., 2014).

Effector functions such as migration and NETosis are glucose dependant (Rodriguez-Espinosa et al., 2015), however neutrophils frequently exist in low nutrient environments and can undergo gluconeogenesis, a function previously thought to be limited to hepatocytes (Sadiku et al., 2017). The importance of the pentose phosphate pathway (PPP) for neutrophil function is clearly observed in patients with G6PD deficiency or impairment, in which the development of infections is common due to dysfunctional neutrophil microbicidal mechanisms (Siler et al., 2017).

Metabolic requirements for neutrophil effector functions

ROS

ROS is a core and crucial mechanism in neutrophil pathogenicity. Britt *et al* explored the metabolic underpinnings following ROS generation in human neutrophils *ex-vivo* with a range of stimuli. Following stimulation with zymosan, TNFα, fMLP, *Pseudomonas aeruginosa* or PMA neutrophils switched from glycolysis at baseline to PPP to regenerate NADPH to supply ROS. There was significant accumulation of all PPP intermediates with accumulation of glycolytic intermediates to a lesser degree. This shift to PPP was also confirmed with carbon tracing. PPP inhibitors abrogated ROS generation to a similar degree as complete NADPH oxidase (NOX) complex inhibitors, which also impaired bacterial killing. Inhibition of PPP also prevents NETosis, as ROS is required for NET generation (Britt et al., 2022).

Oligomycin and CCCP inhibit fMLP induced ROS measured by dihydrohodamine-123 (DHR-123), however DHR123 is not specific for ROS from oxidative burst and includes mitochondrial ROS (Bao et al., 2014).

NETosis

ROS are crucial for NETosis, and PPP is required for NETosis. In human neutrophils *in-vitro* inhibition of G6PD using 6-aminonicotinamide abrogates NETosis. 2-DG also inhibits NETosis. Azevedo *et al* also demonstrated that glucose is required for sustained NETosis by using an alternative energy source. As fructose enters glycolysis below the level of G6PD it cannot contribute to PPP. Use of fructose did not affect overall ATP levels but did reduce NETosis, suggesting that the PPP is crucial for NETosis (Azevedo et al., 2015). The importance of PPP for ROS generation and therefore NETosis is further supported by data from Amara *et al* where activation of phosphofructokinase downstream of G6PD flux to PPP inhibits ROS generation and NETosis (Amara et al., 2021).

NOX dependent NETosis requires glucose, while glycolytic but not mitochondrial inhibitors prevent PMA stimulated NETosis (NOX dependent) (Rodriguez-Espinosa et al., 2015). Inhibition of mitochondrial function prevents NOX-independent NETosis as mROS is needed for NOX independent NETosis in human neutrophils, and mitochondrial uncouplers inhibit NOX independent NETosis (Douda et al., 2015).

Neutrophil metabolism in human disease states

There is very limited data examining metabolism of primary human neutrophils during disease. Borella *et al* examined neutrophil metabolism in a cohort of patients with COVID-19. Participants with COVID-19 had significantly higher rates of basal and stimulated glycolysis compared to controls assessed using extracellular flux.

Intracellular glycogen in neutrophils was significantly higher in in COVID-19 neutrophils compared to healthy controls. They identified reduced oxidative burst in response to PMA. They also identified that stimulation of *ex-vivo* healthy neutrophils with single cytokines and combinations of (G-CSF, TNFα, IL8 and Interferon-γ) upregulated glycolysis. They identified upregulated RNA expression of GLUT1 and LDHA in COVID-19 neutrophils (Borella et al., 2022).

Neutrophils from donors with stable COPD have lower basal ATP levels, reduced glycolytic response to bacterial challenge and impaired bacterial killing (Sadiku et al., 2021). Further Sadiku *et al* investigated the metabolism of human neutrophils from healthy donors in the basal state and following LPS stimulation. Using radiolabelled substrates, they identified high rates of glycolysis in the basal state which was significantly upregulated by LPS stimulation. Abundance of glycolytic and PPP intermediates also increased following LPS treatment. LPS stimulated neutrophils had higher glycogen stores, and inhibition of glycogen degradation impaired neutrophil survival (Sadiku et al., 2021).

RNAsequencing has demonstrated upregulation of glycolytic enzymes in neutrophils from donors with sepsis (Pan et al., 2022). An *in-vitro* sepsis model was generated by treating human neutrophils with 100ng/ml LPS for 4 or 8 hours. 4 hour LPS treatment significantly increased basal glycolysis and glycolytic capacity, while 8 hour LPS treatment did not alter glycolytic rates assessed by extracellular flux (Pan et al., 2022), suggesting that metabolic flux occurs rapidly.

Upregulation of glycolytic enzymes at RNA level in donors with mild CAP compared to controls (Schuurman et al., 2023), however this study enrolled CAP donors with significantly higher rates of COPD than controls. COPD is known to influence

neutrophil function and metabolic status (Elizabeth Sapey et al., 2011; Sadiku et al., 2021).

There are no data examining *ex-vivo* neutrophil metabolism directly in respiratory infection in older adults, or data including young adults.

Neutrophil metabolism in animal disease states

Animal studies have largely demonstrated upregulation of glycolysis in acute infection, some animal models suggest improved outcomes from infectious and inflammatory disorders when glycolysis is suppressed, however, these models are not neutrophil specific. Work by Sadiku *et al* demonstrated that increased glycolysis fuels persistence of neutrophils in inflamed airways in a murine model of *S.pneumoniae* infection (Sadiku et al., 2017)

In a murine model of *S.pneumoniae* mediated otitis media bulk microarray analysis indicated upregulation of glycolytic enzymes following infection, and 2-DG reduced bacterial killing by neutrophils (Fan et al., 2021).

AMPK is a master regulator of cellular energetics; phosphorylation of AMPK occurs in low energy states leading to enhanced anabolism of ATP and reduced catabolism (Herzig and Shaw, 2018). AMPK inhibition inhibits neutrophil migration in murine neutrophils, and treatment with metformin (AMPK activator) enhances migration (Park et al., 2013). Other studies have demonstrated enhanced neutrophil migration during metformin treatment in murine models, reduced bacterial burden, severity of lung injury and reduced inflammatory cytokines in bronchoalveolar lavage were also seen (Zhao et al., 2008).

In a murine model of pemphigoid disease (a neutrophil driven inflammatory disease) both metformin and 2-DG reduced the severity of skin erosions. Treatment with metformin enhanced gene expression of anti-inflammatory cytokines IL-10 and TGF-β (Schilf et al., 2021.

Metabolism clearly plays an important role in neutrophil's ability to carry out their effector functions, and clear alterations in function and expression of key enzymes have been demonstrated in disease models. Given the broad dysfunction in neutrophils in older adults with infection, this study sought to explore whether altered metabolism played a role in neutrophil dysfunction in older adults with CAP and sepsis.

5.3. Hypothesis

Depressed neutrophil function seen in older adults with CAP is due to alterations in glycolysis, and restoring glycolysis will improve function.

5.3.1. Aims:

1) Describe neutrophil function in older adults with community acquired pneumonia and sepsis compared to age matched controls.
2) Assess *ex-vivo* glycolytic metabolism in neutrophils from donors with community acquired pneumonia and sepsis compared to healthy younger adults.

5.4. Materials and Methods

5.4.1. Ethical Approval

The CAP study received favourable ethical approval from Research Ethics Committee (Wales), reference 19/WA/0299. The study was also registered on the National Institute for Health Research (NIHR) Portfolio, ID43632. The Study was sponsored by The University of Birmingham and approved by University Hospitals Birmingham NHS Foundation trust. The healthy young adults were recruited from staff members at QEHB. Written informed consent was given by all healthy young participants in accordance with the University of Birmingham Research Ethics Committee (ERN 12-1185R2). All research was conducted in accordance with Good Clinical Practice guidelines.

5.4.2. Study procedures

Where possible CAP participants were recruited within thirty-six hours of admission to hospital. Remote follow up to assess readmission to hospital and long-term mortality was completed at six and twelve months. Control participants were recruited at a single time point only.

5.4.3. Subject recruitment

CAP cases were recruited from inpatient wards at QEHB and were expected to remain admitted for ≥24 hours. The control cohort was matched as much as possible for age and frailty with no recent acute illness, control participants were recruited from outpatient clinics at QEHB. Participants were recruited between November 2019 and April 2023.

Full inclusion and exclusion criteria for each cohort are in Table 5-2. Exclusion criteria were selected as common conditions or medications known to significantly impact neutrophil function.

Table 5-2: Study inclusion and exclusion criteria

CAP Cohort	
Inclusion Criteria	**Exclusion criteria**
Community acquired pneumonia +/- sepsis admitted to hospital. Age ≥65 years	Asthma Chronic obstructive pulmonary disease Metastatic malignancy Haematological malignancy Participant already enrolled in study of novel/unlicensed treatment. Immunosuppressive treatment in preceding 12 weeks Known immunodeficiency syndrome. Treatment withdrawal imminent Current infection with COVID-19
Control Cohort	
Inclusion Criteria	**Exclusion criteria**
Age ≥65 years Or Age ≤ 35 years with no medical conditions	As above plus: Acute illness in preceding 12 weeks Unable to give informed consent to enrolment

CAP was defined as “signs and symptoms consistent with an acute lower respiratory tract infection associated with new radiographic shadowing for which there is no other explanation” according to British Thoracic Society guidelines(W. S. Lim et al., 2009). Sepsis was defined as a quick sequential organ failure assessment (qSOFA) score of ≥2(Seymour et al., 2016).

Potential CAP participants were identified on weekdays by an automated alert of all adults ≥65 years admitted to general medicine who were also prescribed an anti-

microbial drug. This list was then reviewed to identify participants with CAP. Potential participants were then approached to discuss study enrolment. CAP frequently occurs in people who lack capacity to consent to involvement in research. In this situation the next of kin was approached to act as consultee for study enrolment. If there was no next of kin available, the consultant in charge of the person's care was approached to act as nominated consultee. The professional consultee was always independent to the study. If participants regained capacity to consent to involvement in research retrospective consent was sought.

5.4.4. Blood sample collection

Peripheral blood was drawn into lithium heparin containing vacutainer tubes (BD Biosciences, UK) for neutrophil isolation. Blood was also drawn in Ethylene-diamine-tetra-acetic acid (EDTA) and serum clot activator vacutainer tubes for serum and plasma samples.

5.4.5. Clinical data collection

Study data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at The University of Birmingham (Harris et al., 2009). A bespoke database was designed for this study to include factors which may influence neutrophil function and outcome of CAP. Traditionally studies of CAP have considered binary outcomes such as survived/died, however the public and patient involvement and engagement work demonstrated that a more nuanced approach was preferred. Therefore, information on post discharge mobility, care needs and re-admissions to hospital were collected in addition. Data were only collected if performed as part of usual care. Figure 5-2 summarises the data recorded.

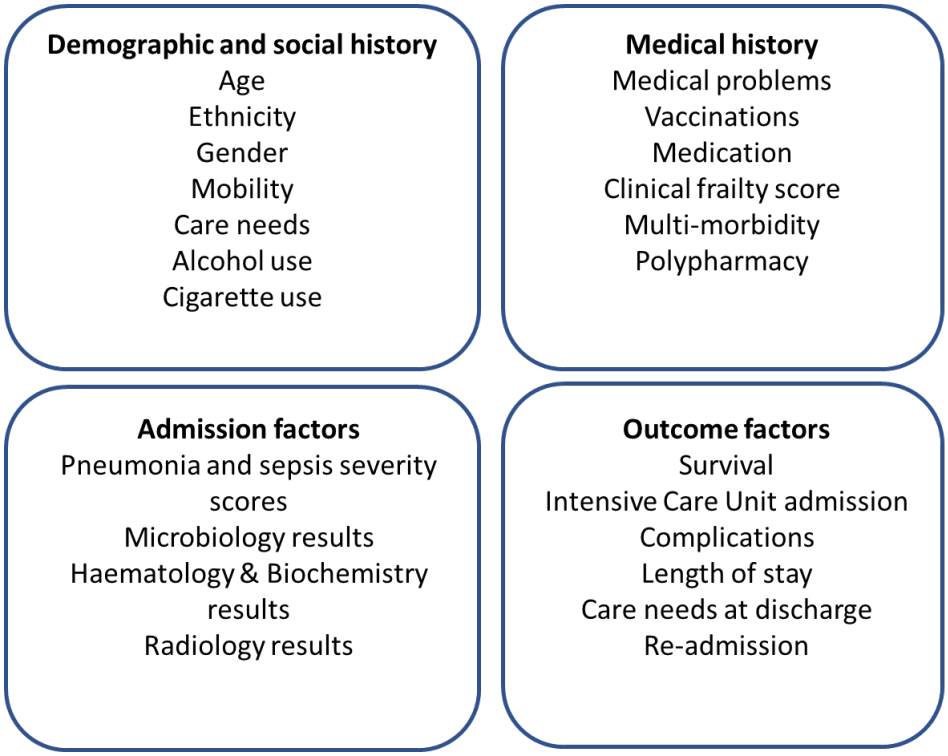

Figure 5-2: Data domains collected for participants.

The four domains of data collected at each study visit: demographic and social history, medical history, admission factors and outcome factors. Data were only collected if routinely available. Taken from (Grudzinska et al., 2023b)

5.4.6. Serum and plasma processing

Lithium heparin (6ml), EDTA (4ml) and serum clot activator (6ml) vacutainer tubes were collected for serum and plasma samples. Samples were allowed to sediment at room temperature for 30 minutes followed by centrifugation at 560x *g* for ten minutes. Serum and plasma were then aliquoted into 500µl samples and frozen at -80°C.

5.4.7. Neutrophil Isolation using Percoll.

Neutrophil isolation was performed as described in Section 4.3.2.

5.4.8. Viability

Isolated neutrophils were resuspended in Annexin V (AnV) binding buffer (diluted 10X buffer solution 0.2µM HEPES, 1.4M NaCl and 25mM $CaCl_2$ 1:10 (v/v) in dH_2O) at $1x10^6$/ml and kept on ice for assay. AnV FITC stain (Miltenyl Biotec, 130-093-060) was added at 1:500 dilution in AnV binding buffer and incubated on ice in the dark for

twenty minutes. Neutrophils were washed twice (250x *g,* 10 minutes, 4°C) in AnV binding buffer and then suspended in 200µl AnV binding buffer. Immediately prior to data acquisition propidium iodide (PI) (Sigma-Aldrich, Poole UK, product no. P4864) was added 1:100 (v/v). Samples were analysed on MACSquant Analyzer 10. Fluidics set to medium and 10,000 neutrophil events gated on forward scatter (FSC) and side scatter (SSC). Gates for positive events were plotted compared to unstained control neutrophils. Proportions of live (viable), early apoptotic, late apoptotic, and necrotic cells were determined based on gating. Viable were determined as AnV-/PI-, early apoptotic were AnV+/PI-, late apoptotic were AnV+/PI+ and necrotic were AnV-/PI+.

5.4.9. Insall Chamber Neutrophil Migration

Insall Chambers were used to assess multiple migration parameters. Freshly isolated neutrophils at $2x10^6$/ml with 0.15% Bovine Serum Albumin (BSA) (Sigma-Aldrich) to prevent aggregation were adhered to a 22mm x 22mm glass coverslip which had been sterilised with 0.4M H_2SO_4 and briefly coated with 400µl 7.5% (w/v) BSA for twenty minutes at room temperature. The Insall chamber was washed three times with sterile RPMI prior to the coverslip being inverted onto the Insall chamber. The RPMI in the outer chamber (Figure 5-3) was then removed and replaced with either sterile RPMI (negative control) or 100nM Interleukin 8 (IL8) (R&D Systems, product no. 208-IL)). For each donor the order of assays- negative and positive control was varied to reduce risk of time on migrationary dynamics. The chemoattractant gradient was allowed to develop for one minute prior to time lapse microscopy using a Leica DMI 6000B microscope with DFL350 FX camera. The 20x lens was used to focus and identify a field in the red square in Figure 5-3 where at least ten neutrophils were

present. An image was captured every twenty seconds for twelve minutes yielding 37 images.

Films were exported as AVI files and analysed using the manual tracking plugin on FIJI Image J (National Institutes of Health, Bethesda, Maryland.(Schneider, Rasband and Eliceiri, 2012). Ten neutrophils were identified at random prior to the film being analysed and the migration tracked as previously described by (Sapey, Greenwood, G. M. Walton, et al., 2014) and validated within the group to be representative of the whole population. Cells were excluded if they collided with another. These pixel position data was inputted into a pre-formatted excel template (M. Hughes, 2021) to generate values for speed, velocity, persistence, chemotactic index, displacement, distance travelled and directness to describe cell movement. These values were averaged across the ten cells tracked. Each of these terms is defined in Table 5-3.

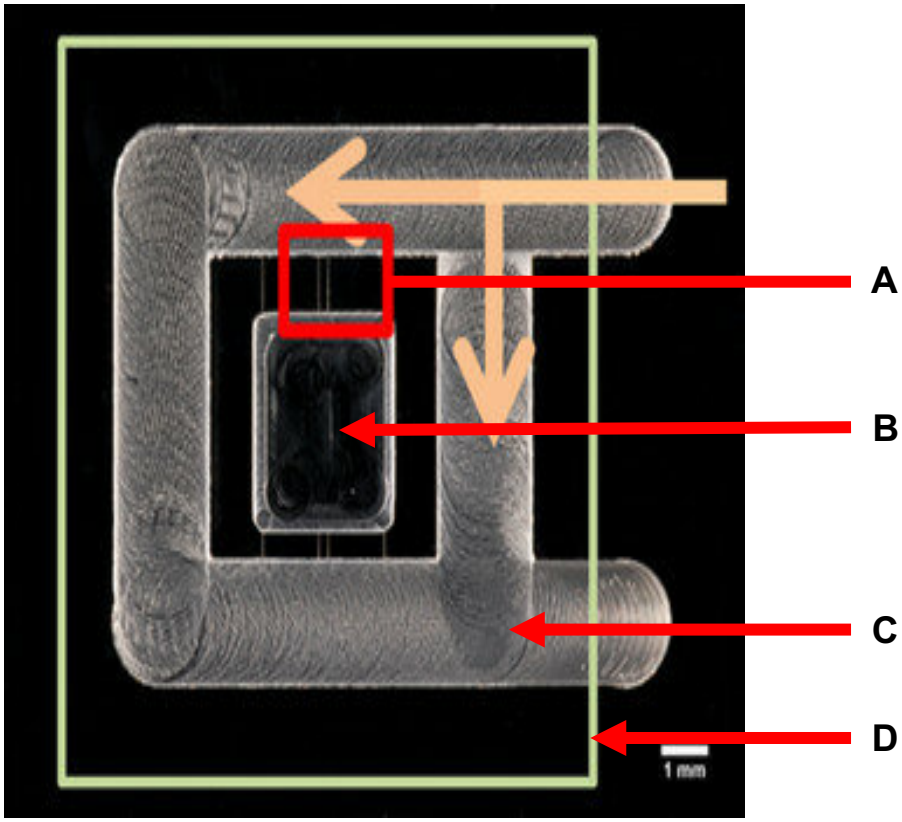

Figure 5-3: Photograph of Insall Chamber

A: area visualised during migration, B: central well, C: outer well and D: coverslip which is inverted onto chamber after allowing neutrophils to adhere. Photograph from Roberts *et al*(Roberts et al., 2015)

Table 5-3: Definitions of migrationary parameters

Per Frame Terms	Definition
Speed	Distance travelled by the cell over time converted to μM/minute using frame length and distance per pixel
Velocity	Speed travelled towards (or away from) the chemoattractant gradient (μM/minute)
Persistence	The orientation of the cell relative to the chemotactic gradient. Where 1 represents a cell moving directly towards the chemotactic index and 0 represents a randomly moving cell.
Whole Time-lapse Terms	**Definition**
Chemotactic index	Measure of accurate movement towards chemoattractant across all frames where 1= movement directly to the chemoattractant and -1 = movement directly away from the chemoattractant gradient
Displacement	Distance between start and end point of cell track independent of distance independent of direction
Distance travelled	Total distance travelled independent of direction
Directness	Measure of straightness of the path taken by the cell calculate by displacement/distance travelled

Definitions taken from (Hughes, 2021)

5.4.10. Extracellular Flux analysis

Performed as described in Section 4.3.3.

5.4.11. Neutrophil Proteinase Activity

Measurement of proteinase 3 (PR3) and neutrophil elastase (NE) activity were completed by Celine Hsi Chen. Aα-Val541 is a specific fibrinogen cleavage product used to assess PR3 activity. Aα-Val541 activity was measured using an indirect ELISA according to Newby *et al* (Newby et al., 2019). Aα-Val360 is an NE specific fibrinogen cleavage product measured in a sandwich ELISA according to Carter *et al* (Carter et al., 2011).

5.4.12. Neutrophil Cell Surface Marker Expression

Neutrophil phenotype was assessed by staining isolated neutrophils with antibodies that bind to extracellular surface markers. Antibodies to detect cluster of differentiation (CD) 11b, CD66b, CD10, CXC chemokine receptor (CXCR) 2, and CXCR4 were mixed as one cocktail, while antibodies that bind CD16, CD11c, CD62L, CD54 and programmed death-ligand (PD-L) 1 were mixed as a second cocktail. The antibody cocktails as well as isotype controls are added to neutrophils and incubated on ice for 30 minutes in the dark. Unstained controls are also included in the incubation. After incubation, neutrophils were washed with 1% bovine serum albumin (BSA) in PBS (FACS buffer) and centrifuged at 300 g for 5 minutes. The neutrophils are resuspended in FACS buffer and analysed using a MACSQuant 10 flow cytometer (Miltenyl Biotec). Data is analysed using FlowJo software. The analysis of the expression of surface receptors will allow for comparison of neutrophil phenotype in terms of their activation, maturity, senescence, reverse migration, respiratory burst, and immunosuppression.

5.4.13. Plasma biomarker assays

EDTA plasma was used to measure Neutrophil elastase (DY9167), Myeloperoxidase (DY3174), e-selectin (DY724) and *p*-selectin (DY137) kits were purchased from R&D Systems. Lactate (MAK064) and CRP (RAB0096) kits were purchased from Sigma-Aldrich. Kits were used according to manufacturer's instructions.

5.4.14. Statistical analysis

Data distribution was assessed using Shapiro-Wilk test where *p*-value >0.05 was considered normally distributed. Normally distributed data was analysed using parametric tests. Paired t-test for paired samples and unpaired for unrelated

samples. If >2 independent comparisons were made a one-way ANOVA was used. For non-normally distributed data non-parametric tests were used. For paired samples Wilcoxon test was used. For independent samples Mann-Whitney U test was used. For categorical demographic data Fishers exact test was used. Analysis of experimental data was performed by GraphPad Prism (Version 9.4.1). Demographic data was analysed using SPSS (Version 29.0).

5.4.15. Power calculation

The primary outcome measure for sample size calculation was based on a reduction in chemotaxis in patients with CAP compared to age matched controls. Pilot data indicates a 62% reduction in chemotaxis in CAP cases with SD 0.079 for CAP and 0.135 for controls. To detect a difference in chemotaxis with $p<0.05$ and 95% power sample size was 8 CAP and 8 controls. This limited sample size was anticipated to inform relationships between clinical outcomes and neutrophil function but is powered to detect a biological endpoint (difference in neutrophil chemotaxis between CAP and controls).

5.5. Results

5.5.1. Screening and Recruitment

Overall, 8064 episodes identified by automatic alerts were screened, of these 840 CAP cases were identified. Recruitment was significantly impacted by COVID-19. Figure 5-4 demonstrates CAP recruitment. The most common reasons for exclusion of CAP cases were chronic respiratory disease (48%), non-severe CAP (23%) and COVID-19 positive/awaiting result (11%). 67 potential participants were identified and 30 approached for study enrolment.

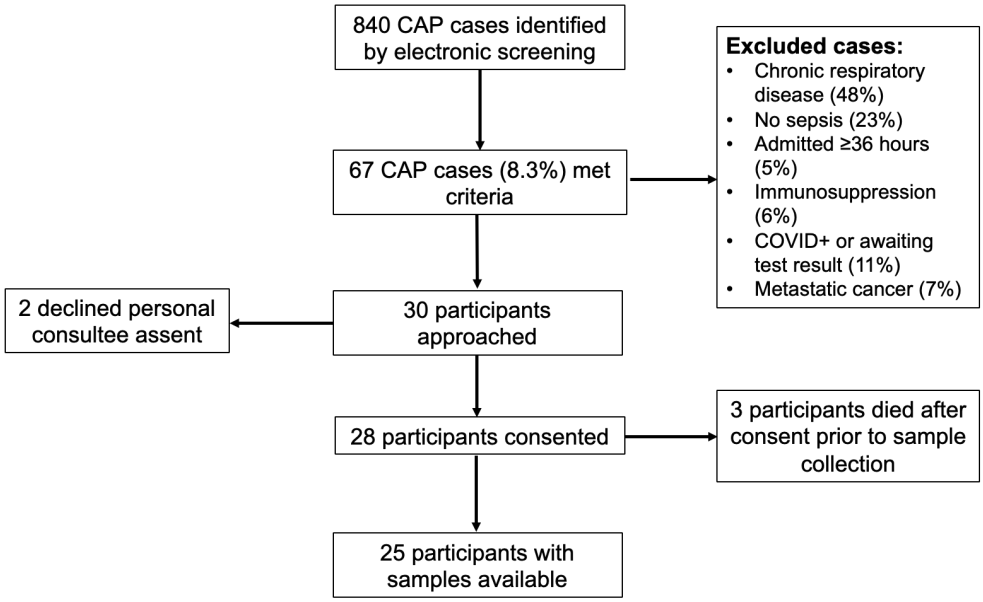

Figure 5-4: Consort Diagram describing participant recruitment.

5.5.2. Participant demographics and outcomes

57 participants were recruited from QEHB, 32 controls and 25 CAP cases. CAP participants and controls were well matched for age, gender, ethnicity, clinical frailty score and major co-morbidities which are known to influence neutrophil function (Table 5-4). Control participants were more likely to have received annual influenza vaccination and pneumococcal vaccination (both p <0.001). CAP participants had

higher prevalence of dementia (p=0.032). Multimorbidity and polypharmacy were prevalent in both groups. More CAP participants lived in supported accommodation (p=0.005). CAP participants had poorer baseline mobility (p=0.027) and had more domiciliary care (p=0.001).

Data regarding CAP severity and outcome is in Table 5-5. CAP participants were recruited at a median of 26 hours following admission and had been symptomatic for a median of 3 days [IQR 3] prior to hospitalisation. A microbiological cause was not identified in any participants, however testing rates were low. CAP cases had severe pneumonia with median CURB65 score 3 [IQR 1] and high acute illness scores demonstrated by median NEWS score 7 [IQR 5]. They had high levels of inflammation; mean CRP 180 [S.D 134.8] and neutrophil count of 13.1 [S.D 5.5]. Outcomes were poor for this group with 36% 30-day mortality and 42% of survivors were readmitted within 90-days. 47% of survivors had increased care needs at hospital discharged. Median length of stay was 10 days [IQR 12].

Table 5-4: Demographics of study participants

	CAP *(n=25)*	**Control (*n=32*)**	***p*-value**
Age (Mean, S.D)	83 (8)	81 (7)	0.053
Female (Count, %)	16 (64.0)	17 (53.1)	0.290
White ethnicity (Count, %)	24 (96.0)	29 (90.6)	0.408
Never smoker (Count, %)	12 (48.0)	18 (56.3)	0.082
Clinical frailty score (Median, IQR)	5 (3)	4 (2)	0.341
Ever had pneumococcal vaccination (Count, %)	**11 (44.0)**	**20 (62.5)**	**<0.001**
Influenza vaccination (Count, %)	**14 (56.0)**	**29 (90.6)**	**<0.001**
BMI (Mean, S.D)	26.6 (6.2)	25.8 (4.2)	0.611
Number of co-morbidities (Median, IQR)	3 (2)	4 (4)	0.449
Number of medications (pre-admission) (Median, IQR)	5 (4)	4 (4)	0.299
Usually resident in own home	**19 (76.0)**	**30 (93.8)**	**0.005**
Independently mobile	**11 (44.0)**	**22 (68.8)**	**0.027**
Domiciliary care	**11 (44.0)**	**2 (6.3)**	**<0.001**

CAP= community acquired pneumonia, S. D= standard deviation, IQR=interquartile range, BMI=body mass index, RH= residential home, NH=nursing home.

Table 5-5: Pneumonia severity indices and outcomes

Pneumonia severity factors	
Median CURB65 score (IQR)	3 (1)
Median NEWS2 score (IQR)	7 (4.5)
Median qSOFA score (IQR)	2 (0)
Median SOFA score (IQR)	2.5 (2)
Mean white cell count ($x10^9$) (S.D)	15.1 (6.1)
Mean neutrophil count ($x10^9$) (S.D)	13.1 (5.5)
Mean CRP (mg/L) (S.D)	169 (132.5)
Mean Creatinine (mmol/L) (S.D)	88.4 (37.5)
Mean Urea (mmol/L) (S.D)	9.54 (4.8)
Mean ALT (units/L) (S.D)	31.4 (26.2)
Mean Lactate (mmol/L) (S.D)	2.7 (1.2)
Delirium present at admission (Count, %)	16 (64)
Mean Respiratory rate/min (S.D)	25 (6)
Mean systolic blood pressure (mmHg) (S.D)	117 (26)
Mean diastolic blood pressure (mmHg) (S.D)	66 (17)
Mean heart rate/min (S.D)	102 (21)
Outcomes	
Inpatient mortality (count, %)	6 (24)
30-day mortality (count, %)	9 (36)
90-day mortality (count, %)	10 (40)
ICU admission (count, %)	2 (8)
90-day readmission if survived index admission (count, %)	8 (42)
Increased care needs at discharge (count, %)	9 (47)
Survivor LoS (median, IQR)	10 (12)

IQR=interquartile range, NEWS2= national early warning score 2, qSOFA= quick sequential organ failure assessment score, SOFA= sequential organ failure assessment score, LoS: length of stay, S. D=standard deviation, CRP= c reactive protein.

5.5.3. Viability of isolated neutrophils is reduced in CAP.

Viability of isolated neutrophils was assessed by flow cytometry using AnV and PI staining in Figure 5-5. Viable neutrophils were deemed to be AnV-/PI-, early apoptotic neutrophils were AnV+/PI- and necrotic neutrophils were AnV-/PI+. In CAP 79.0 [IQR 35.2]% neutrophils were viable compared to 91.4 [IQR 10.8]% in controls, p=0.0025 by Mann-Whitney. Neutrophils from CAP donors exhibited increased early apoptosis (13.7[IQR 12.7] vs. 4.6[10.2]%, p=0.0009 by Mann-Whitney). There were no differences in frequency of necrotic cells between CAP and control donors.

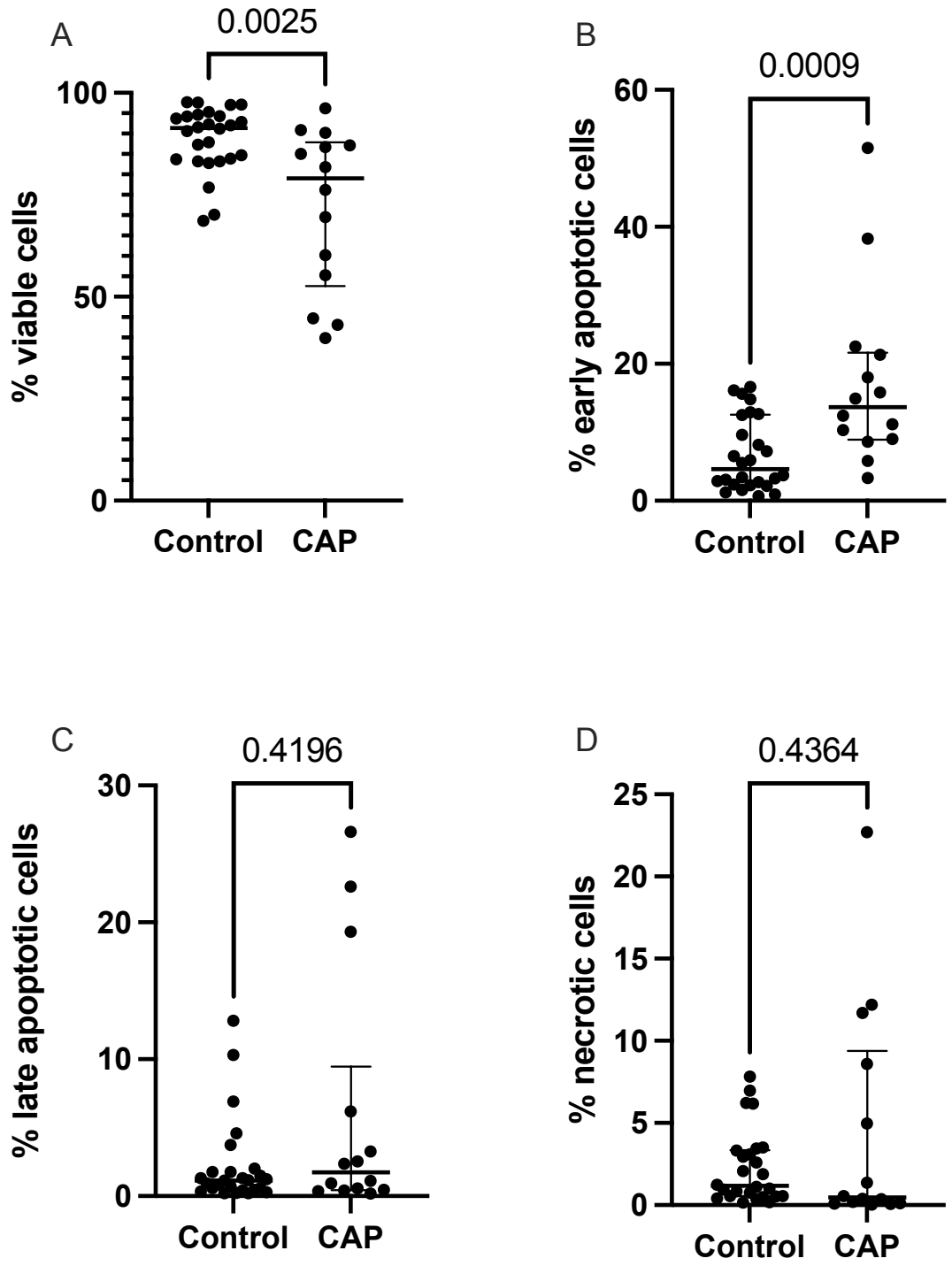

Figure 5-5: Neutrophil viability in CAP

Isolated neutrophils were stained using Annexin V-FITC and Propidium iodide. Viable neutrophils were deemed to be AnV-/PI-, early apoptotic neutrophils were AnV+/PI- and necrotic neutrophils were AnV-/PI+. Dot per participant. Data was not normally distributed (Shapiro–Wilk) and Mann-Whitney tests were performed. Data bars are median and IQR.

5.5.4. Neutrophil migration in Insall Chamber in CAP

To ensure the migration assay was functioning as previously described, migratory parameters for negative control (RPMI) were compared to positive control (IL8) for both CAP and controls. In control participants all metrics were significantly increased in response to IL8 compared to RPMI. In CAP participants speed, velocity and chemotactic index were increased in response to IL8 but directness was not significantly different (Figure 5-6).

Further it was noted that neutrophils from CAP donors appeared to be activated in negative controls as evidence by increased speed of migration of CAP neutrophils to RPMI compared to controls. However directional metrics (velocity, chemotactic index, and directness) assessed to RPMI were not significantly different in CAP compared to controls (Figure 5-7).

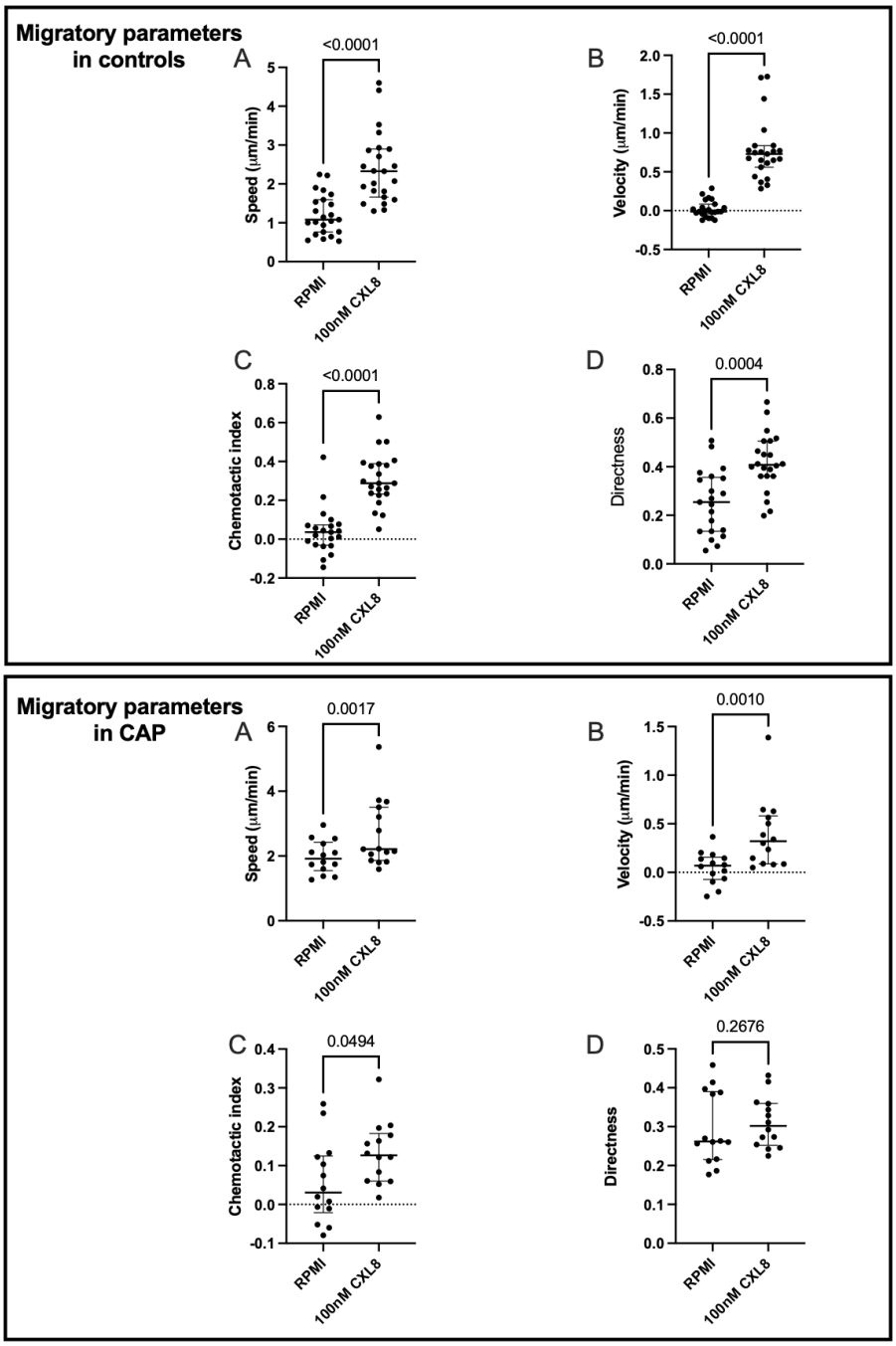

Figure 5-6: Neutrophil migratory parameters in IL8 compared to RPMI.

Isolated neutrophils migrated towards 100nM IL8 or RPMI in a modified Insall chamber. (A) Neutrophil speed in any direction (B) Neutrophil velocity is measured in μM/min towards IL8. (C) Neutrophil chemotactic index is a measure of directional accuracy where 1 is direct migration to the chemoattractant and -1 is directly away. (D) Neutrophil directness is a measure of the straightness of the path taken where 1 is a direct straight line over the entire time lapse. Data presented is dot per participant, bars are median and IQR. Wilcoxon test used for paired comparisons.

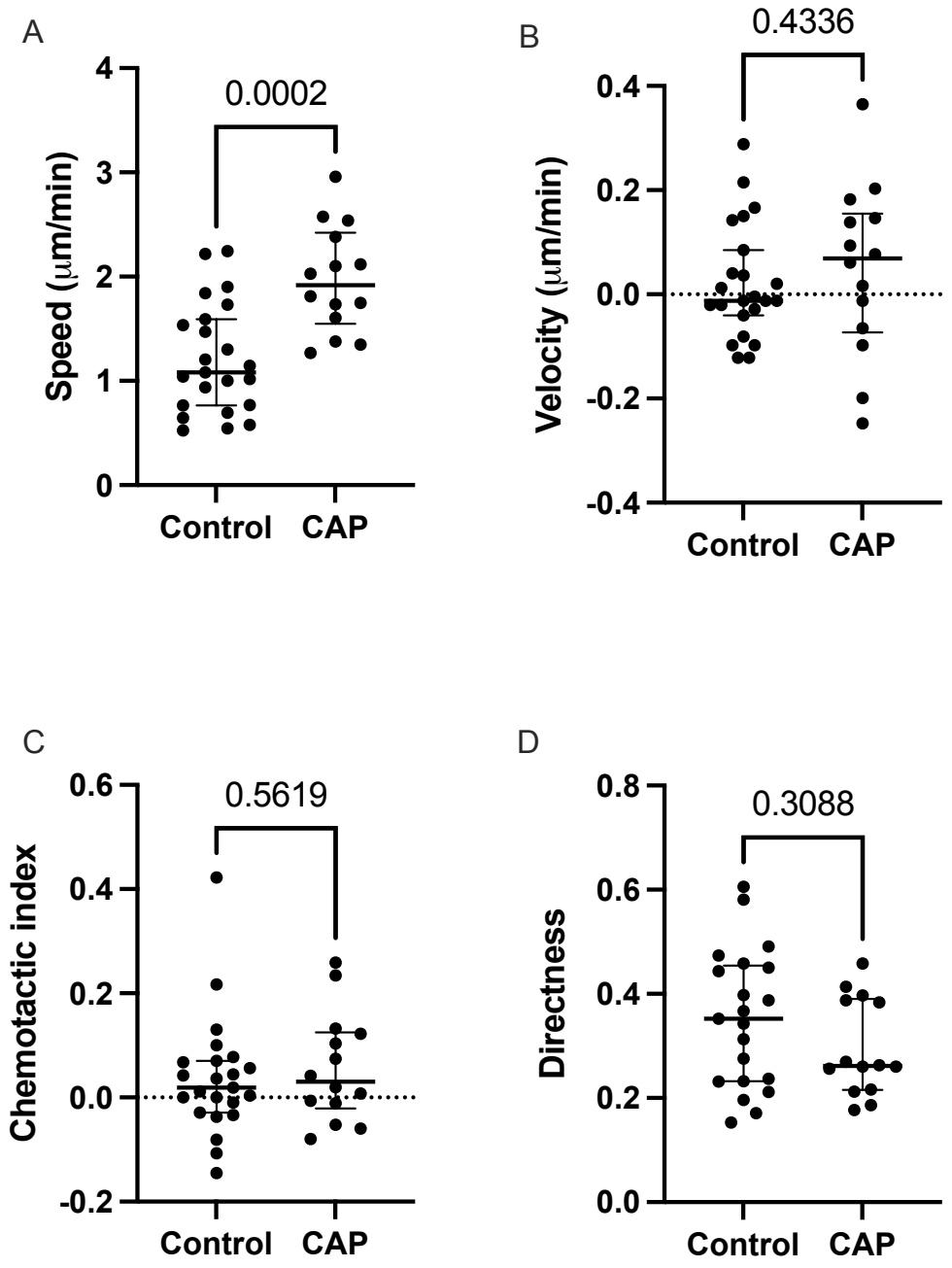

Figure 5-7: Neutrophil migratory parameters towards RPMI in CAP and Controls.

Isolated neutrophils migrated towards RPMI in a modified Insall chamber. (A) Neutrophil velocity is measured in μM/min towards RPMI. (B) Neutrophil speed in any direction. (C) Neutrophil chemotactic index is a measure of directional accuracy where 1 is direct migration to the chemoattractant and -1 is directly away. (D) Neutrophil directness is a measure of the straightness of the path taken where 1 is a direct straight line over the entire time lapse. Data presented is dot per participant, bars are median and IQR. Mann Whitney used for comparisons.

5.5.5. Neutrophils from donors with CAP migrate inaccurately to IL8.

Neutrophil migratory paths showed that cells from CAP donors migrated less accurately towards IL8 than controls (Figure 5-8). Velocity, chemotactic index, and directness are all metrics of directional movement towards the chemotactic gradient. Velocity was reduced in CAP donors (median [IQR], 0.32[0.01-0.58] μm/min in CAP vs. 0.73[0.56-0.83] μm/min; p=0.003 by Mann Whitney). Chemotactic index is measure of directional accuracy where 1=perfect accuracy. Chemotactic index was reduced in CAP (mean[S.D), 0.14[0.03] in CAP vs. 0.31[0.13] in controls; p<0.0001 by t-test). Directness is a measure of straightness of travel where 1 is a direct line. Neutrophil directness was reduced in CAP donors (mean [S.D], 0.31[0.06] in CAP vs. 0.41[0.12] in controls, p=0.0042 by t test). Neutrophil speed in any direction was maintained in CAP donors compared to controls (median [IQR] 2.2[2.0-3.6] μm/min for CAP and 2.3[1.7-2.9] μm/min; p=0.3402 by Mann Whitney).

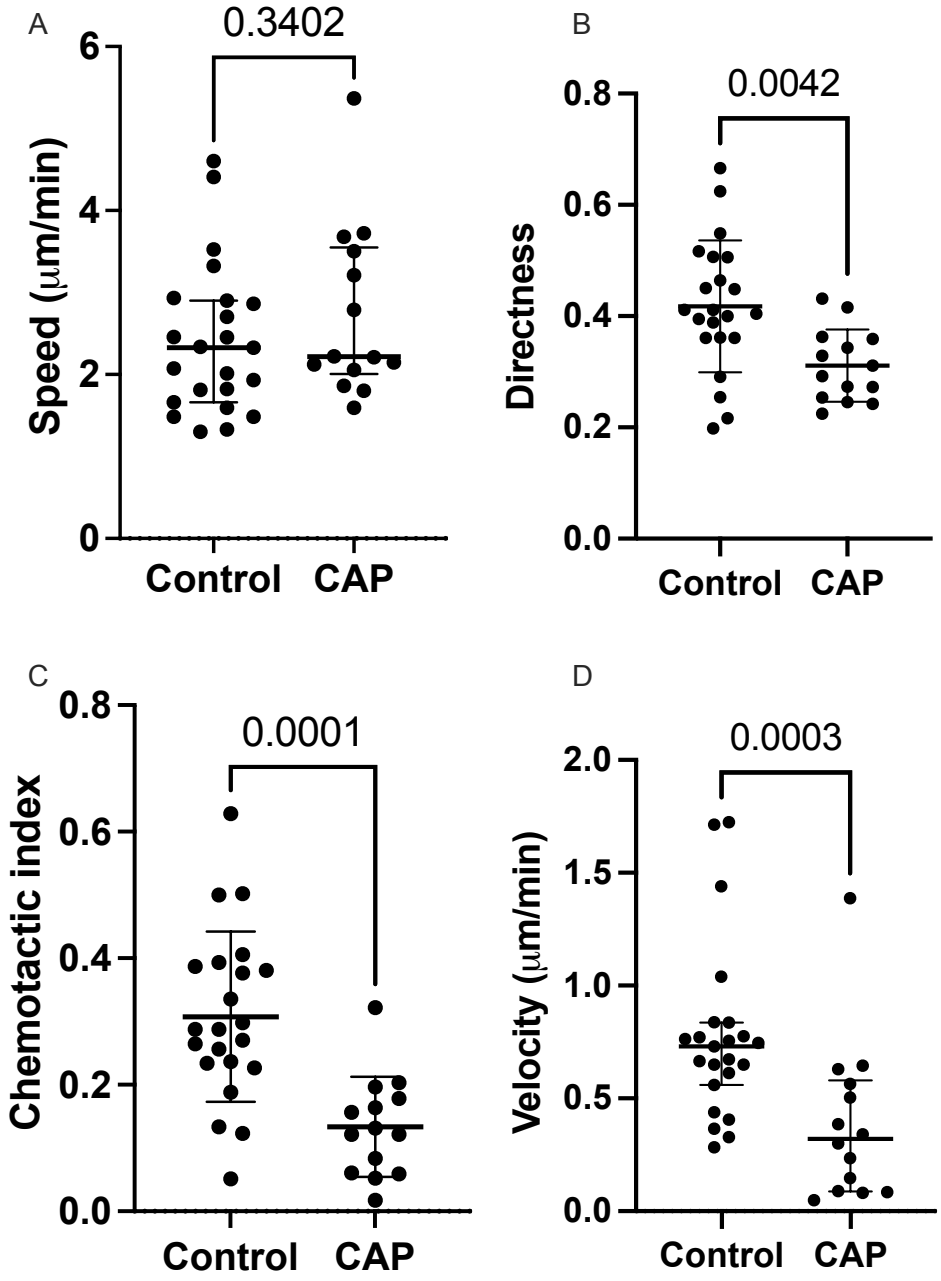

Figure 5-8: Neutrophils from participants with CAP have impaired migratory accuracy towards IL8.

Isolated neutrophils migrated towards 100nM IL8 in an Insall chamber. (A) Neutrophil velocity is measured in μM/min towards IL8. Data shown is dot per participant with median and interquartile range (p= 0.003). (B) Neutrophil speed in any direction was maintained between groups, data shown is median and IQR. (p=0.3402). (C) Neutrophil chemotactic index is a measure of directional accuracy where 1 is direct migration to the chemoattractant and -1 is directly away. Data shown are mean and SD (p<0.0001). (D) Neutrophil directness is a measure of the straightness of the path taken where 1 is a direct straight line over the entire time lapse. Data presented is mean and standard deviation (p=0.0042).

5.5.6. Neutrophil migratory parameters are not different according to 30-day mortality.

Data in Figure 5-9 and Figure 5-10 are exploratory and limited by low sample size. Metrics of directional and non-directional neutrophil migration to IL8 are not different in CAP donors according to 30-day mortality. Median neutrophil velocity in survivors was 0.503[1.23] μm/min compared to 0.235[0.48], p=0.116 by Mann-Whitney. Chemotactic index was 0.135[0.26] in survivors vs. 0.178 [0.179] in those who died within 30-days, p=0.557 by Mann-Whitney. Directness and speed were not significantly different between the two groups (Figure 5-9).

5.5.7. Neutrophil migration is not altered in participants who survive without hospitalisation until 90-days or more.

A composite outcome of hospitalisation free survival at 90-days was generated based on PPIE feedback of readmission being of equal importance to overall mortality in a previous study (Sapey et al., 2019). Neutrophil migratory parameters were not statistically significantly different between those who survived 90 days or more without hospitalisation and those who were readmitted or died within 90 days (Figure 5-10).

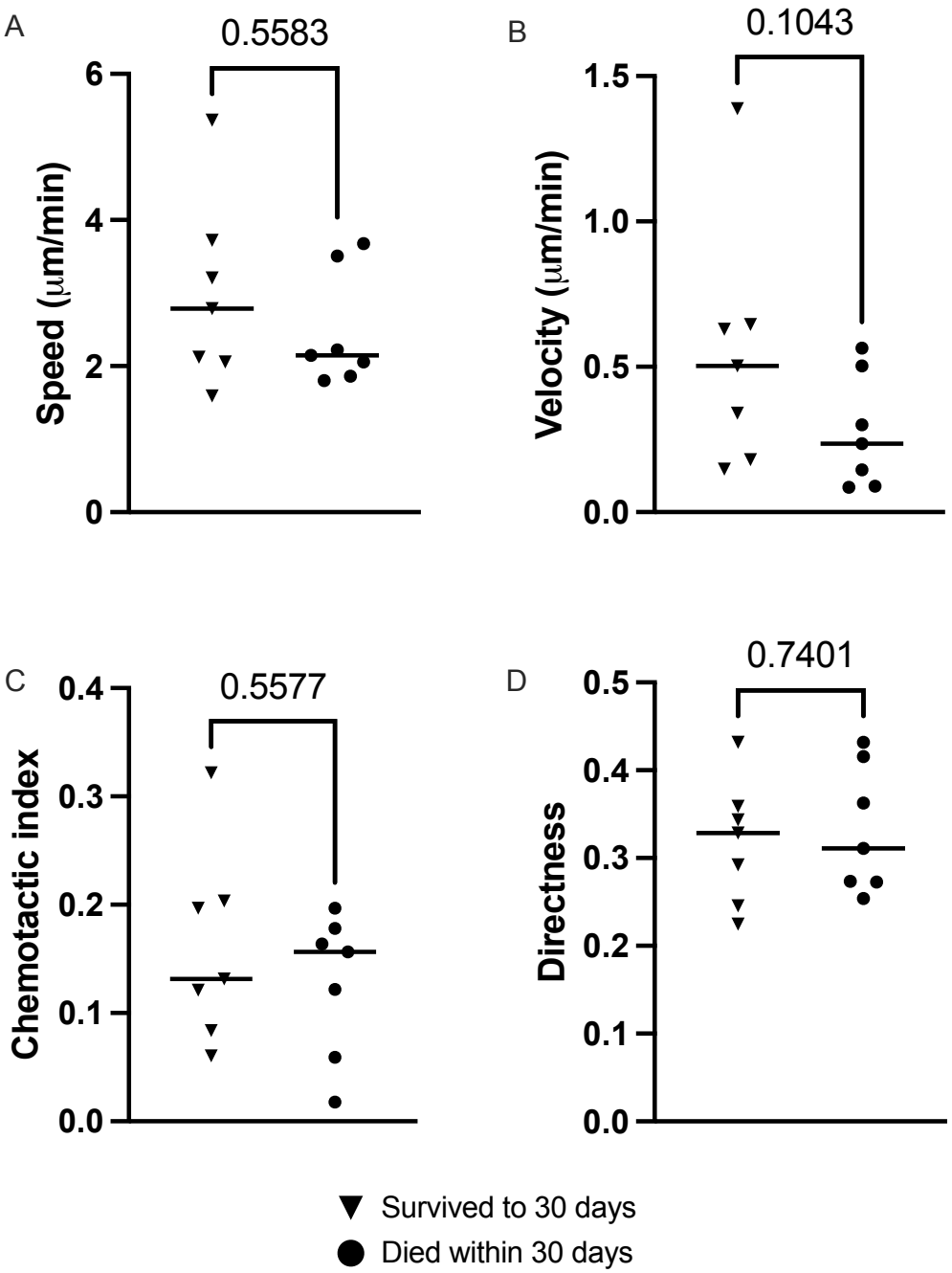

Figure 5-9: Neutrophil migration is not altered in survivors.

Isolated neutrophils migrated towards 100nM IL8 in an Insall chamber. (A) Neutrophil velocity is measured in μM/min towards IL8. Data shown is dot per participant with median and interquartile range (p= 0.104). (B) Neutrophil speed in any direction was maintained between groups, data shown is median and IQR. (p=0.558). (C) Neutrophil chemotactic index is a measure of directional accuracy where 1 is direct migration to the chemoattractant and -1 is directly away. Data shown are median and IQR (p<0.557). (D)Neutrophil directness is a measure of the straightness of the path taken where 1 is a direct straight line over the entire time lapse. Data presented is median and IQR (p=0.7404). Data shown is dot per participant. Comparisons performed using Mann-Whitney.

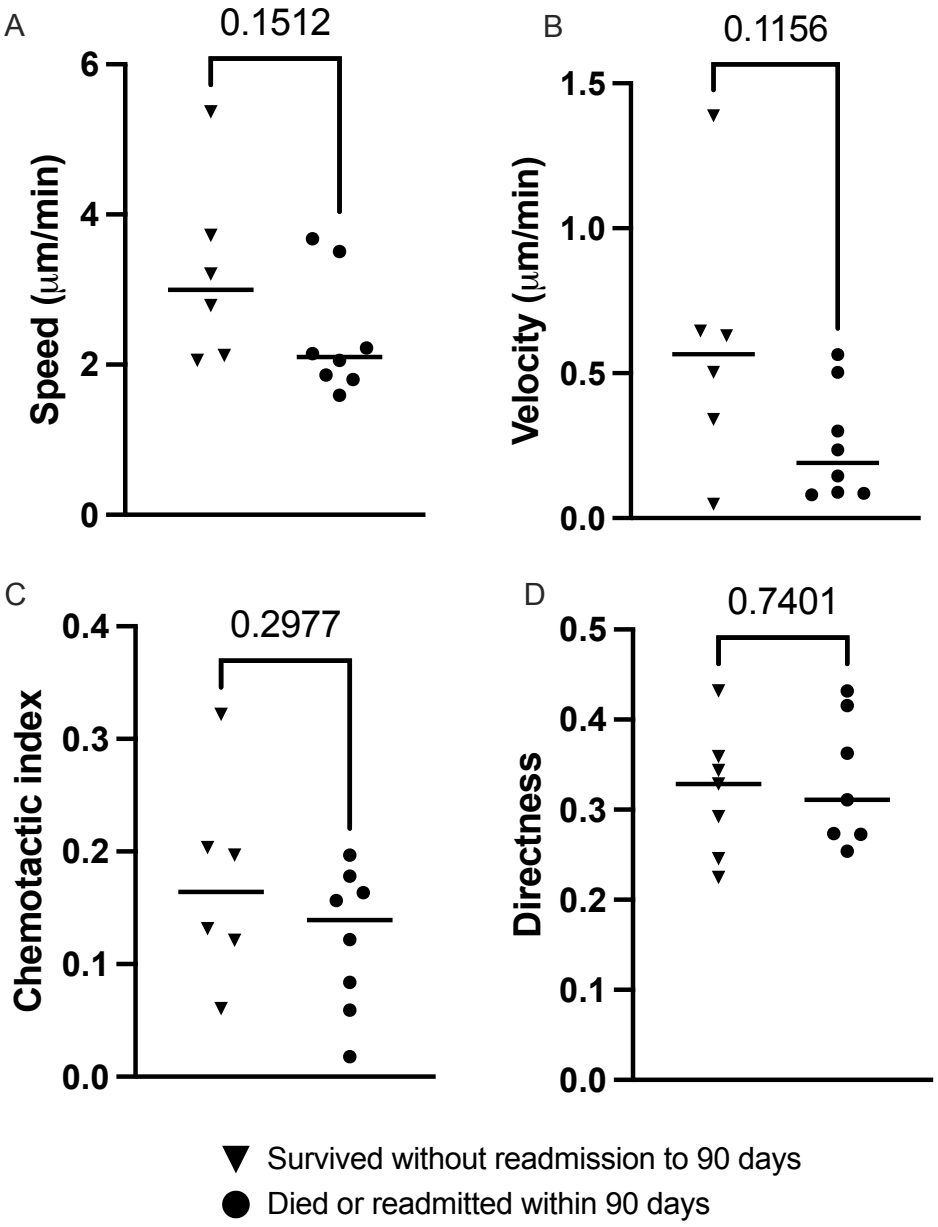

Figure 5-10: Neutrophil migration is not altered according to 90-day hospitalisation free survival.

Isolated neutrophils migrated towards 100nM IL8 in a modified Insall chamber. (A) Neutrophil velocity is measured in μM/min towards IL8. Data shown is dot per participant with median and interquartile range (p= 0.1156). (B) Neutrophil speed in any direction was maintained between groups, data shown is median and IQR. (p=0.1512). (C) Neutrophil chemotactic index is a measure of directional accuracy where 1 is direct migration to the chemoattractant and -1 is directly away. Data shown are median and IQR (p<0.2977). (D)Neutrophil directness is a measure of the straightness of the path taken where 1 is a direct straight line over the entire time lapse. Data presented is median and IQR (p=0.7401). Data shown is dot per participant. Comparisons performed using Mann-Whitney.

5.5.8. Neutrophil degranulation and proteinase secretion is increased in people with CAP.

NE and MPO were measured in plasma (Figure 5-11). NE was significantly increased in CAP donors (median [IQR] 7.71[6.4-10.1] ng/ml vs. 3.96[3.46-4.42]ng/ml, p<0.0001 by Mann-Whitney). MPO was significantly higher in CAP, (median [IQR] 87.1[53.5-147.6]ng/ml vs. 37.5[27.5-53.5]ng/ml, p= <0.0001 by Mann-Whitney). Further, systemic evidence/fingerprint of neutrophil proteinase activity was assessed by measurement of Aα-VAL^{541} (PR3 specific cleavage product) and Aα-VAL^{360} (NE specific fibrinogen cleavage product). Aα-VAL^{541} was higher in CAP donors than controls (mean [S.D] 91.93 [57.44] nM in CAP vs. 51.7 [26.46]; p=0.0044 by t-test). Aα-VAL^{360} was not different between groups (mean [S.D] 5.41 [1.5] nM in CAP vs. 5.48 [1.3] nM; p=0.8817 by t-test).

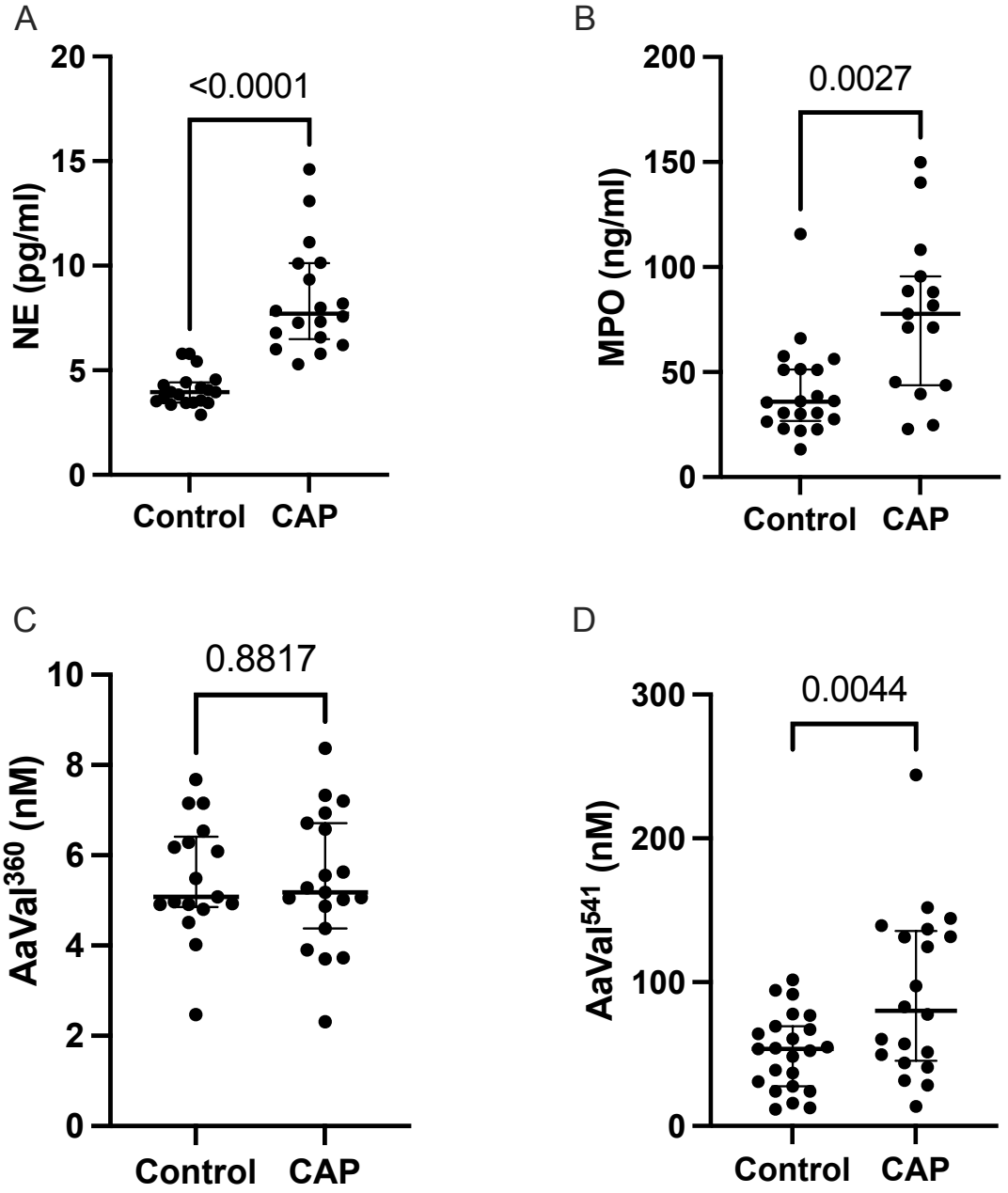

Figure 5-11: Neutrophil degranulation is increased in CAP.

Plasma neutrophil elastase (NE), Myeloperoxidase (MPO), NE was significantly increased in CAP donors (median [IQR] 7.71[6.4-10.1] ng/ml vs. 3.96[3.46-4.42]ng/ml, p<0.0001 by Mann Whitney. MPO was significantly higher in CAP samples, (median [IQR] 87.1[53.5-147.6]ng/ml vs. 37.5[27.5-53.5]ng/ml, p= <0.0001 by Mann Whitney. Aα-VAL^{360} is a neutrophil elastase specific fibrinogen cleavage product. Aα-VAL^{541} is a proteinase-3 specific cleavage product. Cleavage products were measured using ELISA in plasma. Data shown is a dot per participant.

5.5.9. Systemic inflammation is increased in CAP.

CRP, lactate, e-selectin, and p-selectin were measured in plasma. CRP was significantly higher in CAP compared to control (median [IQR] 154[466] vs. 3[5]mg/L), $p<0.0001$ by Mann-Whitney. Plasma lactate was significantly higher in CAP (median [IQR] 2.3[11.2] vs. 1.9[4.3]), $p=0.0057$ by Mann-Whitney. Soluble p and e-selectin are leucocyte receptors expressed on platelets and endothelial cells. E-selectin was significantly higher in CAP compared to controls; levels of p-selectin were not altered (Figure 5-12).

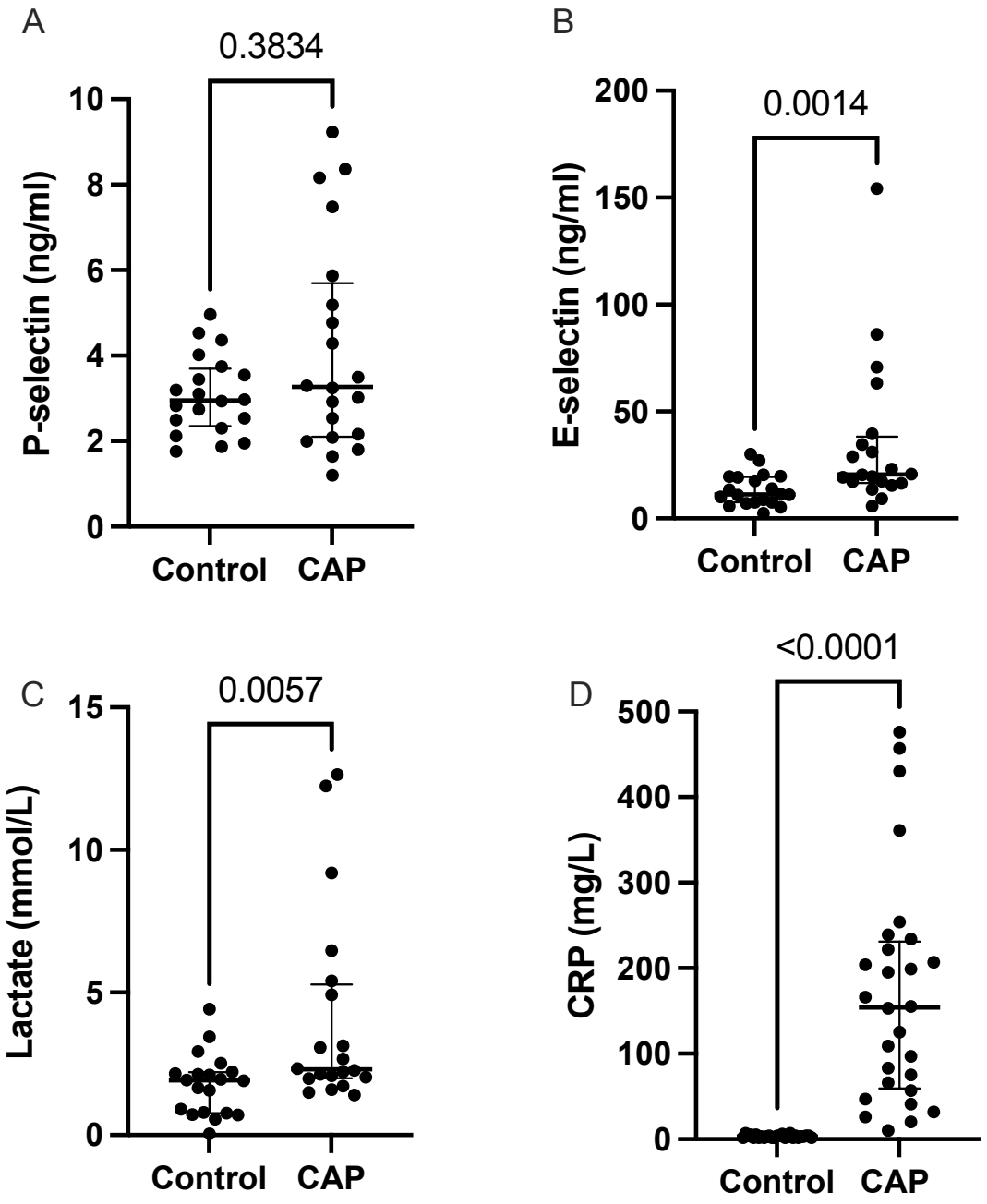

Figure 5-12: Plasma inflammatory markers.

P-selectin, e-selectin, CRP, and lactate were measured in plasma using commercially available kits. Data shown is dot per participant with median and IQR indicated. Comparisons were made using Mann-Whitney test.

5.5.10. Neutrophil oxidative burst in CAP is reduced in response to PMA.

Respiratory burst response to 160nM PMA was assessed using extracellular flux technology to monitor oxygen consumption. Injection of mitochondrial inhibitors enables oxygen uptake to be attributed to generation of ROS. Neutrophils from CAP donors consumed similar levels of oxygen over the entire assay as neutrophils from controls (mean [S.D], 11084[2768] pmol in CAP vs. 12855[3890] pmol in controls; p=0.384 by t-test), however the kinetics and magnitude of oxygen uptake were significantly different (Figure 5-13). Figure 5-13A shows the kinetics of oxygen consumption in response to PMA stimulation. Controls have a 200-fold increase in oxygen consumption shortly after PMA stimulation, where CAP donors display a 120-fold increase nearly 50 minutes later. The peak rate of oxygen consumption was significantly higher in control (mean [S.D] 210.6[71.33] ppmol in controls vs. 128.4 [26.15] pmol in CAP; p=0.0243 by t test) and time to reach peak oxygen consumption was significantly faster in controls (mean[S.D]) (100[17.62] minutes vs. 151[9.83] minutes; p<0.0001 by t-test).

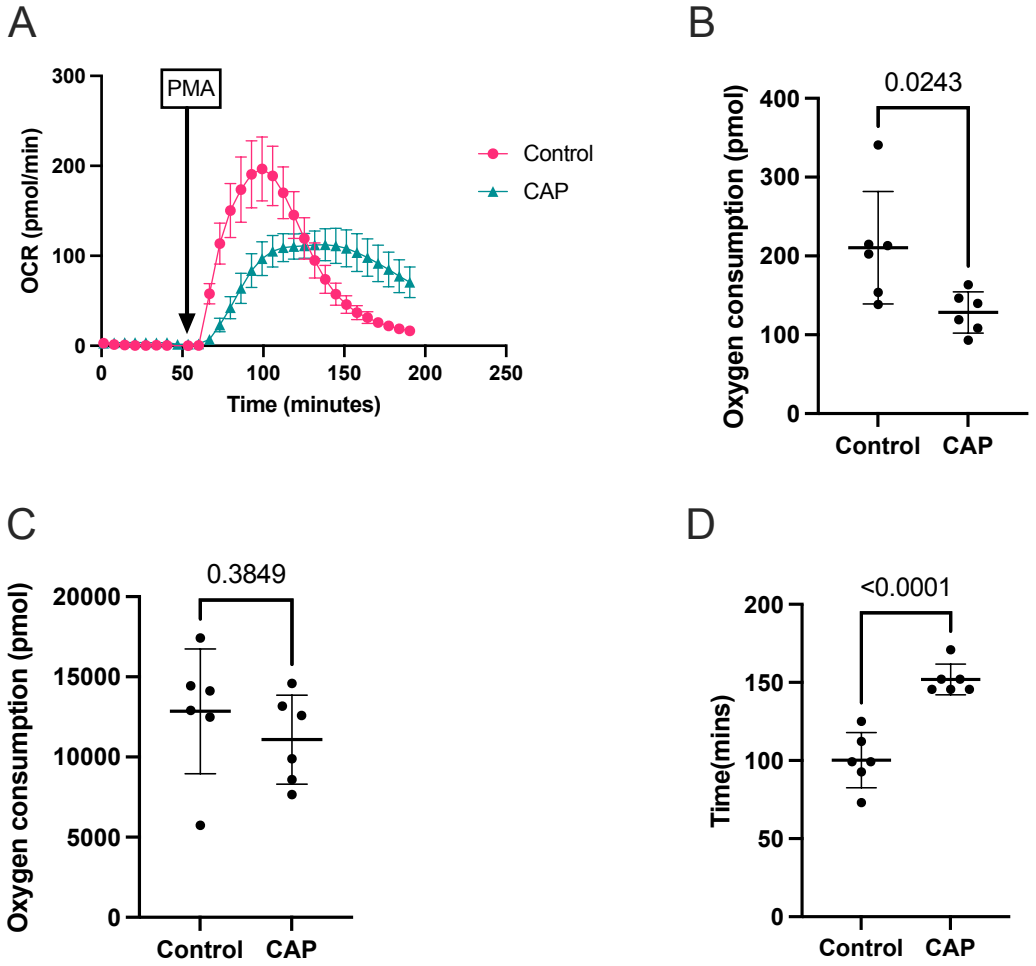

Figure 5-13: Neutrophil oxygen consumption following PMA stimulation.

Isolated neutrophils were seeded at 5x10^4/well on Cell Tak coated XFe96 well plates. Oxidative burst was probed by sequential injection of 2 µM rotenone plus 2 µM antimycin A then 160 nM phorbol 12-myristate 13-acetate (PMA). Oxygen consumption rate (OCR) was monitored over the duration of the test. (A) Oxygen consumption over time, (B) Peak Oxygen Consumption was significantly higher in controls (p=0.0243 by t-test), (C) Time to maximum oxygen consumption was significantly slower in CAP donors (p=<0.0001, by t-test), (D) Total Oxygen consumption was unchanged between groups (p=0.3849, by t-test). Time to max OCR, maximum OCR and total oxygen consumption were calculated from area under the curve analysis. Data presented are dot per participant, mean and standard deviation.

5.5.11. *Ex-vivo* basal and stimulated rates of glycolysis are unchanged in CAP from age matched donors but are increased compared to healthy young donors.

Ex-vivo metabolism was assessed using extracellular flux technology which provides

real time measurement of oxygen consumption and extracellular acidification

(surrogate of glycolysis). There was no difference in basal PER (mean [S.D] 113.8[40.05] ppmol/min in CAP vs. 114.4[28.96] in controls; p=0.9694 by t-test) in Figure 5-14A. There was also no difference in fold change in PER following stimulation with 160nM PMA (mean [S.D] 3.8[1.8] in CAP vs. 3.4[1.4]; p=0.6755 by t-test) in Figure 5-14B.

Glycolysis was also assessed in healthy young donors. Basal PER was significantly lower in healthy young adults compared to CAP and controls (mean [S.D]) 40.82 [11.45] pmol/min in young adults vs. 113.8[40.05] pmol/min in CAP vs. 114.4[28.96] pmol/min in controls; p=0.0001 by ANOVA). Glycolytic rate was significantly increased following PMA stimulation in healthy young adults compared to CAP or older controls (mean [S.D]) 7.4[2.1 vs. 3.8[1.9] in CAP vs. 3.5[1.4] in controls; p=0.007 by ANOVA).

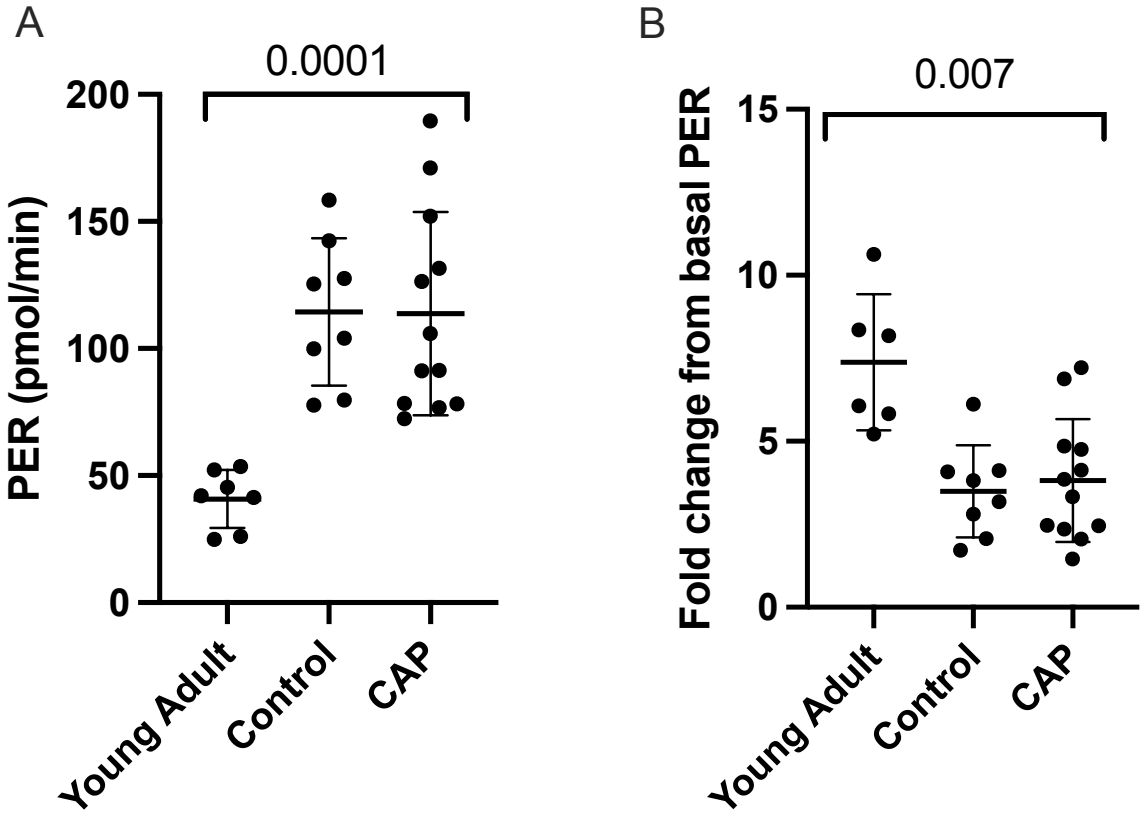

Figure 5-14: Neutrophil PER is significantly different in CAP and controls compared to younger adults.

Neutrophils were seeded at a density of 2 x 10^5 cells/well onto a Cell-Tak™ coated XFe96 microplate. 2 µM oligomycin was injected first, followed by sequential injections of 160nM PMA or finally 50 mM 2-Deoxy-D-Glucose (2-DG) PMA. Extracellular acidification rate (ECAR) was measured across the duration of the test. Following data export, ECAR readings were converted to proton efflux rate (PER) using Seahorse analytics by accounting for the buffer capacity of the media. (A) Basal PER is unchanged in CAP compared to controls (p=0.9694). (B) Fold change in PER after PMA injection is unchanged (p=0.6755). (C) Basal PER is significantly lower in young adults compared to CAP and controls (mean [S.D]) 40.82 [11.45] pmol/min in young adults vs. 113.8[40.05] pmol/min in CAP vs. 114.4[28.96] pmol/min in controls; p=0.0001 by ANOVA. (D) Fold change in PER following PMA stimulation was significantly higher in young adults (mean [S.D] 7.4[2.1] in healthy young adults vs. 3.8[1.9] in CAP vs. 3.5[1.4] in controls; p=0.007 by ANOVA. Data shown is a dot per participant with mean and standard deviation.

5.5.12. Neutrophils from CAP donors are immature but maintain surface expression of adhesion, activation markers and IL8 receptor.

Neutrophil phenotype was assessed using flow cytometry to assess cell surface markers in freshly isolated cells. Neutrophils from CAP are immature, $CD66b^+/CD10^+$

cells were significantly reduced (median[IQR]) 68.0[47.1-83.1]% in CAP vs. 90.9[81.2-95.1]% in controls, *p*=0.0025 by Mann-Whitney There were no significant differences seen in rates of expression of adhesion markers (CD11b, CD66b and CD11c), activation markers (CD62L) or IL8 receptor (Table 5-6).

Table 5-6: Neutrophils cell surface marker

Cellular marker	**CAP** (%)	**Control** (%)	***p*-value**
CD66b+/CD10+ (median [IQR])	68.0 [47.1-83.1]	90.9[81.2-95.1]	0.025
CD62L+ (median [IQR])	83.5[66.5-90.6]	86.0[82.1-89.3]	0.516
CD66b+ (median [IQR])	99.0[98.1-99.6]	98.5[98.5-99.0]	0.362
CD11b+ (median [IQR])	99.0[98.2-99.3]	98.5[98.1-99.1]	0.460
CD11c+ (median [IQR])	94.7[92.2-97.2]	90.8[70.3-95.8]	0.117
CD11b+/CXCR2 bright (median [IQR])	75.4[39.4-85.4]	71.5[12.9-79.5]	0.505

Expressed as percentage of cells expressing marker (median [IQR]). Antibodies to detect cluster of differentiation (CD) 11b, CD66b, CD10, CXC chemokine receptor (CXCR) 2, CD11c, CD62L with isotype controls were incubated with isolated neutrophils. Data were acquired using MACSQuant 10 flow cytometer (Miltenyl Biotec) and analysed using FlowJo software. CAP n=19, controls n=9.

5.6. Discussion

This study provides the first report of *ex-vivo* neutrophil metabolism in older adults with CAP and sepsis, demonstrating altered effector functions, but maintained *ex-vivo* glycolysis assessed by extracellular flux. This study also demonstrates that neutrophil glycolysis is significantly different in healthy young adults compared to older adults with or without CAP.

This study identifies impaired migratory accuracy in combination with increased degranulation, reduced oxidative burst and immature phenotype which is interpreted to be problematic in the early stages of CAP. The cumulative impact of these alterations in neutrophil function would be predicted to impact bacterial clearance while causing increased tissue damage. Others have shown impaired bacterial chemotaxis as well as phagocytosis that were associated with poor patient outcomes, both in terms of mortality and risk of development of secondary infections (Patel et al., 2018; Morris et al., 2011). Therefore, aiming to normalise neutrophil function would be expected to improve outcomes.

CAP places a huge burden on healthcare services, and this is expected to rise over the coming years due to population aging and increased prevalence of multi-morbidity (Ferreira-Coimbra, Sarda and Rello, 2020). The high mortality and readmission rates in this study despite optimal treatment according to current guidelines demonstrate that antibiotics alone are not the answer in CAP. We urgently need new strategies to tackle the burden of CAP in frail older adults.

5.6.1. Apoptosis

Neutrophils from CAP donors had increased rates of early apoptosis compared to healthy donors immediately following isolation, this replicates findings from other studies(Patel et al., 2018). Previous work has indicated that although early apoptosis is increased at an early time point, neutrophils from donors with sepsis have prolonged lifespan compared to those from healthy donors at late timepoints (Patel et al., 2018; Sadiku et al., 2017). This work did not assess apoptosis serially, but this suggests that the apoptotic neutrophils are not being effectively removed and may be contributing to inflammation and tissue damage, while having altered function. In

future, sorting cells by viability and assessing function according to viability stage would inform the relationship between early apoptosis and effector function.

5.6.2. Migration

This study demonstrates impaired *ex-vivo* neutrophil migration to IL8 in older adults with CAP compared to age and morbidity matched controls. There was no difference observed in neutrophil migration according to 30-day mortality, or a composite measure of 90-day hospitalisation free survival, however this study was not powered to detect these differences and the included numbers were low. Based on the data in this study, the indicative sample sizes to detect differences in 30-day mortality would be 112 participants and 46 participants for the 90-day hospitalisation free survival measure. Impaired neutrophil migration in CAP and sepsis has been consistently reported (Sapey et al., 2017, 2019; Patel et al., 2018). Previous studies have been able to demonstrate a stepwise deterioration in neutrophil chemotaxis with increasing severity of infection (Sapey et al., 2017), however this study only recruited severe CAP, so this previous finding is not tested in the current study. The reduced directional accuracy suggests that *ex-vivo* neutrophils from donors with CAP may not be able to accurately migrate to the site of infection and therefore potentially expose an increased area to the injurious contents of an activated neutrophil. Unstimulated neutrophils from donors with CAP exhibit greater speed of migration to negative control than control neutrophils, again suggesting that in the absence of a chemoattractant CAP neutrophils may be “wandering”.

5.6.3. Oxidative burst

Altered oxidative burst in response to PMA stimulation supports the dysfunctional neutrophil phenotype. This study uses a total oxygen consumption assay to

determine neutrophil oxidative burst following stimulation with PMA. This provides advantages over the typically used chemiluminescent assays which are highly selective for individual species of reactive oxygen and suffer from artefacts related to redox recycling, therefore meaning the true picture of ROS generation is challenging to understand (Murphy et al., 2022a). This study shows similar levels of overall oxygen consumption, but significant differences in time taken for oxygen consumption and in peak of oxygen consumption. This demonstrates the importance of considering kinetics of neutrophil responses. Due to their potentially toxic capacity the ideal neutrophil oxidative burst would reach a rapid peak and quickly wane, in contrast we demonstrate a slow burn of oxidative burst which may not be effective as an anti-microbial agent and may increase tissue injury (Worthen et al., 1987). Other studies assessing neutrophil oxidative burst in older adults have demonstrated mixed results, which probably reflects differences in assays- such as chemiluminescent assays which can cross react with mitochondrial ROS, reduced sensitivity of assays, whole blood or isolated neutrophils and age/severity of participants. In CAP specifically there is limited data regarding ROS; using a whole blood assay in a young cohort (50-60 years of age) Fernandez-Botran *et al* demonstrated reduced oxidative burst in severe CAP compared to controls, however, oxidative burst was maintained in non-severe CAP (Fernandez-Botran et al., 2014). In a sepsis study where pneumonia was the most common cause of sepsis, Martins *et al* identified increased ROS in sepsis compared to controls, but this was in a younger adult cohort (40-50 years) than included in the current study (Martins et al., 2008). In COVID-19 infection ROS has been shown to be up and downregulated dependent on the study, and severity of disease (Borella et al., 2022; Loyer et al., 2022; Belchamber et al.,

2022). Despite the mixed picture established in the literature this study shows that abnormal ROS generation is part of the aberrant neutrophil phenotype seen in CAP.

5.6.4. Degranulation

Neutrophils granules contain serine proteinases which are used as anti-microbial agents and to facilitate migration through extra-cellular matrices (Amulic et al., 2012). This study shows increased degranulation evidenced by measurement of total NE and MPO in plasma. Further, proteinase activity was measured using PR3 and NE specific cleavage products, these assays measure the systemic signal generated by proteinase activity in the lung compartment (Newby et al., 2019; Carter et al., 2011). PR3 activity is increased in CAP, but despite increased NE in plasma, NE activity is not increased. This is an important observation, as typically plasma NE levels are reported and purported to be associated with increased tissue damage. The differential activity levels in PR3 compared to NE in this study is attributed to a combination of:

1. Differential inhibition of NE by alpha-1 antitrypsin (AAT) (an acute phase protein) in inflammation (caused by CAP) as NE has a much higher affinity for AAT than PR3 (Beatty, Bieth and Travis, 1980; Rao et al., 1991; Korkmaz et al., 2004).
2. NE can be inhibited by organisms which commonly cause pneumonia (Vered, Dearing and Janoff, 1985)
3. PR3 is much more abundant and shed more rapidly (Campbell, Campbell and Owen, 2000).

NE and MPO are widely reported to be increased during CAP (Matsuse et al., 2007; TAGAMI et al., 2011; Belchamber et al., 2022; Loyer et al., 2022; Schuurman et al., 2023) in keeping with this data, however, this is the first report of PR3 activity in CAP,

or any acute illness. A significant reduction in NE activity (Aα-VAL^{360}) at day four of statin treatment has been reported in an RCT of CAP, this study demonstrated increased hospitalisation free survival in statin treated participants (Sapey et al., 2019). Aα-VAL^{360} activity has been assessed in older adults (both healthy and frail) compared to younger adults where Aα-VAL^{360} was highest in healthy older adults (16.5nM) compared to 9.5nM in frail older adults and 8.8nM in healthy young adults (Wilson et al., 2020). These values are higher than those reported in this study (CAP 5.2[IQR 2.3] vs. 5.1[1.6]), however this assay exhibits a known inter-plate variation of ~20% therefore direct comparisons are not possible.

Overall, these altered neutrophil functions suggest that neutrophils in CAP have impaired accuracy of migration compared with increased degranulation and a slow burn of ROS generation. Together this suggests that CAP neutrophils may be wandering in a nonspecific manner while exposing tissues to increased levels of injurious proteinases with a slow and potentially ineffective oxidative burst.

5.6.5. Metabolism

As neutrophil effector functions are energy intensive (Stjernholm, 1967) and inhibition of glycolysis abolishes neutrophil effector functions (Boxer, Baehner and Davis, 1977; Maianski et al., 2004), it was hypothesised that impaired function in CAP was related to failure of energy generation by glycolysis. Here there are significant impairments in migration and oxidative burst, with increased degranulation, but *ex-vivo* metabolism was unchanged suggesting altered metabolism is not the driver of the functional defects observed.

This data in frail older adults contrasts to other studies in younger cohorts with mild infection and different co-morbidities. In a study of mild pneumonia, metabolic shifts

to glycolysis were identified in neutrophil transcriptome and metabolome, however, they did not directly assess cellular energetics. Further this study is limited by cofounding as the CAP cohort had significantly higher rates of COPD (Schuurman et al., 2023) which is known to influence both neutrophil function and metabolic status (E Sapey et al., 2011; Sadiku et al., 2017).

Extracellular flux analysis of neutrophils from donors with COVID-19 demonstrated increased glycolysis which was confirmed by RNA-sequencing (Borella et al., 2022). A study of the neutrophil transcriptome during intra-abdominal sepsis also demonstrated upregulation of glycolytic enzymes assessed by RNA sequencing (Pan et al., 2022). Important caveats for these studies are younger cohorts, co-morbidity, severity of illness and type of acute illness which are all known to influence neutrophil function and metabolism.

Neutrophil metabolism is significantly different in healthy younger adults, with lower basal rates but increased rates in response to PMA stimulation. This data suggests that neutrophil metabolism changes with age, but in older adults acute respiratory infection does not alter neutrophil metabolism. This demonstrates the importance of defining the cohort at risk and investigating immune cell function in those with closely matched controls. Frail older adults are at the greatest risk of developing CAP (Welte, Torres and Nathwani, 2012), and experience the poorest outcomes in CAP (Cillóniz et al., 2013).

5.6.6. Strengths

These are important findings which warrant further investigation. Particularly the difference seen in glycolytic metabolism between young and aged donors. There is a body of literature investigating immunosenescence and this data suggests that

alterations in metabolism may play a role in altered function; there are no other data assessing neutrophil metabolism either in young vs. older adults or using longitudinal assessment.

This study recruited a pragmatic population of CAP participants and well-matched controls. This is a population underserved by existing immunology research, but who bear the brunt of ill-health related to infectious disease.

There has been increasing interest in targeting immunometabolism in sepsis, this data provides caution that each patient population should be carefully phenotyped with consideration for known factors which influence immune cell function. This data suggests that alongside indirect assessment of metabolism using 'omic approaches, metabolic activity should be directly measured in *ex-vivo* cells as discordance between transcriptome and proteome is well recognised (Wang et al., 2019b), however agreement between directly measured function such as extra-cellular flux and 'omic approaches has not been established.

5.6.7. Limitations

There are limitations to this study. Firstly, only glycolysis was assessed, Chapter Five, section 4.4.6 demonstrates the limitations of extracellular flux in assessment of mitochondrial respiration (Grudzinska et al., 2023b). This is in keeping with the low density of mitochondria in neutrophils and evidence demonstrating that mitochondrial function does not contribute to overall ATP balance (Sadiku et al., 2017; Fossati et al., 2003). Other groups have also demonstrated that mitochondrial respiration is not amenable to measurement by extracellular flux (Chacko et al., 2013). Shifts towards PPP (a key regulator of ROS and therefore NETosis) (Britt et al., 2022) were not examined in this study.

Ex-vivo metabolism was assessed but cellular ATP or glycogen stores were not measured.

This study provides snapshot of neutrophil function and metabolism, an optimal response would be a rapid and robust response early in the disease course, with rapid waning to allow for resolution of inflammation. CAP participants were recruited on average three days after symptom onset meaning the changes in glycolysis may have occurred early and then resolved to prevent over-activation and tissue damage, understanding this would require large numbers of community dwelling participants with longitudinal sampling. All CAP participants had sepsis defined as qSOFA≥2, however 2021 sepsis guidelines have removed this definition, but to ensure a standardised definition was used for the entire study period this definition was used throughout.

The absence of metabolic shift demonstrated here could also be related to neutrophil maturity, Table 5-6 demonstrates that neutrophils from CAP donors were more immature as defined by CD66b+/CD10+. The relationship of neutrophil maturity and metabolism is unknown in humans, however in murine studies mitochondrial respiration appears to play a role in neutrophil bone marrow differentiation (Fossati et al., 2003; T Riffelmacher et al., 2017; Richer et al., 2018). Therefore, it may be that these immature circulating neutrophils have altered metabolism compared to mature neutrophils. This could be addressed by cell sorting according to maturity and assessing metabolism in those sub-groups.

PMA was used throughout this study as a robust stimulator of neutrophil functions. PMA is a non-physiological stimulus. It was chosen as it presented the most robust stimulation of neutrophil oxidative burst and glycolysis in Chapter Five, section 4.4.4.

In Chapter Five physiological stimuli such as fMLP and bacteria were not able to induce oxidative burst even when neutrophils were primed with TNFα. However, to further this work neutrophil responses to more physiological stimuli such as sputum from CAP participants or relevant bacteria should be assessed.

Finally, not all assays were performed in all donors due to limited cell numbers, time restrictions in performing all assays on the same day and access to equipment. This small sample size limits analysis of how neutrophil behaviour relates to clinical outcomes. This approach also introduces a risk of bias, as if specific donors were selected for specific assays this would influence the results presented. However, the variable number of donors used for each assay is as a result of unpredictable factors such as a cell number and access to specific equipment required for assays. In future work ensuring a core assay (such as migration) had complete data available would prevent any bias.

5.7. Summary

Neutrophils from CAP donors exhibit a range of altered effector functions *ex-vivo* in keeping with the published literature, however, despite neutrophil functions being highly energy dependent, *ex-vivo* metabolism was unchanged. Importantly, this study demonstrates altered metabolism in older adults compared to healthy young adults. This study only assessed glycolysis at a single time-point and did not consider other forms of metabolism such as mitochondrial respiration or pentose phosphate pathway. Further studies should assess the age-related change in glycolysis by considering longitudinal sampling alongside including CAP participants at early and late time points, and mild versus severe disease to understand the kinetics of neutrophil metabolism. To develop understanding of the temporal relationship of

neutrophil dysfunction and infectious disease a longitudinal cohort of older adults at high risk of infection, such as people undergoing elective surgery could be established.

6. NEUTROPHIL TRANSCRIPTOME IN COMMUNITY ACQUIRED PNEUMONIA

6.1. Abstract

Neutrophils play a crucial role in responding to infection and inflammation, however aberrant neutrophil responses have been demonstrated in a wide range of conditions, this is particularly true in older adults where even mild infection can cause significant depression of multiple effector functions. The mechanism(s) underlying this dysfunction are unknown. This study sought to test the feasibility of RNAsequencing in isolated neutrophils, and to understand whether transcriptome analysis could be used to validate findings from Chapter Five, but also offer new insights into neutrophils during severe infection.

RNA isolated from 14 participants demonstrated that good quality RNA suitable for RNAsequencing is feasible from isolated neutrophils. There were 760 differentially expressed genes between CAP and control donors. These included non-coding RNA and novel identifications in neutrophils. The top differentially expressed genes are discussed in relation to their likely impact on neutrophil functions. Functional enrichment using pathway analysis and upstream regulator analysis were also performed to provide validation of the phenotype demonstrated in Chapter Five. The computational analysis of RNAseq in neutrophils is challenging due to the paucity of neutrophil RNA data published, as the analysis pipelines rely on published pathways. Nevertheless, this study confirms that RNAseq in isolated neutrophils is technically good and generates a large number of differentially expressed genes for further analysis.

6.2. Introduction

Ribonucleic acid (RNA) is transcribed from DNA and contains the blueprint for protein assembly in all living cells. In humans RNA is single stranded with a backbone of alternating phosphate and ribose sugars . Each sugar has a base attached; bases provide the code for protein assembly. Protein assembly is the primary function of RNA; however, RNA can also directly influence cellular processes via noncoding RNA (ncRNA) (Berg et al., 2019).

Measurement of RNA expression is a cornerstone of molecular biology facilitating understanding of cellular processes, by assessing how RNA expression relates to disease or interventions. RNA expression can be assessed by variations of PCR. Quantitative-PCR (qPCR) is low throughput and requires larger amounts of RNA depending on abundance of the target, for a limited number of targets. Larger numbers of targets can be assessed using microarrays which enable assessment of a predefined set of transcripts. In 2007 RNAsequencing (RNAseq) was developed which can identify novel targets in addition to measurement of known targets to support or refute an existing experimental hypothesis (Hong et al., 2020).

RNAseq has a wider dynamic range for detection of differentially expressed genes (DEGs) compared to microarray ($>10^5$ for RNAseq compared to 10^3 for microarrays), in addition to being able to detect novel transcripts (Rai et al., 2018; Rao et al., 2019b; Zhao et al., 2014; Wang et al., 2009) . However, RNAseq is a relatively expensive technique generating complex datasets which require significant bioinformatic training and analysis time. qPCR costs approximately £10 per gene, whereas RNAsequencing is approximately 10 pence per gene. Therefore, RNAseq can be an economical approach, especially if using precious samples in which precise mechanisms of altered phenotype are unknown (Nonis et al., 2014).

Table 6-1: Comparison of qPCR, microarrays and RNAseq

	q-PCR	Microarray	RNAseq
Transcripts detected	Gene by gene or panels	Custom and off shelf arrays available	All transcripts, including novel findings, variants, and splicing.
Throughput	Low	High	High
Cost	Cheapest	Intermediate	Most expensive
Dynamic range	High	Low	High
Wet lab labour	High	Low	Low
Complexity	Low	Low	High
Background noise	Low	High	Low
RNA required	High	High	Low

Adapted from (Wang 2009, Rai 2018 and Zhao 2014 and Rao 2019)

RNAseq provides a snapshot of how the transcriptome is altered, but does not inform why that change has occurred, or how (or if) that change has influenced cellular function. Further it gives expression data for individual genes, but perturbation of a single gene is unlikely to drive cellular changes seen in disease. Pathway analysis was developed to address the analytical issues of RNAseq datasets. Pathway analysis is a bioinformatic technique used to map altered gene expression to known pathways, however, this technique is open to bias as

1. Pathways need to be established in the literature.
2. Annotation of pathways can be biased by the quality of experimental data used to establish a pathway.
3. This assumes that pathways are conserved across cell types.

Pathways can be experimentally determined in humans, animals, or cell lines, or be predicted based on similarity with expert opinion. Despite its limitations, pathway analysis can be a helpful tool to identify altered signalling pathways for further investigation (Subramanian et al., 2005; Kanehisa et al., 2016; Gene Ontology Consortium, 2015; Culhane et al., 2012; Geistlinger et al., 2021).

Transcript expression does not always mean protein translation, and protein expression does not always correlate with direct assessments of cellular activity (Wang, Liu, and Zhang, 2014; D. Wang et al., 2019). The innate immune system has multiple restraint mechanisms to prevent uncontrolled immune activation and subsequent tissue damage, one of these restraint mechanisms is post-translational modification (PTM) (Liu et al., 2016). PTM describes linkage of proteins to new functional groups, such as phosphate, methyl groups and others to stabilise, activate or repress function (Deribe et al., 2010). PTMs have been shown to regulate immunity through modification of pathogen recognition receptors and other key signalling molecules in immune cells (Liu et al., 2016). Therefore, RNAseq should be used alongside complementary methods which consider protein abundance and direct measurement of protein function and/or the cellular activity.

Despite these limitations, RNAseq is a useful tool for hypothesis testing where altered cellular function has been demonstrated but can also be used as a hypothesis generation tool to provide novel targets for further investigation. This was a useful approach early in the COVID-19 pandemic where it was rapidly established that COVID-19 was a highly inflammatory disease with significant alterations in innate immune cells. Studies used RNAseq to rapidly provide unbiased data on alterations in gene expression to provide a shortlist of implicated or candidate genes for further

investigation (Wilk et al., 2020). The benefit of RNAseq approaches has also been demonstrated by development of gene “signatures” associated with a particular risk or outcome in sepsis. RNAseq data has informed models which then generate a small number of genes that can be used to predict risk of a specific outcome, such as ICU admission or mortality (Baghela et al., 2022). This has specific appeal to research settings where there is increasing recognition of the heterogeneity of sepsis with different cellular phenotypes associated with positive and negative outcomes. Zador *et al*. compared the whole blood transcriptome from participants with CAP, traumatic injury, or intra-abdominal sepsis to healthy controls, and although there were no differences in RNA expression between the different aetiologies of critical illness, they were able to identify clusters of similar RNA expression based on survival. Where survivors had a more immunocompetent phenotype, non-survivors had a more immunosuppressed phenotype based on RNAseq (Zador et al., 2020). This work has been further developed in a secondary analysis of a clinical trial of steroids in septic shock, where participants with the immunocompetent phenotype had increased mortality if given steroid therapy, and this remained significant after adjustment of age and disease severity (Antcliffe et al., 2019). Clearly RNAseq and associated bioinformatic analysis is not practical either clinically or to stratify research studies, so this model has been validated for used by RT-PCR and microarrays to identify participants as immunocompetent or immunosuppressed (Cano-Gamez et al., 2022). This brings the suggestion of personalised or stratified medicine closer to reality and could help reduce the frequency of failed clinical trials by providing a rapid method for study stratification, which in time may inform clinical practice.

6.2.1. Transcriptome in sepsis

There are multiple studies examining alterations in whole blood or PBMC in sepsis or infection (Lei et al., 2021, 2022; Ryan et al., 2022; Baghela et al., 2022; Wilk et al., 2020; Overmyer et al., 2021; Burnham et al., 2017). All these studies demonstrate hundreds of differentially expressed genes between sepsis participants and controls. The aetiology of sepsis or infection source does not appear to influence gene expression in these cohorts, for example there were no differences seen in donors with faecal peritonitis compared to CAP (Burnham et al., 2017). However, a unified finding was that there was differential expression between survivors and non-survivors independent of infection source (Burnham et al., 2017; Zador et al., 2020; Lei et al., 2021, 2022). Other studies have taken an “omics” approach to sepsis or infection where they combine the transcriptome, proteome, and metabolome to attempt to explain alterations seen in the immune system (Overmyer et al., 2021; Palma Medina et al., 2023). Although strengthening the RNAseq data by examining other compartments, these studies lack functional correlation, or longitudinal follow up.

6.2.2. Neutrophil transcriptome

Despite neutrophils being the most abundant leucocyte, there is a paucity of data regarding the neutrophil transcriptome. This is likely to be reflective of dogma regarding the role of neutrophils as terminally differentiated cells with no need for transcriptional activity. It may also reflect the challenges of working with neutrophils and subsequent RNA isolation, including need for rapid isolation from whole blood, short lifespan, high content of proteinases (including RNAses), and propensity to undergo activation and apoptosis.

Neutrophils possess less RNA than monocytes (Maraux et al., 2020), but due to the abundance of neutrophils and sensitivity of modern RNAseq platforms this does not preclude RNAseq. Neutrophils RNA expression is measurable in the quiescent phase (Itoh et al., 1998; Kobayashi et al., 2002), and expression is rapidly altered following *ex-vivo* exposure to physiological stimuli (Subrahmanyam et al., 2001; Wright et al., 2013; Chiewchengchol et al., 2015).

Concern has been reported over contamination of neutrophil isolations with other leucocytes which possess more RNA and therefore may bias RNAseq data. However, there was no alteration in RNAseq data from “ultrapure” (>99.9% pure) neutrophils isolated using immunomagnetic methods to the more typical 95% purity from density gradient isolations (Thomas et al., 2015). Neutrophils are particularly sensitive to activation and apoptosis compared to other leucocytes; therefore, the RNA expression of these cells could be altered upon manipulation. However up to 6 hours at 4°C following isolation, RNA expression was unchanged, but after 6 hours there were significant changes in activation, apoptosis, and cell control signalling (Xing et al., 2021).

It is widely acknowledged that neutrophils respond to different stimuli using a diverse range of signalling pathways, although there is redundancy. This diversity in signalling pathways is reflected in RNAseq datasets, where neutrophils were primed with TNFα or GM-CSF for one hour prior to RNA isolation. TNFα stimulation led to 605 DEGs, where as GM-CSF stimulation led to 1843 DEGs, with ~500 overlapping DEGs between both stimuli (Wright et al., 2013). It is helpful to know that the diversity known from direct assessment and functional analysis in neutrophils is reflected in RNAseq data to inform novel targets for further investigation.

6.2.3. Neutrophil transcriptome in disease

Neutrophil RNAseq has not been widely completed in human disease studies. The studies in sepsis or CAP have mainly used whole blood or PBMCs rather than isolated neutrophils. Single cell RNAseq is even less studied in neutrophils where neutrophil populations could be investigated from whole blood, but the algorithms and bioinformatic analysis to determine which cells are neutrophils is limited by the lack of neutrophil specific studies in the literature (Wigerblad et al., 2022). The ability to perform neutrophil scRNA would represent an advance and reduce the risk of change of transcriptome relating to isolation methods.

Studies of the neutrophil transcriptome have been performed in Diabetes Mellitus (Type 2) (Lin et al., 2020a), Bechet's Disease (Yu et al., 2022), Tuberculosis (Geng et al., 2022), and stroke (ischaemic and haemorrhagic) (Cai et al., 2020; Carmona-Mora et al., 2023), however, these studies mostly lack functional correlation.

The neutrophil transcriptome in sepsis in young and aged donors compared to healthy volunteers has been explored using microarray, this study enrolled older adults aged 76-78, with all the sepsis participants admitted to ICU and no data available regarding co-morbidity or frailty, factors which are known to influence neutrophil function. Presence of sepsis influenced higher rate of DEGs than ageing alone. In older adults (+/- sepsis) the most dysregulated canonical pathways were oxidative phosphorylation and mitochondrial dysfunction compared to young cases. There were also pathways which were dysregulated in older adults with and without sepsis, but not altered in young adults with and without sepsis (Vieira da Silva Pellegrina et al., 2015). This data demonstrates that the neutrophil transcriptome is influenced by disease state and age meaning that matching cohorts for factors which are known to influence neutrophil function is key.

Pan *et al* undertook a combined transcriptome and metabolome study of neutrophils in sepsis. They identified genes involved in glycolysis and hypoxia inducible factor 1α to be upregulated in sepsis, then used untargeted metabolomics to demonstrate that aerobic glycolysis was the most upregulated pathway confirming similar pathways are altered at both transcript and metabolite level (Pan et al., 2022).

The neutrophil transcriptome and direct assessment of metabolism using extracellular flux following *in-vitro* infection with Leishmaniasis has been explored. At six hours post infection, infected neutrophils had significantly upregulated glycolysis as assessed by both RNAseq and extracellular flux, demonstrating a synergy and correlation between transcriptome and direct methods of assessment (Ohms et al., 2021).

RNAseq provides an economical approach to assessing gene expression in a disease state: it can be used to test a specific hypothesis or generate altered expression data for further investigation. Neutrophils possess RNA at levels which makes testing feasible, and neutrophils rapidly alter their transcriptome to *ex-vivo* stimuli and demonstrate alterations with disease state and age. However, many studies in sepsis have used a bulk whole blood approach, or PBMCs. Neutrophil specific studies have not been completed in older adults with CAP and sepsis with functional correlations to support or refute changes seen in transcriptome.

6.3. Hypothesis

Neutrophils from older adults with community acquired pneumonia exhibit dysfunctional migration, generation of reactive oxygen species and degranulation. This aberrant effector function will be associated with alterations of the neutrophil transcriptome detectable by RNA-sequencing.

6.3.1. Aims:

1) Isolate high-quality RNA from neutrophils for downstream processing.
2) Identify altered gene expression in neutrophils using RNAseq from donors with community acquired pneumonia compared to controls.
3) Perform pathway analysis to identify differentially regulated pathways in neutrophils from CAP donors.

6.4. Materials and Methods

6.4.1. Participants

Participants were recruited and neutrophil isolation undertaken as detailed in Chapter Five. Given the expense of RNAseq and technical issues discussed around using neutrophils a sample size of seven CAP and seven controls was selected to test feasibility and provide exploratory data. There are no validated methods for power calculation for RNAseq, the literature suggests a minimum of six biological replicates (Schurch et al., 2016). Isolated neutrophils were used, cells were immediately resuspended in lysis buffer after cell counting to avoid any artefact associated with activation or apoptosis.

6.4.2. RNA extraction and sequencing

RNA isolation was completed using RNAqueous- Micro Kit (Thermo-Fisher Scientific) according to the manufacturer's protocol. $5x10^6$ freshly isolated neutrophils were pelleted (1,000 x *g*, 10 minutes) in an RNAase free micro-centrifuge tube then resuspended in 1ml lysis solution from kit and stored at -80°C until batched RNA extraction.

100µl of ethanol was added to 200µl lysed neutrophil suspension and loaded into a micro-filter cartridge tube and centrifuged at 17,200 x *g* for 10 seconds resulting in the RNA being bound to the filter in the micro-tube. Then the filter was washed with 180µl of wash buffer 1 and then twice with 180µl wash buffer 2/3. The filter tube was then centrifuged at maximum speed for 1-2 minutes to remove residual fluid.

The filter was then transferred to a fresh tube and 10µl of heated elution solution added and stored for one minute at room temperature prior to centrifuging at

maximum speed for 30 seconds to elute RNA from the filter, this step was repeated once.

DNase treatment was completed by adding 2µl DNase I buffer and 1µl DNase I to the 20µl RNA sample mixing well prior to incubating at 37°C for twenty minutes. Then 2µl DNase inactivation reagent was added and stored at room temperature for two minutes with mixing. The tube was then centrifuged for 1.5 minutes at maximum speed prior to removal of the RNA to a fresh RNAase free tube and stored at -80°C for long-term storage.

RNA sequencing was carried out by the Genomics department at University of Birmingham. RNA quality was measured using the Agilent 2200 Tapestation using 5ng RNA. The polyA+ mRNA fractionation was isolated and cDNA libraries prepared using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina with 5ng input. Samples were then subjected to 75bp, single-end sequencing using Illumina NextSeq 500 sequencing machine.

6.4.3. RNA analysis workflow

The RNA analysis pipeline shown in Figure 6-1 was adapted from Batut *et al* (Batut et al., 2021). Sequence data in .fastq form was uploaded to Galaxy web platform at usegalaxy.org (Afgan et al., 2016). FastQC and Cutadapt were used to trim adapter sequences (Martin, 2011; Babraham Bioinformatics, 2023) and perform quality control of trimmed sequences. Each read was aligned to the human genome (GRCh38) using RNA STAR (Dobin et al., 2013). Gene counts were calculated using FeatureCounts (Liao et al., 2014). Differential expression was generated using DESeq2 (Love et al., 2014), DESeq2 uses the Benjamini-Hochberg correction for multiple testing. Upstream regulator analysis (URA) was performed using Searchlight

2 (Cole et al., 2021) using the TTRUST database URA was performed using IPA method (Ingenuity Systems, 2012). URA enables assessment of which genetic regulators may be influencing differential expression of the measured genes; by comparing the differentially expressed genes to a predefined dataset of regulators and genes which they influence and typical direction of influence. The URA algorithm then determines for each upstream regulator how closely the observed pattern of differential expression matches the expected pattern, expressed as a z-score. Gene annotations were obtained from STRING database (Szklarczyk et al., 2023). Pathway analysis was performed using Gene Set Enrichment Analysis (GSEA) comparing gene expression against Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology (GO) curated gene sets (Subramanian et al., 2005; Gene Ontology Consortium, 2015; Kanehisa et al., 2016). GSEA also uses Benjamini-Hochberg correction for multiple testing. Data visualisation was completed using R (R Foundation for Statistical Computing, 2021) (v4.3.1, Vienna, Austria).

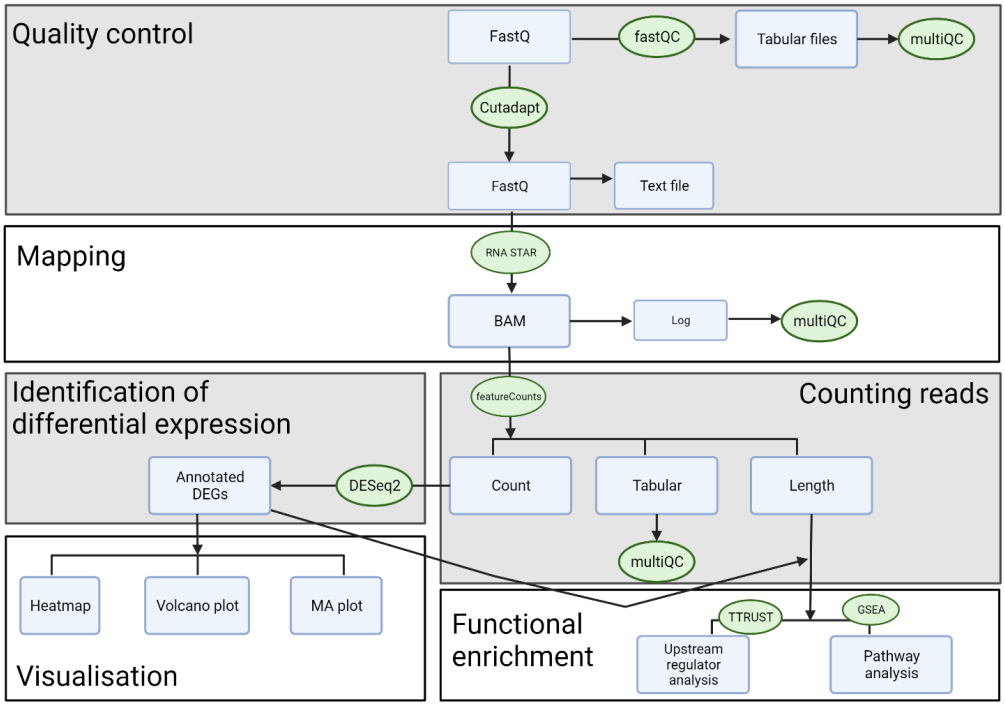

Figure 6-1: RNA Analysis Pipeline

Raw sequencing files in .fastQ form were uploaded to galaxy web server, where fastQC and cutadapt were used to trim sequences of adapter reads and ensure quality control (QC). Each read was then mapped using RNA STAR, further QC was performed using multiQC. After mapping gene expression was counted using feature counts. This expression data was then used in differential expression analysis using DESeq2 and to normalise differentially expressed genes (DEGs) for functional enrichment. Outputs of DESeq2 were used for data visualisation and functional enrichment. Blue boxes are file types or outputs, green ellipses are tools used to analyse data. Quality control, mapping, read counts and identification of DEGs was completed using galaxy server. Visualisation was completed using R. Upstream regulator analysis was completed using searchlight 2 and pathway analysis was completed using GSEA. Created using biorender.com.

6.5. Results

6.5.1. Demographics of participants

RNAseq was performed in neutrophils isolated from 7 CAP and 7 matched control donors from the overall cohort described in Chapter Five. The demographics of this subset are in Table 6-2. These donors were well matched for age, frailty, burden of existing medical conditions and polypharmacy as in the overall cohort. The CAP participants had severe CAP (median CURB65 3), and high 30-day mortality (42%). 30-day mortality was higher than the overall cohort (42 vs. 36%). All CAP participants in this study were recruited within 24 hours of hospitalisation. Individual comorbidities and medications for each participant are listed in Appendix One. The most common comorbidities were cardiovascular disease and diabetes mellitus. The prevalence of dementia was significantly higher in the CAP group.

Table 6-2: Participant demographics

	Control	**CAP**	***P*-value**
Number of participants	7	7	
Median Age (IQR)	87.5 (84-91)	88 (80-95)	0.859
Female Sex (%)	5 (71)	5 (71)	0.792
White ethnicity (%)	7 (100)	6 (85)	0.714
Mean Body Mass Index (S.D)	23.34 (2.8)	23.1 (3.4)	0.441
Median Clinical Frailty Score (IQR)	4 (4-4)	5 (4-6)	0.067
Median number of co-morbidities (IQR)	4 (2-6)	3 (3-4)	0.697
Cardiovascular disease (Count, %)	5 (83.3)	6(100)	1.000
Dementia (Count, %)	***0***	***3 (50)***	***0.045***
Diabetes Mellitus	2 (33.3)	1 (16.6)	0.505
Median number of medications (IQR)	4 (2-4)	4 (3-5)	0.494
Median CURB65 score (IQR)	-	3 (3-3)	-
30-day mortality (%)	-	3 (42)	-

Chi-square test for categorical variables, Mann-Whitney test for non-parametric variables and t-test used for parametric variables. IQR: interquartile range, S.D: standard deviation.

6.5.2. RNA quality assessment

All samples demonstrated good integrity and were suitable for RNAseq, typically a RIN score >7 is deemed acceptable for RNAseq (Farrell, 2023) in Figure 6-2. There were no differences in integrity score between controls and CAP participants (*p*=0.688, by Mann-Whitney).

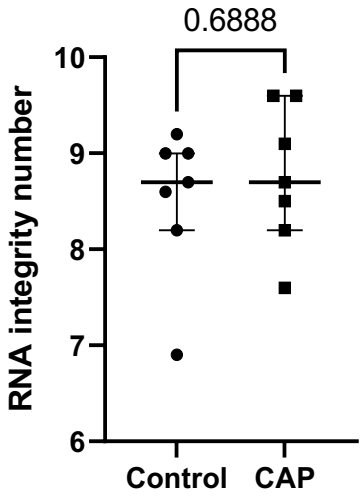

Figure 6-2: Neutrophil RNA Integrity Number in controls and CAP

RNA quality was measured using the Agilent 2200 Tapestation. Median RIN score was 8.7 in both groups. Scores were equivalent across both groups (*p*=0.6888 by Mann-Whitney)

The technical quality of RNAsequencing can also be assessed by distribution of expression values. Outlier samples with atypical distribution can be considered suggestive of technical issues during sequencing. Figure 6-3 demonstrates similar distribution across all samples regardless of sample type.

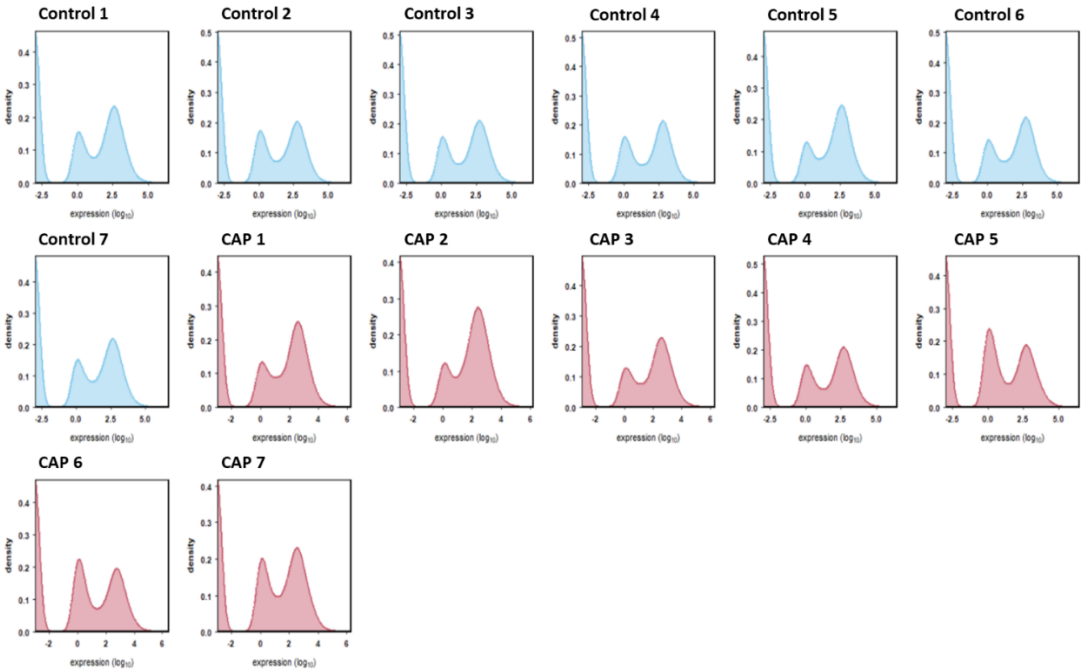

Figure 6-3: Distribution of expression values across samples

Density plots showing the overall distribution of expression values for each sample. Expression values are given on x-axis and are on a log 10 scale. The gene density is given on the y-axis. CAP samples are in red and control samples in blue.

6.5.3. RNA sequencing

All samples had >30 million reads which has been defined as optimal for RNAseq (Ji and Sadreyev, 2018). Between 87-94% reads were mapped to the human genome.

Table 6-3: Quality Assessment of RNAsequencing

Sample ID	Total Reads	Average read length	Mapped reads (%)	Uniquely mapped reads (%)
Control 1	3.49E+07	75	88.68	73.09
Control 2	3.68E+07	75	93.03	76.43
Control 3	3.56E+07	75	91.58	71.68
Control 4	3.25E+07	75	93.84	76.1
Control 5	3.64E+07	75	87.21	68.98
Control 6	3.22E+07	75	90.46	76.03
Control 7	3.32E+07	75	88.67	74.12
CAP 1	3.09E+07	75	91.95	74.29
CAP 2	3.51E+07	75	94.51	68.05
CAP 3	3.76E+07	76	91.97	76.49
CAP 4	3.19E+07	75	94.04	74.71
CAP 5	3.47E+07	75	93.92	74.44
CAP 6	3.64E+07	75	92.6	77.59
CAP 7	3.26E+07	75	92.75	77.69

After trimming for adaptors using Cutadapt alignment to the human genome was performed using RNAstar. Output from RNAstar was assessed using multiQC to generate the above parameters.

6.5.4. Differential expression

There were 760 differentially expressed genes between CAP and controls.

Differential expression was defined as adjusted p or false discovery rate (FDR) <0.05 and absolute fold change >0.5. Of the 760 differentially expressed genes 446 were upregulated and 314 were downregulated. Of the differentially expressed genes 517 (72%) were mapped to protein coding genes, the remainder mapped to non-coding regions of RNA(Szklarczyk et al., 2023). The top 100 most differentially expressed genes are in Appendix 1.

Principal component analysis (PCA) was performed to assess the variation between CAP and control samples. Figure 6-4 demonstrates that there is separation between

CAP and controls in both PCA1/2 and PCA3/4, indicating that neutrophil RNA expression in CAP is different to controls.

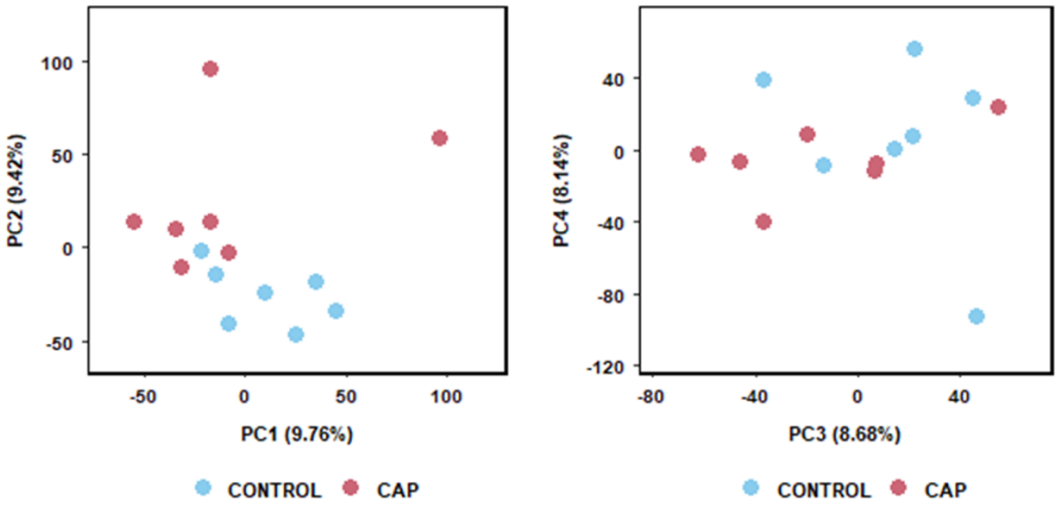

Figure 6-4: Principal component Analysis of CAP and Controls

Gene expression data Principal Component Analysis (PCA) scatterplots. showing PC1 vs PC2 (left) and PC3 vs PC4 (right). Individual samples are represented by dots. The percentage of total variation explained by each component is given in the x and y-axis as appropriate. To control over representation of very highly expressed genes, all gene expression values were scaled on a gene-by-gene basis using the z-score transformation, prior to the PCA.

Overall gene expression is shown in Figure 6-5. This volcano plot follows the expected pattern that genes with increasing fold change have increasingly significant *p*-values.

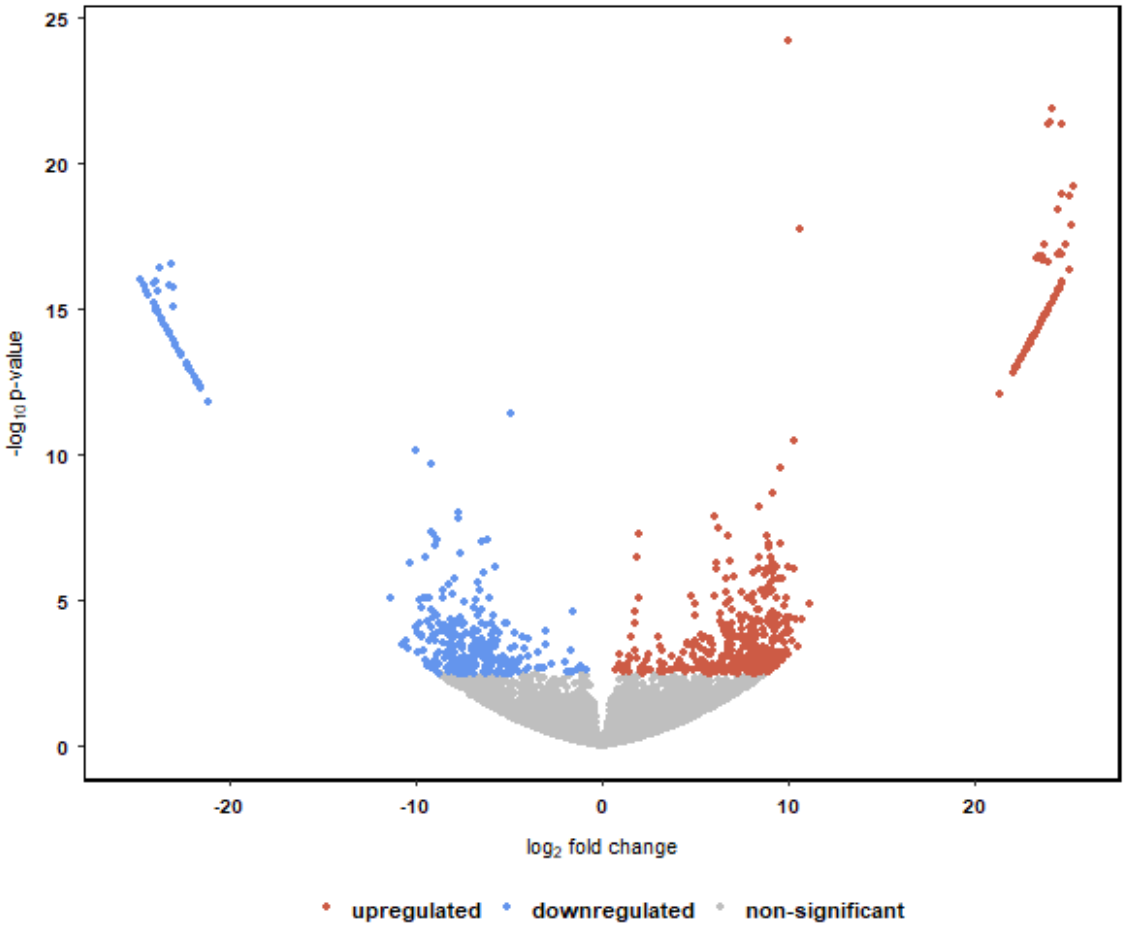

Figure 6-5: Volcano plot demonstrating differential expression of genes.

Volcano plot for the comparison between cap and control. Significantly differential genes (*p*.adj < 0.05, absolute log2 fold > 0.5) are shown in red (upregulated) or blue (downregulated) and non−significant genes in grey. A positive fold change indicates higher expression in CAP than in control.

The clustered heatmap in Figure 6-6 demonstrates clear groups of significantly differentially expressed genes between control and CAP samples.

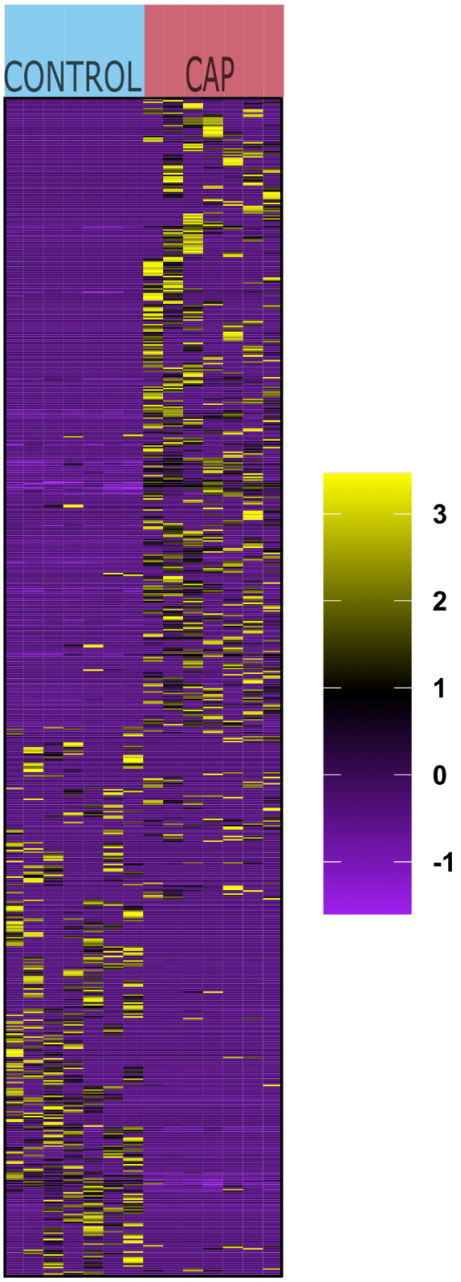

Figure 6-6: Clustered heatmap demonstrating significantly differentially expressed genes.

Hierarchically clustered heatmap of the significantly differentially expressed genes (*p*.adj < 0.05, absolute log2 fold > 0.5) between CAP and control, for all samples in the sample groups: control and CAP. Samples are on the x axis and genes on the y axis. Colour intensity represents expression level, with purple representing low expression, and yellow representing high expression. Expression levels have been row scaled into z−scores. The y-axis (both plots) and x-axis (right plot) have been hierarchically clustered using, Spearman distances, with UPMGA agglomeration and mean reordering.

6.5.5. Upregulated genes

Of the 446 significantly upregulated genes 91 (20%) were upregulated with a log2 fold change >20, and 301 (67%) are upregulated with a log2 fold change between 5-20. The top five upregulated genes according to *p*-value and log2 fold change are shown in Table 6-4 and Table 6-5.

Table 6-4: Most upregulated genes in CAP according to Fold Change

Gene ID	Annotation	log2 Fold change	FDR
RAB11FIP5	Rab effector- protein trafficking	25.2	1.8E-16
FBXO40	Protein-ubiquitin ligase	24.7	4.7E-14
EGR2	Early growth response protein 2 Transcription factor	24.7	4.7E-14
ADCY7	Membrane bound adenylate cyclase	24.7	1.8E-16
ACOX3	Peroxisome function	24.7	9.4E-15

Table 6-5: Most upregulated genes in CAP according to *p*-value

Gene ID	Annotation	log2 Fold change	FDR
EXT1	Glycosyltransferase	10.0	6.5E-21
GOLGA5	Formation of Golgi stacks and ribbons	24.1	9.2E-19
ADCY7	Catalyses formation of cAMP	24.7	1.8E-16
RAB11FIP5	Rab effector- protein trafficking	25.2	1.8E-16
MYC	Transcription factor for growth related genes	24.5	4.5E-16

6.5.6. Downregulated genes

There were 314 significantly downregulated genes in the CAP group. Of these 49 (16%) had a log2 fold change of >negative 20, and 224 (71%) had a log2 fold change between negative 5-20.

Table 6-6: Most downregulated genes in CAP according to Fold Change

Gene ID	Annotation	log2 Fold change	FDR
SLC25A38	Mitochondrial glycine transporter	-24.82	4.3E-14
FSCN1	Actin binding protein	-24.63	5.6E-14
BRD3OS	Putative uncharacterised protein	-24.49	7.0E-14
PRPSAP2	Phosphoribosyl pyrophosphate synthase-associated protein 2	-24.39	8.8E-14
PPM1L	Suppressor of the SAPK signalling pathways	-24.11	4.7E-14

FDR: false discovery rate.

Table 6-7: Most downregulated genes in CAP according to *p*-value

Gene ID	Annotation	log2 Fold change	FDR
AKAP11	Protein kinase A binder	-23.8	1.7E-14
SLC25A38	Mitochondrial glycine transporter	-24.63	4.3E-14
CDH26	Cadherin	-24.0	4.6E-14
PPM1L	Suppressor of the SAPK signalling pathways	-24.1	4.7E-14
FSCN1	Actin binding protein	-24.63	5.6E-14

FDR: false discovery rate.

6.5.7. Upstream Regulator Analysis

Most activated upstream regulators.

There were two significantly activated upstream regulators in CAP, SP1 and SP3 (Figure 6-7). Both are transcription factors capable of activation or repression dependent on stimuli.

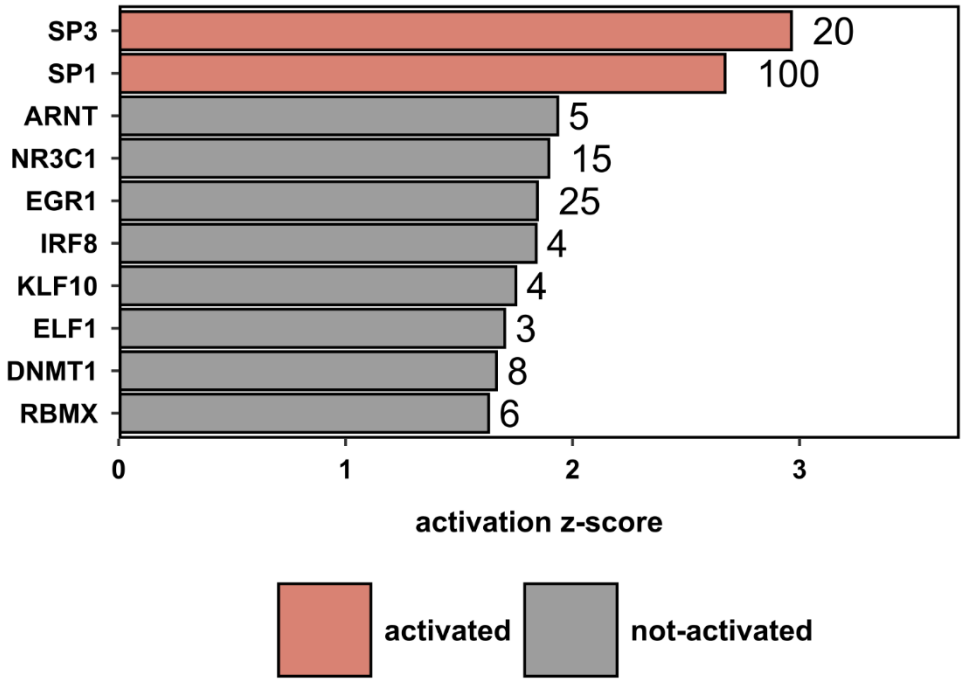

Figure 6-7: Summary bar charts of the ten most activated upstream regulators

Activation was calculated using the IPA-like algorithm based on all differential expression values. The activation score is given on the x-axis and the upstream regulator name on the y-axis. Significantly activated upstream regulators (activated > 2.0) are coloured red and non-significant grey. Upstream regulator analysis was performed on the databases TRRUST. The number adjacent to each bar represents the number of genes in the dataset relating to that regulator.

Most inhibited upstream regulators

The significantly inhibited upstream regulators were *FOS, PPARA, GATA2* and *JUN* (Figure 6-8). *FOS* encodes c-FOS which forms a complex with *JUN/AP-1* transcription factor. *PPARA* encodes Peroxisome proliferator-activated receptor alpha which influences fatty acid oxidation. *GATA2* regulates haematopoietic differentiation. *JUN* forms a complex with *FOS* making *AP-1* a transcription factor which displays diverse responses to a variety of stimuli (Szklarczyk et al., 2023).

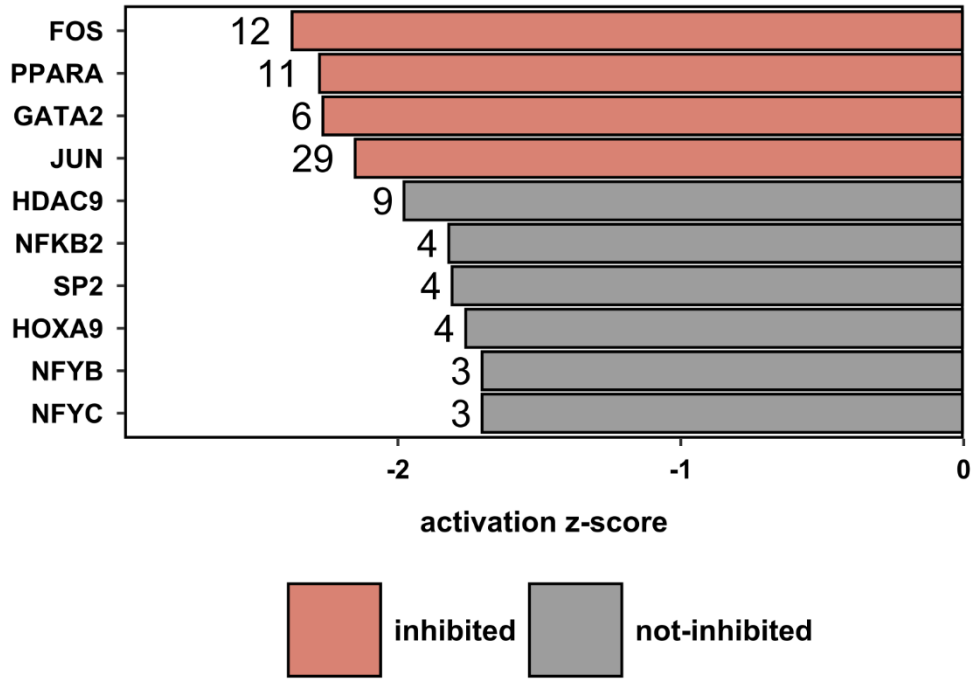

Figure 6-8: Summary bar charts of the ten most inhibited upstream regulators

Inhibition was calculated using the IPA-like algorithm based on all differential expression values. The activation score is given on the x-axis and the upstream regulator name on the y-axis. Significantly inhibited upstream regulators (inhibited <2.0) are coloured red and non-significant grey. The number adjacent to each bar represents the number of genes in the dataset relating to that regulator. Upstream regulator analysis was performed on the databases TRRUST.

6.5.8. Pathway analysis

Pathway analysis using all expression data.

Gene set enrichment analysis using KEGG terms against all expression data demonstrated 110 positively enriched pathways, but none reached statistical significance. There were 44 downregulated pathways, again none reached statistical significance. Table 6-8 shows the top enriched pathways.

Table 6-8: GSEA in all genes using KEGG pathway.

KEGG Pathway	Normalised enrichment score	FDR
Positively enriched in CAP		
Epithelial cell signalling in *H. Pylori* infection	1.403	0.363
MTOR signalling	1.404	0.379
NOD like receptor signalling	1.375	0.388
Glycerophospholipid metabolism	1.407	0.392
Apoptosis	1.378	0.397
Negatively regulated in CAP		
Spliceosome	-1.683	0.432
Ribosome	-1.614	0.372
Antigen processing and presenting	-1.607	0.264
Proteasome	-1.515	0.378
Cell adhesion molecules	-1.442	0.489

FDR: false discovery rate, CAP: community acquired pneumonia.

Using GO terms in the whole expression matrix there were 2941 upregulated terms, but none reached FDR <0.05. There were 1343 negatively regulated terms, but none reached statistical significance (Table 6-9).

Table 6-9: GSEA in all genes using GO terms.

GO Term	Normalised enrichment score	FDR
Positively enriched in CAP		
Tertiary granule	2.280	0.056
Tertiary granule lumen	2.168	0.058
Mature B cell differentiation	2.170	0.085
T Helper Cell differentiation	2.061	0.092
Tertiary granule membrane	2.093	0.092
Negatively regulated in CAP		
Ribosome subunit	-1.750	0.520
Oxidoreduction active transmembrane transport	-1.757	0.535
Cytoplasmic translation	-1.780	0.546
Protein folding chaperone	-1.758	0.593
Translational elongation	-1.686	0.594

FDR: false discovery rate, CAP: community acquired pneumonia.

GSEA considers strength or rank of gene expression but does not consider whether a gene is up or downregulated (Hong et al., 2014). Therefore, GSEA was repeated with both upregulated (log2 fold change >0) and downregulated (log2 fold change <0) genes considered alone.

Pathway analysis of upregulated genes

Using upregulated genes there were 104 upregulated KEGG pathways, of which 1 had FDR <0.05: Haematopoietic cell lineage. There was only one negatively regulated KEGG pathway identified (Cell Adhesion Molecules), with normalised enrichment score -0.898 and FDR 0.534.

Table 6-10: GSEA in upregulated genes using KEGG pathway.

KEGG Pathway	Normalised enrichment score	FDR
Positively enriched in CAP		
Hematopoietic cell lineage	2.236	0.001
RIG1 like receptor signalling	1.880	0.085
GNRH Signalling pathway	1.837	0.087
Adipocytokine signalling pathway	1.827	0.073
Small cell lung cancer	1.824	0.060
Negatively regulated in CAP		
Cell adhesion molecules	-0.898	0.534

FDR: false discovery rate, CAP: community acquired pneumonia.

Using GO terms in the upregulated gene expression data there were 2966 upregulated terms, of these 14 had FDR <0.05 (Table 6-11). There were 68 downregulated GO terms, of which none of the terms were statistically significant.

Table 6-11: GSEA in upregulated genes using GO terms.

GO Term	Normalised enrichment score	FDR
Positively enriched in CAP		
Inflammatory response to antigenic stimuli	2.276	0.025
Response to leukaemia inhibitory factor	2.068	0.030
Regulation of sterol transport	2.070	0.031
Specific granule	2.087	0.033
Tertiary granule lumen	2.075	0.033
T cell differentiation during immune response	2.100	0.033
Cellular response to glucocorticoids	2.104	0.035
Cellular response to corticosteroids	2.104	0.041
T cell activation	2.028	0.043
Skin epidermis development	2.015	0.046
Transcription factor binding	1.887	0.048
Activation of immune response	1.888	0.048
Centrosome duplication	1.884	0.049
Mononuclear cell differentiation	1.888	0.049
T cell differentiation	1.890	0.049
Negatively regulated in CAP		
Morphogenesis of polarised epithelium	-1.418	1.000
Condensed nuclear chromosome	-1.216	1.000
Ciliary transition zone	-1.106	1.000
Establishment of tissue polarity	-1.102	1.000
Inner ear receptor cell development	-1.100	1.000

FDR: false discovery rate, CAP: community acquired pneumonia.

Pathway analysis of downregulated genes

There were three positively enriched KEGG pathways identified in the downregulated genes, none were statistically significant. There were 91 negatively regulated pathways identified in CAP, none had an FDR <0.05 (Table 6-12).

Table 6-12: GSEA in downregulated genes using KEGG pathways.

KEGG Pathway	Normalised enrichment score	FDR
Positively enriched in CAP		
Melanoma	0.768	1.000
Neuroactive ligand receptor interaction	0.767	1.000
Calcium signalling pathway	0.573	0.945
Negatively regulated in CAP		
Chemokine signalling pathway	-1.550	0.234
Huntingdon's disease	-1.417	0.245
Axon Guidance	-1.459	0.246
Cardiac muscle contraction	-1.556	0.248
Toll-like receptor signalling	-1.392	0.249

FDR: false discovery rate, CAP: community acquired pneumonia.

Using GO terms against the downregulated genes there were 48 upregulated GO terms, none reached statistical significance. 2629 GO terms were negatively regulated, none had FDR <0.05.

Table 6-13: GSEA in downregulated genes using GO terms.

GO Term	Normalised enrichment score	FDR
Positively enriched in CAP		
Vesicle cargo loading	1.477	0.969
Respiratory gas exchange	1.086	1.000
Clathrin coat	1.052	1.000
Vesicle coat	1.002	1.000
Nucleotide mediators	0.980	1.000
Negatively regulated in CAP		
RRNA processing	-1.492	0.185
RNA 3 end processing	-1.492	0.186
Nuclear membrane endoplasmic reticulum membrane	-1.492	0.186
Intracellular bridge	-1.492	0.186
Leucocyte chemotaxis	-1.491	0.186

FDR: false discovery rate, CAP: community acquired pneumonia.

6.5.9. Glycolytic enzyme gene expression

Chapter Five demonstrated dysfunctional neutrophil responses in CAP but showed that *ex-vivo* glycolysis was not different (Chapter 5.4.11), therefore glycolytic genes and pathways were specifically analysed to further validate the *ex-vivo* functional data. No glycolysis related pathways for either KEGG or GO terms were significantly altered in the whole gene analysis, or the downregulated or upregulated gene analysis. Most of the steps of glycolysis are reversible, controlled by the concentration of their substrates. The irreversible and therefore key rate limiting enzymes are hexokinase (*HK2*), phosphofructokinase (*PFKM*) and pyruvate kinase (*PKM*) (Figure 5-1). There are other key genes which control glucose transport into the neutrophil (*Glut1* or *SLCA1*) and lactate efflux (*MCT4* or *SLC16A3*) (Berg et al.,

2019). Expression of these key glycolytic genes was unchanged between CAP and controls (Figure 6-9).

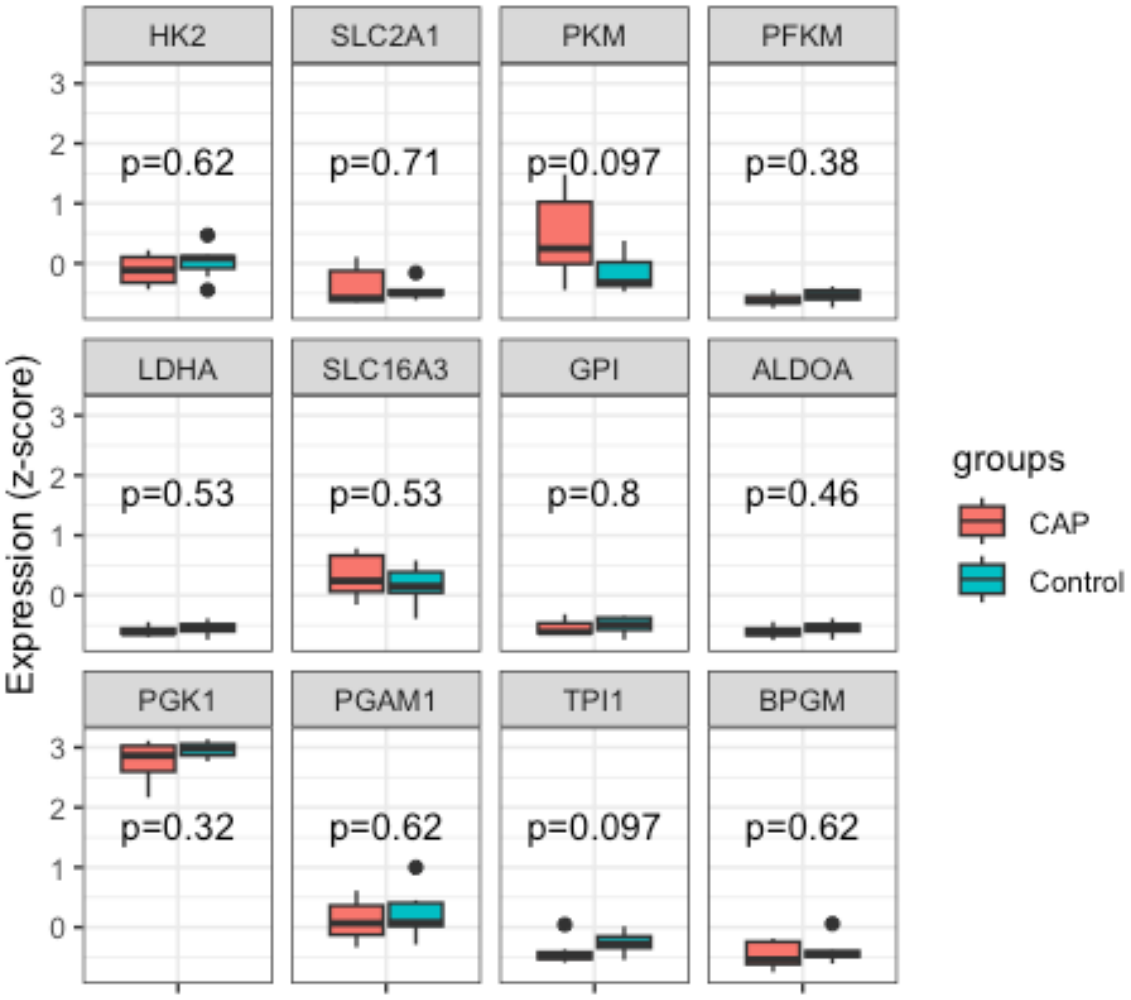

Figure 6-9: Expression of glycolytic genes

Expression levels have been scaled into per gene z−scores. Box and whisker plot demonstrating median in bold line, coloured box=interquartile range, vertical line, or whiskers IQR * 1.5 while any data points beyond are dots (outliers). N=6 in CAP and controls. Comparisons made using Mann-Whitney test. KH2: hexokinase, SLC2A1: Glut1, PKM: pyruvate kinase, PFKM: phosphofructokinase, LDHA: lactate dehydrogenase. SLC16A3: MC4 (lactate efflux), GPI: glucose-6-phosphate-isomerase, ALDOA: Aldolase, PGK1: phosphoglycerate kinase, PGAM: phosphoglycerate mutase, TPI1: triosephosphate isomerase 1 and BPGM: bisphosphoglycerate mutase.

6.5.10. Gene expression relating to neutrophil effector genesets.

There are 45 KEGG pathways and GO terms which directly relate to neutrophil functions (Table 6-14). Given the phenotypic differences demonstrated in this thesis

(migration, degranulation, and respiratory burst) combined with other effector functions known to be abnormal in critical illness (Conway Morris et al., 2012; Belchamber et al., 2022; Patel et al., 2018; Sapey et al., 2019) , a further GSEA analysis was performed to assess whether geneset analysis reflected the phenotype observed, using only terms relevant to the observed cellular phenotype. This would be expected to increase the number of significantly altered pathways by reducing the correction for multiple comparisons.

Table 6-14: KEGG and GO terms relating to neutrophil phenotype.

Granule	**Migration**
Tertiary granule	Granulocyte migration
Specific granule	Neutrophil migration
Specific granule membrane	Granulocyte chemotaxis
Specific granule lumen	Neutrophil chemotaxis
Azurophilic granule membrane	Chemokine binding
Azurophil granule	Neutrophil extravasation
Azurophilic granule lumen	Positive regulation of neutrophil extravasation
Ficolin 1 rich granule	Regulation of neutrophil extravasation
Regulation of neutrophil degranulation	**Respiratory burst**
Phagocytosis	Positive regulation of ROS metabolic process
FC gamma R mediated phagocytosis	Regulation of respiratory burst
Regulation of neutrophil mediated cytotoxicity	Respiratory burst
Opsonisation	ROS metabolic process
Phagocytosis recognition	Regulation of ROS biosynthetic process
Activation & differentiation	Negative regulation of ROS metabolic process
Granulocyte activation	ROS biosynthetic process
Regulation of granulocyte differentiation	Respiratory burst in inflammatory process
Negative regulation of neutrophil activation	Respiratory burst in defence response
Neutrophil activation involved in immune response.	
Positive regulation of neutrophil activation	
Regulation of neutrophil activation	

In the whole gene dataset, there were 22 positively enriched pathways/terms with the 5 significantly enriched terms relating to degranulation and activation (Table 6-15). There were four negatively regulated pathways/terms, none reached statistical significance.

Table 6-15: Neutrophil GO/KEGG terms in the whole dataset

GO term/KEGG pathway	Normalised enrichment score	FDR
Positively enriched in CAP		
Tertiary granule	2.28	<0.001
Specific granule	2.07	<0.001
Specific granule membrane	1.88	0.01
Specific granule lumen	1.84	0.01
Azurophil granule membrane	1.79	0.01
Neutrophil activation	1.72	0.02
Positive regulation of ROS metabolic process	1.63	0.05
Ficolin 1 rich granule	1.58	0.06
Granulocyte activation	1.44	0.14
Azurophil granule	1.41	0.15
Negatively regulated in CAP		
Chemokine binding	-1.485	0.182
Regulation of granulocyte chemotaxis	-0.997	0.758
Regulation of neutrophil chemotaxis	-0.885	0.720
Regulation of neutrophil migration	-0.793	0.688

FDR: false discovery rate, CAP: community acquired pneumonia.

In the upregulated subset of genes there were 24 positively enriched terms/pathways, of these 9 were significantly enriched with FDR<0.05. These pathways substantially overlapped with those demonstrated to be significantly

enriched in the whole dataset. The pathways represented degranulation, ROS, and activation (Table 6-16). There were no negatively regulated pathway or terms in the upregulated subset.

Table 6-16: Neutrophil GO/KEGG terms in the upregulated subset

GO term/KEGG pathway	Normalised enrichment score	FDR
Positively enriched in CAP		
Tertiary granule	2.08	0.008
Specific granule	2.04	0.006
Specific granule lumen	1.92	0.011
Specific granule membrane	1.84	0.020
Azurophil granule membrane	1.78	0.027
Positive regulation of ROS metabolic process	1.76	0.027
Ficolin 1 rich granule	1.69	0.040
Respiratory burst	1.66	0.046
Granulocyte activation	1.65	0.044
Regulation of ROS metabolic process	1.55	0.074
ROS metabolic process	2.08	0.008

FDR: false discovery rate, CAP: community acquired pneumonia and ROS: reactive oxygen species.

In the downregulated subset there were no positively enriched pathways. There were 18 negatively regulated pathways, of which none had FDR <0.05.

6.6. Discussion

This chapter demonstrates that RNAsequencing using isolated neutrophils is feasible, resulting in high quality RNA, and after undergoing downstream processing can be used to identify a high number of differentially expressed genes.

This data confirms the observed metabolic phenotype from Chapter Five where metabolism was not different in CAP when measured *ex-vivo*. This section discusses how the identified genes may be implicated in control of neutrophil functions. There are large shifts in fold change expression with 20% and 15% of up and downregulated genes having a log2 fold change >20, indicating high probability of biological significance.

6.6.1. Upregulated genes in CAP neutrophils

There were 446 significantly upregulated genes in CAP. The most upregulated gene was *RAB11FIP5*, followed by *FBOX40, ERG2, ADCY7, ACOX3, EXT1, GOLGA5* and *MYC*.

RAB11FIP5

RAB11FIP5 gene encodes Rab11 family- interacting protein 5. Rab GTPases belong to the small GTPase family. Rab GTPases control endocytic recycling in cells through control of downstream mediators such as motor proteins (Rodriguez-Boulan et al., 2005). As part of their ability to control motor proteins Rab GTPases also play a role in cellular migration by regulating F-actin protrusions and recycling of integrins which are crucial for cell adhesion (Wilson et al., 2023). In macrophages over expression of *Rab11a* inhibits efferocytosis of apoptotic neutrophils (Jiang et al., 2017) and prevents recycling of CXCR2 (a crucial chemokine receptor) to the plasma membrane after internalisation (Fan et al., 2003). *RAB11FIP5* overexpression

demonstrated here may explain in part the migratory inaccuracy in CAP neutrophils. Alterations in *Rab27a* have been shown to cause a wide range of aberrant neutrophil functions (Catz, 2014), given the overlap in Rab GTPase signalling pathways this indicates that Rab11 family should be investigated in neutrophils. Small GTPases have can be targeted by statins, satins inhibit Rab GTPase prenylation, a post translational modification by lipids required for activity (Parks et al., 2019). *In-vitro* simvastatin treatment of neutrophils from older adults with CAP improved migratory accuracy, but not if sepsis was present. This study also demonstrated that two weeks of simvastatin treatment improved neutrophil migration in older adults without acute illness (Sapey et al., 2017). This work was further developed in an RCT of high dose simvastatin in CAP, high dose simvastatin treatment reduced NETosis, reduced systemic neutrophil elastase and improved chemotaxis (Sapey et al., 2019). In this study 4/7 CAP participants and 3/7 control participants were taking statins; however, none were receiving high dose statins (80mg simvastatin) as used in the RCT (Sapey et al., 2019).

FBXO40

FBXO40 encodes F-box protein 40, which participates in the identification of target proteins for ubiquitination and degradation by the ubiquitin 26S proteasome system (UPS). UPS plays an important role as an immune regulator in pathogen recognition signalling and activation of DCs and T cells (Hu and Sun, 2016), but there is very little neutrophil specific data on the role of UPS. Targeted degradation of proteins plays a key role in many cellular functions (Kerscher et al., 2006). There is very limited data on F-box proteins specifically in immune cells but given their capacity to control

protein degradation (and therefore antigen presentation) they could be implicated in control of apoptosis, recycling of receptors and or protein expression in neutrophils.

EGR2

EGR2 is a transcription factor, upregulation of *EGR2* has also been observed in neutrophils primed *ex-vivo* with a range of compounds, including TNFα and GM-CSF (Wright et al., 2013; Kobayashi et al., 2005). Expression of *EGR2* is upregulated in CD4+ T cells and PBMCs from people with Multiple Sclerosis (Riveros et al., 2010) and in human monocytes following *in-vitro* adherence, where addition of cytochalasin D diminished the increase in *EGR2* (Shaw et al., 1990). Differentiation of THP-1 monocytes to macrophages using PMA upregulates EGR2 expression (Liu et al., 2023). In animal models of neuroinflammation, *EGR2* enhanced myeloid cell recruitment to the brain by upregulating genes associated with pathogenesis of T cells, and chemokines. T cell specific deletion of *EGR2* led to reduced neuroinflammation without reduced ability to control infection (Gao et al., 2023). Taken together this suggests that *EGR2* is required for neutrophil activation, but that overactivation may be associated with high levels of inflammation.

ADCY7

ADCY7 encodes for a membrane bound adenyl cyclase which catalyses formation of cyclic AMP (cAMP) from ATP. In general cAMP suppresses the inflammatory immune response, including suppression of inflammatory cytokines, suppression of opsonin dependent and independent phagocytosis and modulation of ROS (Serezani et al., 2008; Scott et al., 2016). Impairment of neutrophil phagocytosis associated with cAMP can be partially restored by inhibition of AKAP which is involved in directing cAMP to protein kinase A (PKA) (Scott et al., 2016), *AKAP11* is downregulated in this

dataset, possibly implying that the neutrophils are attempting to restore cAMP homeostasis. In murine bone marrow derived macrophages *ADCY7* supressed release of TNFα in response to zymosan, suggesting *ADCY7* may play a role in limiting inflammatory response (Jiang et al., 2013). Elevated expression of *ADCY7* may be a compensatory feature to attempt to reduce inflammation.

ACOX3

ACOX3 encodes Acyl-Coenzyme A oxidase 3, which catalyses the first step of β oxidation of branched fatty acids in the peroxisome. Peroxisome β oxidation does not contribute to energy generation in the same way as mitochondrial β oxidation, instead it provides H_2O_2 and regenerates NADH+ key substrates for ROS generation and scavenging, while also degrading prostaglandins and leukotrienes which are inflammatory mediators (Demarquoy, 2015; Di Cara et al., 2019). While the role of *ACOX3* in neutrophils has not been defined, ROS is a key pathogenic mechanism for neutrophils and upregulation of *ACOX3* may support altered ROS demonstrated in Figure 5-13.

EXT1

EXT1 encodes exostosin-1 which is required for heparan sulfate synthesis. Heparan sulfates mediate cellular interactions with a wide range of proteins at the cell surface (Leisico et al., 2022). Heparan sulfate is present in NETs, and mice lacking heparan sulfate are more susceptible to infection and their neutrophils produced fewer NETs, which were less bactericidal, but ROS, phagocytosis and degranulation were unchanged (Xu et al., 2015).Therefore, altered expression of *EXT1* may influence NETosis, neutrophil adhesion and or retention at sites of inflammation.

GOLGA5

GOLGA5 encodes Golgin subfamily A member 5, a protein which localises to the Golgi surface and is a Rab effector protein, and thought to play a role in membrane tethering, reaching into the cytoplasm to capture vesicles (Wong and Munro, 2014). Other members of the Golgin family act as cytoskeletal tethers linking the Golgi to the polarised cell edge during migration to enable directed secretion of mediators (Witkos and Lowe, 2016). Again, the role of Golgins in neutrophils is undefined, but polarisation of neutrophils is impaired during migration and directed secretion of mediators is important for migration.

MYC

MYC encodes c-myc a transcription factor which is thought to influence 15% of all genes (Gearhart et al., 2007). *MYC* is widely overexpressed in human cancer cells, and inhibition in animal models results in tumour regression (Madden et al., 2021). In neutrophils *MYC* is involved in granulopoiesis, where under emergency conditions *MYC* expression is increased to generate and mobilise neutrophils from the bone marrow. *MYC* is also implicated in expression of MAPK cascades following neutrophil activation (Ai and Udalova, 2020) Therefore increased expression in CAP may reflect emergency granulopoiesis, or activation status of neutrophils.

6.6.2. Most downregulated genes in CAP

FSCN1

FSCN1 encodes fascin actin bundling protein-1 which organises F-actin into parallel bundles to generate actin protrusions (Szklarczyk et al., 2023). The role of *FSCN1* has been widely demonstrated in migration of cancer cells (Li et al., 2022). In

migratory neural crest cells silencing of *FSCN1* results in abnormal migration(Boer et al., 2015). F-actin has been long established as an important protein in neutrophil shape, deformability, and migration (Watts et al., 1991; Ekpenyong et al., 2017), increased levels of F-actin polymerisation have been identified in neutrophils exposed to e-cigarette vapour which induces impaired migration, phagocytosis and ROS generation (Jasper et al., 2023). While in this study *FSCN1* is downregulated, it could be that there is an optimal level of F-actin polymerisation for migration.

CHM

CHM expression was significantly reduced in CAP neutrophils. *CHM* encodes *CHM* Rab Escort protein, which binds unprenylated Rab proteins. Rab proteins must be prenylated for activation, prenylation is a form of post translational modification by lipids (Pereira-Leal et al., 2001). Mutations in the *CHM* gene cause a degenerative disease of retinal epithelium. Silencing of *CHM* in retinal epithelial cells results in reduced phagocytosis and increased chemokine expression *in-vitro* (Gordiyenko et al., 2010). The most upregulated gene in this dataset is *RAB11FIP5*, a Rab GTPase which relies on prenylation by the Rab escort proteins. The differential regulation of a GTPase and the required escort protein reflects the challenges of interrogating intra-cellular signalling pathways. It could be that although upregulation of *RAB11FIP5* would be expected to improve intracellular trafficking and therefore phagocytosis, failure of the escort protein (*CHM*) to facilitate post translational modification may explain there are phenotypic failures of intra-cellular trafficking during CAP.

AKAP11

AKAP11 (also known as *AKAP220*) encodes A-Kinase Anchoring protein 11 (*AKAP*), AKAPs place PKA to various intra-cellular locations. APAKs are also capable of

binding other signalling proteins, thus integrating multiple signalling pathways. *APAK11* spatially restrict protein kinases and microtubule formation at the leading edge of migrating cancer cells, where depletion of *APAK11* reduced velocity of migration (Logue et al., 2011). Other AKAPs have been identified which localise to the cytoskeleton to determine cell shape, movement, and division, such as WAVE1 (Scott, 2003). In this dataset downregulation of *AKAP1*1 may contribute to reduced chemotaxis in CAP neutrophils.

SLC25A38

SLC25A38 encodes a mitochondrial glycine transporter. *SLC25A38* is upregulated in neutrophils from participants with juvenile arthritis (Omoyinmi et al., 2016). *SLC25A38* has been implicated in sideroblastic anaemia (Guernsey et al., 2009) and control of apoptosis (Zhang et al., 2012). Maintenance of mitochondrial function is crucial for control of apoptosis in neutrophils (Bao et al., 2015; Fossati et al., 2003), therefore downregulation of *SLC25A38* would be anticipated to impair mitochondrial function and potentially impact control of apoptosis.

CDH26

CDH26 encodes Cadherin 26. Cadherins are a family of proteins which mediate cellular adhesion via regulation of cell polarisation and migration (Szklarczyk et al., 2023). Identification of *CHD2*6 is novel finding in neutrophils, as its expression has previously been demonstrated in airway and gastrointestinal epithelial cells. In airway epithelial cells, knockdown of *CHD26* results in abnormal actin organisation causing loss of cell polarity (Lachowicz-Scroggins et al., 2018). In gastric epithelial cells *CHD26* bind α-integrins which are required for leucocyte trans endothelial migration (Caldwell et al., 2017). However, less is known about leucocyte expression of

cadherins, especially the more recently discovered cadherins. Cadherins have been shown to be expressed in lymphocytes and monocytes, where their expression has been hypothesised to relate to retention of the cell in tissue (Alexander et al., 2001). Neutrophil expression of cadherins is not well understood, and their downregulation in CAP may contribute to abnormalities of cytoskeleton and may contribute to impaired migration and or retention at inflammatory sites.

PPM1L

PPM1L encodes protein phosphate 1L a suppressor of stress activated protein kinase (SAPK) signalling pathway. SAPK is a member of the MAP kinase signalling family which can be activated by a wide range of stimuli. *PPM1L* can supress apoptotic signalling in response to cytotoxic stress (Szklarczyk et al., 2023). Overexpression of *PPM1L* in murine model of myocardial infarction (MI) resulted in less cardiac dysfunction and reduced inflammatory cytokine secretion in macrophages; in wild-type mice there was downregulation of *PPM1L* following MI. *In-vitro* silencing of *PPM1L* led to increased levels of inflammatory cytokines in bone marrow derived macrophages (B. Wang et al., 2019). A further study in macrophages has suggested that silencing of *PPM1L* leads to persistence of an inflammatory phenotype (Goto et al., 2021). There is no direct evidence of the role of *PPM1*L in neutrophils, but based on the macrophage data and ability of the SAPK signalling pathway to enable cells to respond to stressors it would be predicted that downregulation of *PPM1L* leads to ongoing neutrophilic inflammation in CAP.

PRPSAP2

PRPSAP2 encodes Phosphoribosyl pyrophosphate synthase-associated protein 2. *PRPSAP2* is part of the 5-phosphoribosyl 1-pyrophosphate (PRPP) pathway which

salvages nucleotides and is involved in de novo nucleotide synthesis including those used in the synthesis of DNA/RNA and cofactors such as NAD, FAD, coenzyme A and ATP(Kamal and Christopherson, 2004). The crucial role of PRPP is demonstrated by the disease states caused by single mutations in phosphoribosyl synthase (PRS) genes (de Brouwer et al., 2010). Overactivity of PRS genes is associated with gout and a broad range of neurological disease including forms of Charcot-Marie Tooth disease. Underactivity of PRS genes is termed Arts syndrome, an x linked disorder which multiple neurological manifestations and a significant immunodeficiency meaning most males do not live beyond five years of age. Death is predominantly due to respiratory tract infection; however, the mechanism of immunosuppression is not established (de Brouwer et al., 2010). There are only a few known families with Arts syndrome(orpha.net, n.d.), therefore no published immunological studies. However, reduced expression of *PRPSAP2* would be expected to suppress PRPP and therefore nucleotide/cofactor biosynthesis. Cofactors such as NAD are of crucial importance in neutrophil pathogenicity. Therefore, the observed inhibition may reflect reduced synthesis and salvage of cofactors crucial to neutrophil functions, thereby supressing ROS/NETosis and bacterial killing among others.

These genes are the most substantially altered in the cohort and given the magnitude of fold change and FDR have a high likelihood of being biologically important in the observed phenotype of immaturity, increased degranulation and altered migration. They all require further biological testing and validation in human neutrophils to understand their roles and whether targeting them influences function.

Investigating single genes is time consuming and prone to investigator bias in considering the available literature. Further, alterations in a single gene are unlikely to cause the aberrant phenotype seen in Chapter Five, therefore exploring the genes which regulate differentially expressed genes, and considering whether expression is altered at a pathway level can inform further biological testing.

6.6.3. Upstream regulators

Activated upstream regulators.

There were two significantly upregulated upstream regulators: *SP1* and *SP3*. *SP1* and 3 are ubiquitously expressed transcription factors (Szklarczyk et al., 2023), and overexpressed in a range of tumours and neurodegenerative disorders (O'Connor et al., 2016). *SP1* is required for secretion of CXCL1 by peritoneal cells in an *in-vitro* model of peritonitis demonstrating its role in orchestrating immune responses (Catar et al., 2020). Other studies have demonstrated the role of *SP1* in monocyte/macrophage arrest in tissues (Speranza et al., 2013) by repressing phospholipase D2 (*PLD2*). In animal models of sepsis *PDL2* knockout improves survival and reduces organ damage with altered cytokine production and neutrophil function (Lee et al., 2015). Therefore, increased activation of *SP1* could be involved in attempting to dampen inflammatory responses during CAP. *SP1* has also been demonstrated to play a role in granulopoiesis (Friedman, 2002), therefore activation could also represent the emergency recruitment of neutrophils from bone marrow during CAP. There is less evidence for the role of *SP3* in myeloid cells, however it has been linked with release of bactericidal/permeability increasing protein from azurophilic granules (Lennartsson et al., 2003) perhaps reflecting the increase in

degranulation seen in Figure 5-11 and associated upregulation of pathways relating to degranulation in Table 6-16 in this Chapter.

Inhibited upstream regulators.

Both *FOS* and *JUN* are predicted to be inhibited in CAP neutrophils. *FOS* dimerises with *JUN* proteins to form the *AP-1* complex (Chinenov and Kerppola, 2001). *AP-1* expression has been associated with neutrophil lipocalin 2 (NGAL), where *AP-1* expression had a pro-resolving inflammatory effect. *AP-1* deficient mice demonstrate increased neutrophilic infiltration (Cao et al., 2021). *FOS* has also been implicated in neutrophil secretion of cytokines where neutrophils with low level of *FOS* secreted higher levels of TNFα than neutrophils with high level of *FOS* (Shikama et al., 2016) further evidencing the role of these multi-functional transcription factors in infection and inflammation.

JUN deficient neutrophils were generated using CRISPR technique in HoxB8 myeloid cells subsequently differentiated into neutrophils, these cells impaired neutrophil phagocytosis, ROS generation, bacterial killing and NETosis. However neutrophil recruitment to inflammatory sites was not influenced by *JU*N deficiency, and importantly neutrophil specific *JUNB* deletion limits pathological tissue damage in murine models (Khoyratty et al., 2021). Interestingly mRNA levels of *JUN/FOS* were not altered following activation of human neutrophils with LPS, TNFα, fMLP, GM-CSF or PMA (Cloutier et al., 2003), or following zymosan stimulation, but J*UNB* had significantly higher rates of phosphorylation (Khoyratty et al., 2021). This demonstrates the benefit of upstream regulator analysis where although RNA expression is not altered, they control downstream effector genes. This is known for *AP-1* complex family members (including *FOS* and J*UN*), where activity of *AP-1* is

dependent on formation of the complex and post-translational modification (Angel and Karin, 1991; Khoyratty et al., 2021). The combined results of both *JUN* and *FOS* inhibition suggest that *JUN/FOS AP-1* complex may play an important role in regulation of neutrophil function shown in Figure 6-10.

AP-1 can be targeted by use of macrolide antibiotics (Kikuchi, 2002).Macrolides have been long recognised to have anti-inflammatory properties alongside their anti-microbial mechanisms (Kricker et al., 2021). Macrolides are commonly used in treatment of CAP (Lim et al., 2009), and observational data suggests improved short- and long-term CAP outcomes if macrolides are used (independent of pathogen identified) (Arnold et al., 2018; Reyes et al., 2023). A small study which investigated neutrophil function in hospitalised CAP receiving macrolide or no macrolide, demonstrated no difference in neutrophil function (Arnold et al., 2016). However, *in-vitro* use of macrolides has been demonstrated to influence neutrophil cytokine release (Sugihara, 1997). *In-vivo* administration of azithromycin to healthy subjects suppressed plasma MPO and ROS generation in response to fMLP, in contrast ROS generation in response to PMA was enhanced by azithromycin treatment and neutrophil apoptosis was delayed (Čulić et al., 2002). This predicted downregulation of *AP1* is likely to represent a shift towards pro-resolving phenotype with attempts to reduce inflammation.

Pathways relating to steroid treatment of immune cells were also upregulated in the geneset analysis. *AP-1* is supressed by glucocorticoids (Biddie et al., 2011), this action is suggested to play a role in their immunosuppressive functions. Other commonly used drugs with immunomodulatory effects such as ketamine and morphine have been demonstrated to suppress *ex-vivo* function of human neutrophils

with associated suppression of *AP-1* signalling (Welters et al., 2010; de Oliveira et al., 2015).

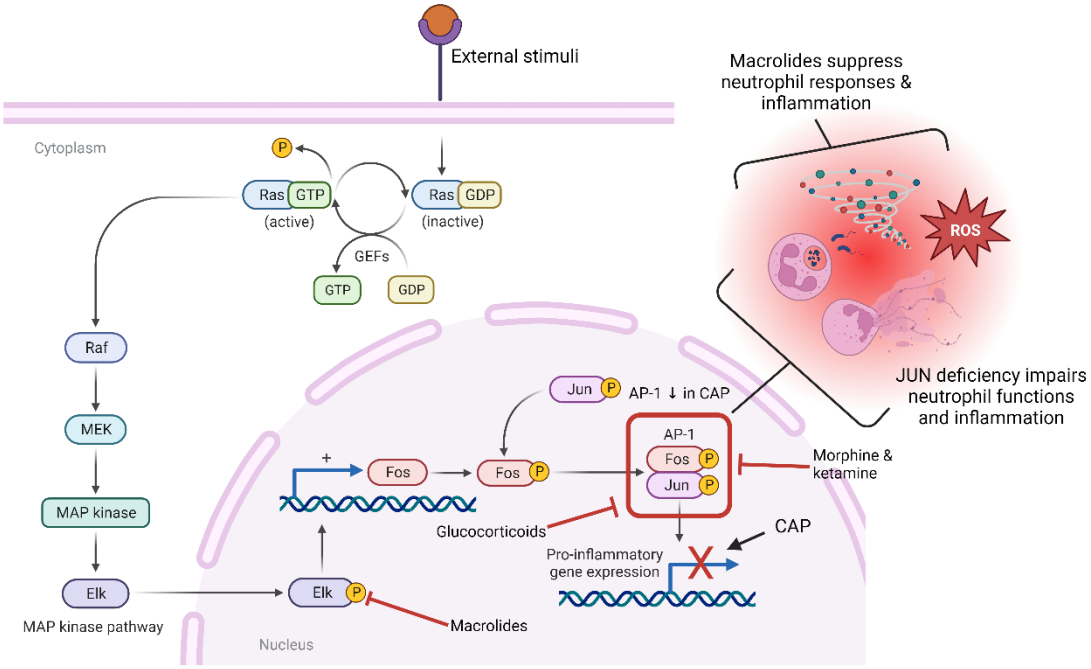

Figure 6-10: AP-1 signalling in neutrophils.

AP-1 is a transcription factor activated by MAPK signalling pathways in response to external stressors. JUN and FOS combine to make the AP-1 complex which then influences expression of pro-inflammatory genes. In CAP the AP-1 complex is predicted to be downregulated. AP-1 can be modulated using macrolides, glucocorticoids, ketamine, and morphine. *In-vitro* deletion of JUN in HoxB8 cells suppresses phagocytosis, NETosis, ROS and reduces cytokines. *In-vivo* neutrophil specific JUN deficiency reduces tissue damage, without impairing neutrophil recruitment to inflammatory sites. Created with biorender.com.

Peroxisome proliferator activated receptor alpha (PPARα) encoded by *PPARA* is predicted to be significantly inhibited in CAP neutrophils. PPARα is a transcription factor thought to be a master regulator of lipid metabolism (Kersten, 2014). PPARα has been shown to have multiple anti-inflammatory roles in human macrophages, and to influence ROS generation (Rigamonti et al., 2008). PPARα is the main target of fibrates, a group of drugs used in management of dyslipidaemia. Fibrates restore neutrophil migration in murine sepsis models (Tancevski et al., 2014), the role of statins in sepsis and neutrophil functions (another class of drugs for dyslipidaemia)

has already been discussed. There are multiple animal models of fibrates in sepsis, but no human interventional or observational data discussing outcomes from infection in relation to fibrates (Morel and Singer, 2014). However, the existence of approved drugs with established safety profiles suggests that in this cohort the role of fibrates in neutrophils should be examined.

GATA2 is a transcription factor crucial for haematopoiesis. *GATA2* deficiency is a primary immune deficiency characterised by severe/recurrent infections, cytopenias and high rates of haematological malignancy (Calvo and Hickstein, 2023). Patients with *GATA2* experience pulmonary infection with non-tuberculous mycobacteria (Marciano et al., 2021), and *GATA2* deficiency is associated with impaired phagocytosis by alveolar macrophages (Tang et al., 2000; Lasbury et al., 2003). Neutropenia has been associated with various mutations in *GATA2* (Fabozzi et al., 2022), but no data examining neutrophil function in *GATA2* deficiency were identified. However, *GATA2* deficiency in this data could represent the fine balance of transcription factors in control of haematopoiesis, where suppression of *GATA2* may be predicted to suppress monopoiesis to prioritise granulopoiesis in the face of significant infection. Figure 6-11 summarises the anticipated effect of gene and regulator expression on neutrophils.

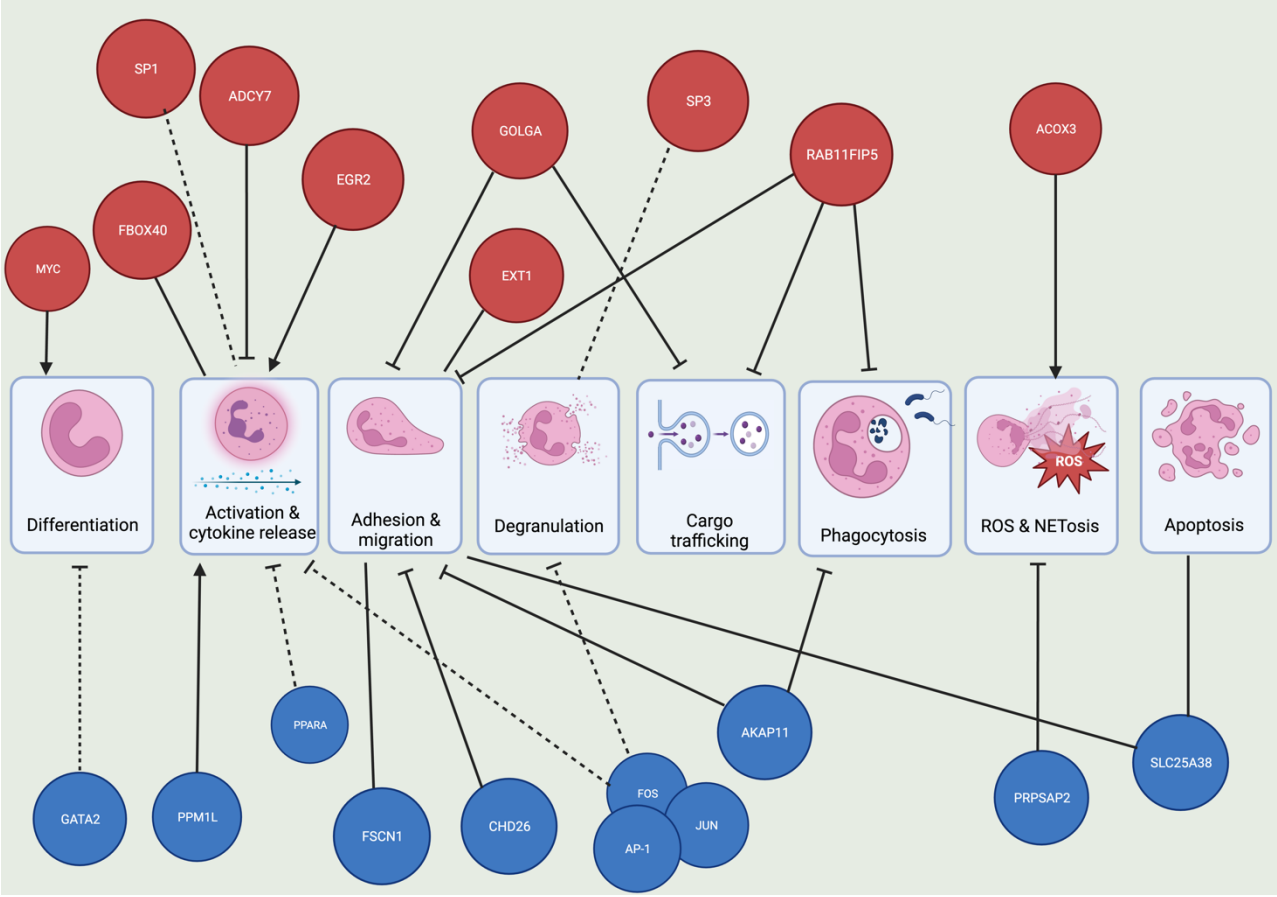

Figure 6-11: Predicted effects of altered gene expression on neutrophil function.

Upregulated genes are in red, downregulated genes in blue. Activation of a function is denoted by an arrow, inhibition by a flat line, if the direction of the relationship is unclear there is an open-ended line. Genes measured in the dataset are solid lines, genes predicted to be dysregulated are marked with dashed line. References as per main text. Created with biorender.com.

6.6.4. Pathway analysis

Using the whole geneset there were no significantly differentially expressed pathways, despite >700 differentially expressed genes. This a well-recognised limitation of GSEA, where although the strength of differential expression is considered during the analysis the direction of fold change is not considered. GSEA ranks all observations using a combination of fold change and adjusted *p* value. Therefore, with equivalent *p*-values a fold change of 20 or -20 are interpreted as the

same. To overcome this GSEA can be repeated with only up or downregulated genes (Hong et al., 2014).

In the upregulated genes there were 15 pathways that were significantly activated (FDR <0.05). One KEGG term (haematopoietic cell lineage), and 14 GO terms. There were two GO terms specific to neutrophils (tertiary granule and specific granule membrane). Chapter Five demonstrated increased degranulation of primary (azurophilic) granules but did not assess specific or tertiary granule contents, however given that primary granules require the highest degree of activation for mobilisation specific and tertiary granules are likely to be impacted too (Lacy, 2006). Most of the other terms reflect general immune activation and or immune cell differentiation. It is important to note that there is significant overlap between GO terms, and that GO terms are biased by the literature. There is a paucity of RNA data regarding neutrophils, therefore there are fewer pathways regarding neutrophils, and therefore pathways are more likely to be annotated in cell types which have a greater number of publications. This limits use of pathway analysis tools in neutrophil RNAseq, however, due to the methodology used in pathway annotation, pathway analysis validates existing findings rather than identifying novel pathways or roles for implicated genes.

The identification of sterol transport being upregulated in CAP neutrophils is interesting given previous discussions of statin and fibrate treatment in CAP (6.6.1). The sterol transport GO term has significant overlap with lipid transport, and general cellular transport GO terms (Culhane et al., 2012). The identification of this term as being perturbed alongside the identification of Rab proteins and Golgin proteins (involved in intracellular trafficking) as upregulated, the prediction of PPARα

downregulation is suggestive that there are overall alterations in intracellular trafficking, and that lipid transport/metabolism are plausible targets for further biological testing.

Leukaemia inhibitory factor (LIF) pathway is upregulated in CAP. LIF is a member of IL6 cytokine family, detectable in pleural fluid in pleural effusions and in BAL from ARDS (Heymann, 1996; Jorens et al., 1996). In murine models of lung infection LIF inhibition results in increased lung injury and inflammatory cytokines with bacterial dissemination (Quinton et al., 2012; Foronjy et al., 2014) , suggesting that upregulation of LIF in CAP neutrophils is a protective factor. LIF is a neutrophil chemoattractant *in-vitro*, but also induces secretion of IL8 from monocytes suggesting it is a pro-neutrophilic chemokine (Musso et al., 1995).

The pathway analysis supports altered degranulation and ROS, as well as maintained glycolysis demonstrated in 5.5.11, but does not entirely correlate with the observed phenotype of shown in Chapter Five. Lack of correlation between observed cellular function and pathway analysis has been observed in other neutrophil studies. In a study of preterm neonates with impaired chemotaxis compared to term neonates, there were >3000 DEGs, but alternations in neutrophil migration genesets were not observed (Raymond et al., 2017). The lack of some cellular functions not being altered at pathway level could reflect several scenarios:

1) Genes involved in neutrophil migration undergo a significant degree of PTM resulting altered cellular function without changing gene expression.
2) This study was underpowered to detect changes at a pathway level, this could be considered as playing a role as terms relating to migration were downregulated but did not reach statistical significance. May groups advocate

for use of an FDR cut off of 0.25 to generate targets for further validation as this indicates ¾ targets are likely to be correctly identified as dysregulated (Subramanian et al., 2005). However, given the relatively small sample in this study it was felt using a less stringent FDR cut-off would not be appropriate. There is no clear guidance available on how to power an RNAseq experiment to inform future experimental design.

3) Finally, the annotated pathways used by KEGG and GO terms may not accurately reflect neutrophil biology, particularly considering the heterogeneity of neutrophil response depending on priming stimuli, activation state and pathogen encountered (Vogt et al., 2018). The concept of bias in gene ontology is well recognised, as genesets rely on published experimental data which are lacking in neutrophils compared to other cells such as lymphocytes. In conserved pathways such as glycolysis there are relatively few genes which play defined roles, and this has been shown to be consistent across organism and cells. However, for complex functions such as migration which require multiple steps to be co-ordinated annotating a geneset to cover all eventualities is very challenging, especially when the experimental data does not exist for neutrophils.

6.6.5. Glycolysis

Chapter Five demonstrates a broad range of altered effector functions but maintained *ex-vivo* glycolysis assessed by extracellular flux. RNA expression of glycolytic enzymes and pathways was also unchanged in this dataset. Further, associated pathways such as gluconeogenesis and glycogenolysis were not differentially regulated in this dataset. Other studies have demonstrated altered metabolic

pathways and enzymes during sepsis (Pan et al., 2022; Schuurman et al., 2023), however these studies did not directly measure cellular energetics. Increased neutrophil glycolysis assessed by extracellular flux and confirmed at transcriptome level has been demonstrated in COVID-19 infection (Borella et al., 2022). These studies had much younger cohorts, mild infection and different types of acute illness which are all known to influence neutrophil function.

This study has demonstrated a wide range of differentially expressed genes, of which multiple are likely to play a role in altered neutrophil effector function. The first steps towards using this data to improve outcomes in CAP will be establishing the role these genes play in neutrophils, and as discussed earlier this may involve use of animal models or neutrophil like cell lines to interfere with RNA expression. Recurring themes throughout this chapter are intracellular trafficking, cytoskeleton and lipids. These functions are generally governed by small GTPases. *AP-1* has also been highlighted as a regulator of neutrophilic inflammation and given the capacity of macrolides to modulate AP-1 combined with observational data suggesting improved outcomes in CAP treated with macrolides this is a highly favourable route of targeting neutrophil function.

6.6.6. General limitations & future work

The findings in this chapter require biological testing to validate their role in neutrophil dysfunction in CAP. The nature of neutrophils means that methods used in other cells to interfere with RNA expression are not currently feasible in primary human cells. Targets identified in this chapter could be examined using RNA interference techniques in neutrophil-like cell lines such as HoxB8 murine myeloid progenitors used to assess deletion of transcription factors by Khoyratty (Khoyratty et al., 2021),

or in murine models, however these models have significant shortcomings. The short-lived nature of neutrophils and their propensity for activation means that only short-term drug treatments are possible. However, as discussed above targeting of AP-1 alone can be achieved through use of steroids and macrolide antibiotics- drugs in common clinical use with established safety records meaning that use in clinical trials could be considered. The challenges of neutrophil RNAseq are outlined in Table 6-17.

Table 6-17: Challenges associated with RNA sequencing.

RNAseq challenges	
Neutrophil specific	**General**
Short lifespan & propensity for activation	Expense
Limited RNA content	Determination of sample size
High levels of proteases including RNAases	Snapshot of transcriptome
Limited ability to interfere with RNA expression in human cells.	Unclear relationship with protein abundance +/- function
Paucity of published datasets for analysis	Computationally challenging analysis

This work represents a small sample of human neutrophils at a single time point in a disease state. It is likely that many of the targets discussed are in flux over the course of infection, for example *AP-1* downregulation appears to have immune-suppressive properties which would be anticipated to have benefit in the later stages of infection but may be less helpful in the early stages of infection where a robust neutrophil response is required.

There is a further question to answer regarding what “optimal” neutrophil function is. There are data demonstrating increased risk of adverse outcomes with both the highest and the lowest levels of NETosis (Lefrancais et al., 2018), but that this is also

likely to be pathogen specific as some pathogens have mechanisms to evade NETs (Beiter et al., 2006; Wartha et al., 2007). “Optimal” neutrophil function is likely to vary from person to person, dependent on aetiological agent and timeline of infection or inflammation.

This data also fails to address the chicken or the egg question- do people who develop CAP have neutrophil dysfunction prior to the onset of CAP- hence why they develop CAP, or do they develop neutrophil dysfunction as a result of CAP. Longitudinal studies recruiting participants who are highly likely to develop a critical illness- such as those older adults undergoing major elective surgery with serial sampling to achieve both pre-morbid neutrophil function and transcriptome with early stage assessment- where neutrophil function could be assessed prior to the clinical recognition of infective complications and participants followed up to observe how the neutrophil response changes over time would support understanding of this.

This study may not have appropriate power to detect change at gene or pathway level, however, there are no well-established methods for estimation of sample size in RNAseq. Typically, ≥6 biological replicates are recommended (Schurch et al., 2016), however the sepsis studies by Burnham *et al* and Zador *et al* used >100 biological replicates (Burnham et al., 2017; Zador et al., 2020) to derive risk signatures. Despite matching for age, sex, frailty and burden of comorbidity there is significant heterogeneity within groups and across the cohorts in this study, plus the known heterogeneity during sepsis, in future studies increased sample size should be used.

Not all DEGs are discussed in this chapter. The most significantly differentially expressed genes and pathways have been discussed as they likely represent the most biologically important genes, there were 140 genes with fold change of >20 or

>-20, discussing each of these individually is beyond the scope of this thesis. In addition, there were 243 non protein coding genes identified as differentially expressed. ncRNA is an umbrella term covering microRNA, short interfering RNA, long noncoding RNA and others, however these ncRNAs are often functional (Mattick and Makunin, 2006). ncRNAs have been identified to play a role in neutrophil function during sepsis (Liu et al., 2021), but there is very limited literature discussing ncRNA in neutrophils and or in infection as compared to cancer, therefore ncRNA was not discussed further.

Genes are generally multifunctional (Pritykin et al., 2015), and this further impacts the analyses used here. Upstream regulator analysis and pathway analysis relies on published literature regarding each gene and regulator, but the literature is biased towards cancer fields and non-neutrophil cells. Therefore, pathways and networks discussed here may not have been directly shown in a primary human neutrophil, but extrapolated from an animal or cell line studies which have their own limitations. Therefore, these findings should be directly verified in human neutrophils by measuring protein expression and assessing functional activity following modulation of expression by agonists/antagonists. Equally as pathway analysis techniques rely on published data, no novel findings can be inferred from this analysis.

The multifunctional nature of genes means that choosing which gene to target and understanding the off-target effects of modulation in immunology are challenging. For example, targeting *AP-1* in neutrophils would be expected to reduce neutrophil chemokine secretion therefore limiting neutrophilic influx to that area, but in the same tissue niche chemokine secretion by macrophages for example can be crucial to resolution of inflammation. The contrast in expression of *RAB11FIP* which would be

predicted to improve phagocytosis, with downregulation of a Rab GTPase escort protein demonstrates the complexity of intra-cellular signalling. Despite the limitations, RNAseq approaches are highly informative and powerful as whole transcriptome expression can be considered, in contrast to a few chosen targets for q-PCR.

6.7. Summary

There are many differentially expressed genes in CAP neutrophils compared to controls. The differentially expressed genes have high fold change and *p*-values indicating they are highly likely to have biological effects. The most differentially expressed genes have limited neutrophil specific data published to allow precise assessment of how they may impact function, but again are plausibly implicated in the abnormal phenotype seen in Chapter Five. Experimental RNA silencing in human neutrophils is not currently technically feasible, but this chapter discusses established drugs known to target dysregulated genes in this study. Pathway analysis confirms altered expression of pathways implicated in the phenotype demonstrated in chapter five. This study confirms that regulation of neutrophil glycolysis is not altered during CAP at the transcriptome level. This work requires biological validation, including longitudinal studies to understand the temporal nature of the transcriptome in CAP. The whole transcriptome approach to neutrophils has the potential to identify novel targets and be used to identify high risk gene “signatures” which may facilitate stratification of clinical trials aimed at normalising neutrophil function.

7. THESIS SUMMARY

CAP is a leading cause of emergency hospitalisation (Public Health England, 2019) and is associated with high short- and long-term mortality (Welte et al., 2012). CAP predominantly affects people at the extremes of age, adults hospitalised with CAP are typically >75 years old and frail with multiple comorbidities (Cillóniz et al., 2013). In the UK only 5% of adults hospitalised with CAP are admitted to ICU (Lim and Lawrence, 2019b), despite this 90% of the CAP clinical trials in the past decade focus on CAP in the ICU.

30-day mortality in CAP has fallen by 50% in the past decade reflecting the advances in CAP care. However, over that time 30-day readmission to hospital has risen by 50% (Lim and Lawrence, 2019b). There are no large studies addressing importance of outcome measures to people with CAP, small pilot studies suggest that for older adults maintaining independence is more important than mortality (Sapey et al., 2019).

Most CAP research focuses on antibiotic treatment, yet death in CAP is rarely due to antimicrobial resistance (Klapdor et al., 2012). There is very little published research in non-antibiotic management of CAP, the evidence sources used for guidance around management of respiratory failure, nutrition, fluid management, mobilisation and discharge follow up are predominantly of low quality (Lim et al., 2009).

Contrast this to stroke care, stroke is again a disease of older frail adults. Implementation of stroke units to deliver gold standard care of incorporating nutrition, mobilisation, and post discharge management with integrated research has significantly improved outcomes including mortality (Stroke Unit Trialists' Collaboration, 2013).

There is a nihilistic view of CAP mortality as being unavoidable, especially in older adults, but the strides made in the last decade and geographic variation in outcomes (Lawrence et al., 2020a) suggests that the nihilism is misplaced.

This thesis aimed to use a bench to bedside approach to understanding factors which influence outcome from CAP in older adults.

The following questions are addressed in this thesis:

1. How did the COVID-19 pandemic influence epidemiology of non-COVID CAP?
2. Can severity assessment tools in CAP be updated to improve prediction of mortality?
3. Can extracellular flux technology be used to interrogate neutrophil metabolism?
4. Does aberrant metabolism drive the neutrophil dysfunction seen in CAP?
5. Can the neutrophil transcriptome in CAP inform mechanisms for the cellular dysfunction?

The work explored in this thesis is summarised in Figure 7-1:

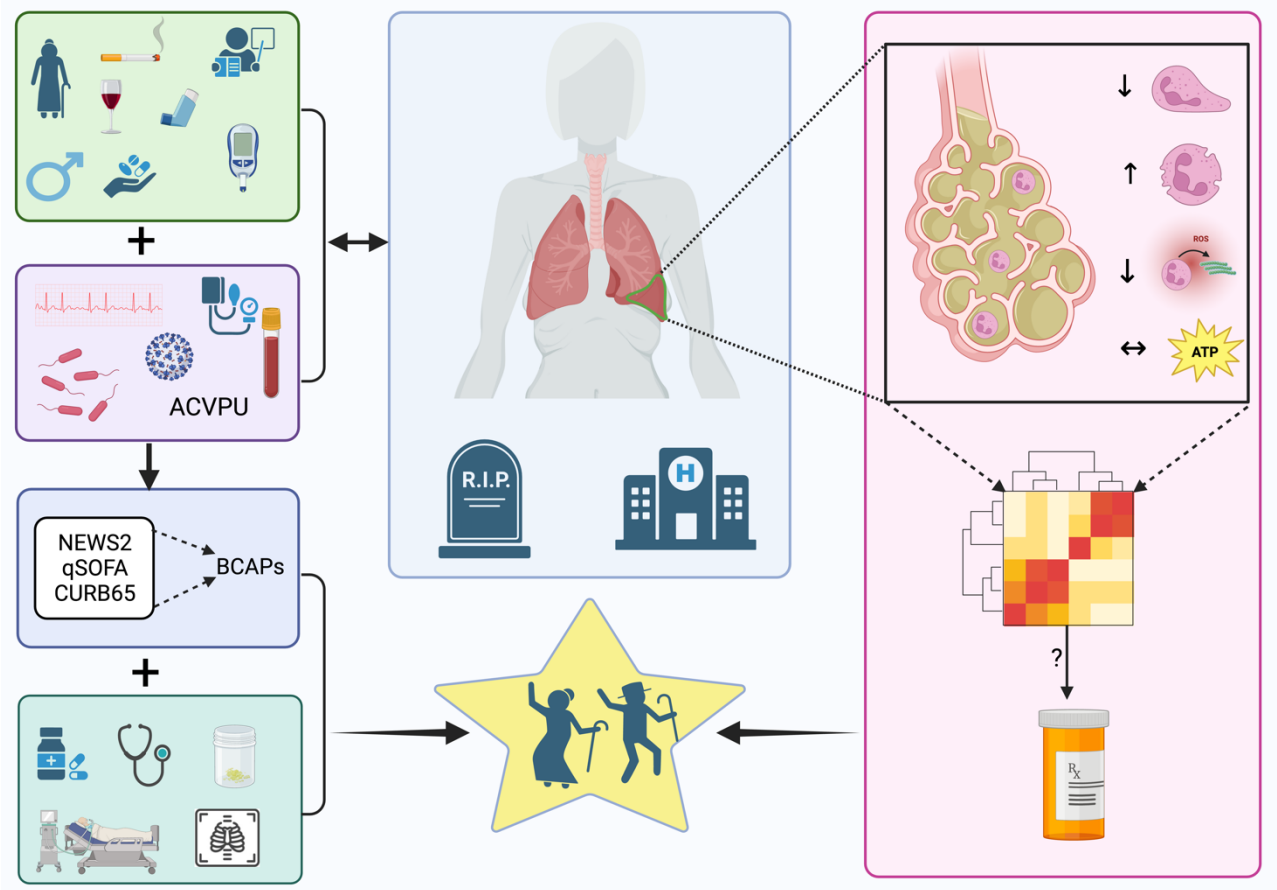

Figure 7-1: Investigating factors influencing outcomes in CAP

Figure 7-1 summarises the interplay between the clinical and immunological factors investigated in this thesis. Chapter 2 explores the epidemiology of CAP and how COVID impacted mortality, Chapter 3 demonstrates that severity assessment tools can be updated to provide better stratification of risk. Chapters 4-5 validates metabolic assessment of neutrophils and shows impaired effector function of neutrophils but with maintained rates of glycolysis. Chapter 6 provides a whole transcriptome overview of neutrophils during CAP, confirming lack of change in metabolism, but also providing potential targets to improve outcomes. By taking a holistic bench to bedside approach to CAP multiple factors influencing outcome can be targeted to maximise benefit for people with CAP.

7.1. Epidemiology of hospitalised community acquired pneumonia between 2019 and 2022 in Birmingham.

This study used health research data hub PIONEER to investigate the epidemiology of CAP during the pandemic. During the pandemic there were large falls in hospitalisation due to CAP, associated with significant increases in mortality up to 360-days, although the percent difference in mortality at 180 and 360-days was smaller than at early time points. During the pandemic CAP cases had a greater

burden of comorbidity and had more severe CAP and higher rates of sepsis. There was no change in delivery of care according to BTS or Society of Acute Medicine standards. This work replicates the findings of others in reduced admissions associated with increased mortality but is the first to use granular data to demonstrate shifts in CAP severity and data that suggests cases were presenting late to hospital. This work demonstrates that in the event of a future pandemic event public health campaigns should focus on encouraging people to seek medical advice to avoid non pandemic excess death.

7.2. Development of a novel severity assessment tool in community acquired pneumonia.

Severity assessment is a cornerstone of CAP management, guidelines advocate for use of severity assessment tools to guide microbial testing strategy, antimicrobial therapy and place of care (Lim et al., 2009; NICE, 2014). Severity assessment tools have been shown to wane in accuracy over time as patient factors and treatment strategies evolve (Steyerberg et al., 2013). Chapter Three demonstrates that CURB65 has greater utility in prediction of 30-day mortality in CAP compared to generic scores such as NEWS2 and qSOFA. Chapter Three also describes development of a novel severity assessment tool BCAPS, which was then validated in a separate cohort. BCAPS displayed greater accuracy for prediction of 30-day mortality compared to CURB65, the current gold standard. This tool requires further validation and refinement but demonstrates that given the changes in CAP outcomes the current tools should be updated. Hospitals are increasingly paper free and with widespread use of handheld devices, computational ease should not limit predictive modelling in 2023. Additionally, refinement of risk prediction is possible to continuous outcomes such as probability of death for an individual for example or, special

attention could be paid to developing models which predict adverse outcomes other than death- such as secondary infection, or readmission as these could be used to stratify for clinical trials and or inform discussions with patients and families regarding their likely outcome.

7.3. Real-time assessment of neutrophil metabolism and oxidative burst using Extracellular Flux Analysis

Neutrophils are crucial in CAP; however, they are broadly dysfunctional in CAP, this thesis hypothesised that this was due to impaired metabolism.

Immunometabolism is a rapidly expanding field which investigates the interplay of cellular metabolism and function. Extracellular flux (XF) enables *ex-vivo* cellular metabolism to be assessed by measurement of oxygen consumption to assess mitochondrial respiration and acidification of media as a surrogate of lactate efflux in glycolysis. Despite the abundance and importance of neutrophils in acute and chronic diseases there is relatively little understanding of their metabolism. XF technology was developed for use in adherent cells which can be maintained in culture, not short-lived primary cells which are prone to activation; therefore, extensive validation of this technique was required for use in neutrophils. Chapter Four demonstrates that assessment of neutrophil glycolysis in basal and stimulated states is feasible and demonstrates the steps taken in optimising this assay. This chapter also shows that assessment of mitochondrial respiration in neutrophils by XF is not possible, this is likely due to the low mitochondrial mass of neutrophils combined with limited sensitivity of XF technology at low oxygen consumption rates. Mitochondrial respiration does not appear to be important for maintenance of overall ATP balance in neutrophils, or most effector functions, but is required for control of apoptosis (Fossati et al., 2003; Maianski et al., 2004; Sadiku et al., 2021). Finally, measurement

of ROS in response to a range of stimuli was validated using XF, this assay measures total oxygen uptake which in the presence of mitochondrial inhibitors can be attributed to ROS generation. Assessment of ROS using this method avoids issues relating to selectivity of oxygen species, intra vs. extracellular ROS and redox recycling associated with chemiluminescent probes (Murphy et al., 2022b). This validation work was crucial for participant data presented in Chapter Five.

7.4. Neutrophil function and metabolism in older adults with community acquired pneumonia and sepsis.

Chapter Five is an observational cohort study examining neutrophil function and metabolism in older adults with CAP compared to age and frailty matched older adults. This chapter hypothesised that the broad neutrophil dysfunction seen in CAP was due to impaired ability to generate ATP for effector functions. This chapter shows that in a cohort of severe CAP with sepsis compared to age and frailty matched controls, *ex-vivo* neutrophils from CAP donors migrate inaccurately towards IL8, have an altered ROS response to PMA, undergo increased rates of degranulation, but do not display differences in basal or stimulated glycolysis. However, neutrophils from healthy young donors have a significantly different pattern of glycolysis, with lower basal rates and increased magnitude of response to stimulation. This suggests that glycolysis does not play a role in the aberrant neutrophil function seen in CAP in older adults but may play a role in the age-related decline seen in neutrophil function. Importantly this contrasts to published reports of increased glycolysis during sepsis in younger adults (Pan et al., 2022), demonstrating the significance of investigating immune responses in the population of interest.

7.5. Neutrophil transcriptome in community acquired pneumonia.

RNAseq facilitates whole transcriptome analysis of neutrophils to provide insight into function, however RNAseq cannot inform why a change has happened, or even if a change in RNA expression results in cellular changes. RNAseq has been widely used in studies of whole blood, or peripheral mononuclear cells in sepsis, but there are few studies examining neutrophils only. Nevertheless, understanding how the whole neutrophil transcriptome changes in response to sepsis rather than changes in a few genes will facilitate greater understanding of neutrophil function during infection. Chapter Six demonstrates that RNA isolation from neutrophils, from donors with and without CAP is feasible and results in high quality RNA for RNAseq. There were 760 differentially expressed genes in CAP compared to controls, the top differentially expressed genes are discussed in the context of how they may influence neutrophil function. Chapter Six also confirms at the RNA level there is no change in expression of individual glycolytic genes or changes in glycolysis pathway.

There is very little data on roles of specific genes in neutrophils, which probably relates to a combination of a view of neutrophils as transcriptionally inactive, but also the challenges of isolating RNA from neutrophils. Bioinformatic analysis enables identification of upstream regulators which are likely to be affected by the DEGs shown. These analyses are helpful as it is well established that RNA levels are not always altered, despite altered function. Upstream regulator analysis identified that AP-1 transcription factor is likely to be inhibited. AP-1 governs expression of pro-inflammatory genes. AP-1 deficient neutrophils have abnormal phagocytosis, NETosis and ROS generation (Khoyratty et al., 2021). AP-1 is targetable by several licenced drugs (Kikuchi, 2002). Pathway analysis was performed identifying degranulation as increased in CAP reflecting the *ex-vivo* identification of increased

degranulation in CAP shown in Chapter Five. Other phenotypic abnormalities shown in Chapter Five were not replicated in pathway analysis, which may reflect limited neutrophil specific data used to annotate pathways, or that diverse signalling pathways such as those used to respond to chemotactic cues are not well understood at gene level. This could also reflect that altered gene expression is not needed to perform chemotaxis. This chapter provides an insight into the transcriptional control of neutrophils during CAP but requires validation of these findings to integrate with established knowledge of neutrophil function during infection.

7.6. Limitations

The data studies presented in Chapters 2 and 3 are limited by their single centre retrospective nature. Although presenting granular data regarding CAP epidemiology and severity assessment their interpretation is challenged by the single site nature. This reflects a larger problem in CAP clinical data research, where despite the wealth of electronically held data in the NHS and allied organisations, the diagnostic inaccuracy of ICD coding severely limits use of this data. Typically, ICD coding is inaccurate in 40-50% of cases, typically due to lack of radiological features (Lim and Lawrence, 2019b) which limits usefulness of these large datasets. Manual radiology review is time consuming, and natural language processing methods to analyse existing reports challenging due to the diverse way in which reports are written and the wide array of radiological findings associated with CAP.

Chapter Four presents the validation of extracellular flux for assessment of neutrophil glycolysis, however, XF was not able to measure mitochondrial respiration in neutrophils. It is possible there is some flux to mitochondrial respiration which was

unmeasured, further, as measurement of glycolytic enzymes, end products and substrates or metabolites was not completed, the lack of change in rates of glycolysis may reflect that cells relied on stored ATP or other methods of energy generation. Non-physiological stimuli were used to assess stimulated glycolysis and ROS generation as physiological stimuli in these assays generated a much more limited response.

In Chapter Five neutrophil function was assessed alongside metabolism. Only a single time point was assessed, and between CAP participants the stage of infection was likely to be variable as people attend hospital with varying duration of symptoms. Although the recruitment target was met for the primary outcome of chemotaxis, there were limited numbers of replicates in other assays. Recruitment was challenging due to the impact of the COVID-19 pandemic, Chapter Two shows a significant reduction in CAP admissions over the period this study was recruiting. There are two major questions in neutrophil biology during sepsis that these studies do not address:

1. Is the neutrophil dysfunction seen here purely due to the impact of CAP, or do people who develop CAP have poorer baseline neutrophil function?
2. What is optimal neutrophil function and how does this alter in relation to stage of infection or inflammation?

Finally, the paucity of neutrophil RNA data, combined with the limited ability to modify RNA expression in human neutrophils makes the interpretation of Chapter Six challenging. However, demonstrating the feasibility of the technique combined with the potential of repurposing existing drugs to target function is an exciting avenue for neutrophil biology.

7.7. Future directions

CAP outcomes are dire for older adults, the importance of CAP at a policy level is reflected by the recent NCEPOD enquiry and Getting it Right First-Time reports. There is a clear opportunity to improve outcomes from this common condition and remove the nihilistic view of CAP in older adults. The following questions have arisen during this thesis:

1. What outcomes are important to older adults with community acquired pneumonia?

Despite bearing the brunt of CAP older adults are seldom involved in research studies (Petrovsky et al., 2022), and even fewer studies have investigated what is important to them. Pilot data seems to indicate maintaining independence (avoiding readmission) is more important than mortality (Sapey et al., 2019). Therefore, the first step should be asking people who are at highest risk of CAP acquisition and adverse outcomes what is important, research can then be focussed to those outcomes. A core outcome set for CAP is currently in development (Mathioudakis et al., 2023).

2. How existing data be better used to improve outcomes in CAP?

Most NHS health data is now held electronically, but there are significant barriers to integrating these data silos (Zhang et al., 2023). PIONEER is an example of how routinely collected data can be curated and used in research (Gallier et al., 2021). Of specific relevance to use of routine data in CAP research is identifying ways to validate radiographic features of CAP. Development of AI techniques to interpret thoracic imaging are likely to play a role for use of data in research (Ahn et al., 2022). Large multicentre CAP datasets will be able to focus on identifying those at high risk of adverse outcomes, whether that be mortality, readmission or important outcome

identified by patients. This data can then be used to stratify for clinical decision making and research.

3. How does premorbid neutrophil function relate to function during infection?
    a. What does optimal neutrophil function look like in older adults and how does this change over the course of infection?

A longitudinal cohort study of older adults who are highly likely to experience an infective disease, such as nursing home residents would facilitate answers to this. If baseline function were established participants could undergo repeated sampling if they developed an infection. This would provide insights into whether “depressed” neutrophil function is associated with increased risk of infection, and how neutrophil function changes over the course of infection, and whether neutrophil function is associated with adverse outcomes during infection. This would be a highly ambitious study but could integrate a whole immune system approach to establish the role of immunosenescence during infection. This work has the potential prevent infection by offering immune stimulation with an mRNA type vaccine to target immune cell progenitors, this type of vaccine is in early phase trials for cancer immunotherapy (Lorentzen et al., 2022).

4. How can RNA expression be experimentally altered in human neutrophils, and does this result in altered function?

Neutrophils are notoriously hard to experimentally modify due to them being terminally differentiated, having a short lifespan and prone to activation. To date neutrophil RNA interference techniques have been carried out in animal models and murine neutrophil like cell lines (Khoyratty et al., 2021). Establishing whether short-term inhibitor treatments of primary neutrophils are capable of inducing alterations in

RNA expression, and whether this is associated with changes in effector functions is a first step. Alternatively, determination of a “most” neutrophil like cell line such as HL60 in which RNA modification could be performed. More ambitiously, haemopoietic stem cells from human umbilical cord blood could undergo modification of RNA prior to differentiation into neutrophils. These studies would progress the understanding of transcriptome in neutrophils and may offer new therapeutic targets for inflammatory diseases.

7.8. Conclusion

The burden of CAP morbidity and mortality falls on older adults, who are rarely involved in research. Epidemiology of CAP has shifted over the past decade with significant falls in short-term mortality but significant increases in readmission rates- the relationship between these two factors is unclear. What is clear is that CAP (outside ICU) has very limited evidence to guide clinical management, and even less understanding how modulating immune response may be of therapeutic benefit.

This thesis undertook a bench to bedside approach to understanding the factors which influence adverse outcomes in CAP, incorporating clinical data and statistical modelling through to an in-depth analysis of neutrophil function and metabolism followed by an unbiased analysis of the neutrophil transcriptome in CAP.

The key findings are:

1. Public health measures to reduce spread of COVID-19 infection significantly impacted rates of CAP hospitalisation, with a more severe phenotype associated with increased mortality.
2. Better risk stratification is possible in CAP using contemporary datasets which more accurately reflect the current epidemiology of CAP.

3. Extracellular flux technology can be harnessed for interrogation of neutrophil glycolysis and as an alternative method for assessing ROS but cannot be used to assess neutrophil mitochondrial respiration.
4. Neutrophils from donors with CAP and sepsis display impaired migratory accuracy, ROS and increased degranulation, but rates of glycolysis are not altered between CAP and controls. However, glycolysis is significantly different in younger adults without infection compared to older adults suggesting that metabolism plays a role in the age associated decline in neutrophils.
5. RNAsequencing with CAP neutrophils is feasible and results in technically accurate data, with significant shifts in gene expression. Bioinformatic analysis is limited by a paucity of neutrophil data but suggests that there are novel and biologically plausible targets to modulate neutrophil function.

Together these data demonstrate that to improve outcomes from CAP a translational approach must be used. Integration of clinical datasets to identify shifts in epidemiology and recognise those at high risk for adverse events, combined with discovery science delivering understanding of how immunology influences outcome. These approaches should be streamed together with clinical interventions to deliver rapid impact now, but discovery science alongside to provide targets for development of novel therapies.

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9. APPENDIX ONE

This appendix contains supplementary data for Chapter Six

Table S1: Comorbid conditions and medications in RNAseq cohort

Participant	Comorbid conditions	Medications
CAP1	Ischaemic Heart Disease Hypertension Hypothyroidism Anaemia	Tramadol Ferrous Sulfate Cyanocobalamin Furosemide Paracetamol Perindopril Lansoprazole Clopidogrel Bisoprolol Folic Acid Atorvastatin Clexane Digoxin Tazocin Sando K
CAP2	Transient Ischemic Attack Aortic stenosis Hypertension	Atorvastatin Levothyroxine Lansoprazole Amlodipine Docusate Sodium Carbocisteine Clexane Furosemide Laxido Co-amoxiclav Clarithromycin
CAP3	Atrial Fibrillation Alzheimer's Disease Hypertension	Amlodipine Bisoprolol Lansoprazole Clexane Amoxicillin Clarithromycin
CAP 4	Hyperthyroidism Diverticular Disease Stroke	Atorvastatin Ramipril Clopidogrel Bisoprolol Clexane Co-amoxiclav Clarithromycin
CAP 5	Vascular Dementia Hypertension Diabetes Mellitus (Type 2) Hyperthyroidism	Carbimazole Ramipril Simvastatin Paracetamol

		Clexane Co-amoxiclav Clarithromycin
CAP 6	Heart Failure Hypertension Chronic Kidney Disease - Stage 4 (CKD4) Vascular Dementia Osteoporosis	Senna Evacal D3 Amlodipine Lansoprazole Amoxicillin Co-amoxiclav
CAP 7	Hypertension	Amlodipine Co-codamol
Control 1	Hypertension Senile Macular Degeneration	Amlodipine Indapamide Candesartan
Control 2	Atrial Fibrillation Hypertension	Digoxin Ramipril Aspirin
Control 3	Ischaemic Heart Disease Atrial Fibrillation Diabetes Mellitus (Type 2)	Adcal-D3 Bisoprolol Candesartan Rivaroxaban Simvastatin Glyceryl Trinitrate
Control 4	Age related macular degeneration. Depression	Zopiclone Citalopram
Control 5	Atrial Fibrillation Congestive Heart Failure Hypothyroidism Stroke	Digoxin Furosemide Levothyroxine Apixaban
Control 6	Parkinson's Disease Osteoarthritis Osteoporosis Hypertension Subdural Haematoma Cardiac Pacemaker	Amlodipine Omeprazole Alendronic acid Atorvastatin Vitamin D Co-careldopa
Control 7	Ischaemic Heart Disease Diverticular Disease of Intestine Diabetes Mellitus (Type 2) Cataract	Atorvastatin Bisoprolol Aspirin

Table S2: Top 100 differentially expressed genes.

Gene Name	Gene annotation	log2 Fold Change	*p*-value	FDR
EXT1	exostosin glycosyltransferase 1	10.01	5.89E-25	6.49E-21
AC069549.1	novel transcript	24.19	1.25E-22	6.88E-19
GOLGA5	golgin A5	24.06	3.75E-22	9.21E-19
RN7SKP127	RN7SK pseudogene 127	24.71	4.08E-22	9.21E-19
GASAL1	growth arrest associated lncRNA 1	23.93	4.18E-22	9.21E-19
ENSG00000274799	Metazoan signal recognition particle RNA	25.32	5.78E-20	1.06E-16
ADCY7	adenylate cyclase 7	24.69	1.15E-19	1.75E-16
RAB11FIP5	RAB11 family interacting protein 5	25.16	1.27E-19	1.75E-16
MYC	MYC proto-oncogene, bHLH transcription factor	24.45	3.71E-19	4.54E-16
PXN-AS1	PXN antisense RNA 1	25.28	1.39E-18	1.53E-15
OLAH	oleoyl-ACP hydrolase	10.6	1.71E-18	1.71E-15
LINC01772	long intergenic non-protein coding RNA 1772	24.9	6.37E-18	5.48E-15
AC010761.3	novel transcript, antisense to SUPT6H	23.78	6.47E-18	5.48E-15
DRC1	dynein regulatory complex subunit 1	24.58	1.12E-17	8.78E-15
SEMA6A	semaphorin 6A	24.53	1.2E-17	8.85E-15
ACOX3	acyl-CoA oxidase 3, pristanoyl	24.68	1.37E-17	9.4E-15
AC091132.4	novel transcript	23.46	1.49E-17	9.64E-15
ATRIP	ATR interacting protein	23.66	1.62E-17	9.91E-15
NAA40	N-alpha-acetyltransferase 40, NatD catalytic subunit	23.39	1.83E-17	1.06E-14
AC011773.1	novel transcript, antisense to E2F5	23.68	2.05E-17	1.13E-14
CHRNB4	cholinergic receptor nicotinic beta 4 subunit	23.99	2.27E-17	1.19E-14
AKAP11	A-kinase anchoring protein 11	-23.81	3.53E-17	1.69E-14
AL035458.1	density-regulated protein (DENR) pseudogene	25.11	4.21E-17	1.93E-14
SLC25A38	solute carrier family 25 member 38	-24.82	9.68E-17	4.27E-14
CDH26	cadherin 26	-23.99	1.08E-16	4.59E-14

FBXO40	F-box protein 40	24.74	1.19E-16	4.68E-14
PPM1L	protein phosphatase, Mg2+/Mn2+ dependent 1L	-24.11	1.23E-16	4.68E-14
EGR2	early growth response 2	24.73	1.23E-16	4.68E-14
TREML3P	triggering receptor expressed on myeloid cells like 3, pseudogene	24.71	1.3E-16	4.76E-14
AC079807.1	novel transcript	-23.3	1.58E-16	5.61E-14
FSCN1	fascin actin-bundling protein 1	-24.63	1.63E-16	5.61E-14
AC087239.1	novel transcript	-23.09	1.7E-16	5.67E-14
C5ORF63	chromosome 5 open reading frame 63	24.6	1.76E-16	5.71E-14
SCN9A	sodium voltage-gated channel alpha subunit 9	24.56	1.99E-16	6.26E-14
U73169.1	novel transcript, sense intronic to GNAI2	24.55	2.05E-16	6.26E-14
FRG1GP	FSHD region gene 1 family member G, pseudogene	-23.87	2.38E-16	6.98E-14
BRD3OS	BRD3 opposite strand	-24.49	2.45E-16	6.98E-14
LRRC14	leucine rich repeat containing 14	24.48	2.47E-16	6.98E-14
PRPSAP2	phosphoribosyl pyrophosphate synthetase associated protein 2	-24.39	3.2E-16	8.81E-14
CD44-AS1	CD44 antisense RNA 1	24.37	3.36E-16	9.03E-14
GNMT	glycine N-methyltransferase	24.34	3.73E-16	9.79E-14
AC005899.6	novel transcript	24.25	4.75E-16	1.22E-13
PRRT3	proline rich transmembrane protein 3	24.18	5.73E-16	1.41E-13
AL008729.1	novel transcript, antisense to PHACTR1	24.18	5.75E-16	1.41E-13
AC026124.2	novel transcript, sense intronic to LEMD3	24.16	5.99E-16	1.43E-13
AC115102.1	novel transcript, antisense to SEC11A	24.16	6.13E-16	1.44E-13
Z95118.2	novel transcript	-24.14	6.46E-16	1.48E-13
RMI2	RecQ mediated genome instability 2	24.13	6.64E-16	1.49E-13
SFMBT1	Scm like with four mbt domains 1	24.12	6.77E-16	1.49E-13

TSPAN18	tetraspanin 18	24.08	7.6E-16	1.64E-13
AL512343.2	H3-3A divergent transcript	-23.06	8.02E-16	1.7E-13
AC079944.2	CTDNEP1 pseudogene 1	-24.03	8.54E-16	1.78E-13
Z85996.2	novel transcript	24.01	9.28E-16	1.89E-13
AC009318.4	novel transcript, antisense to FAR2	23.97	1.02E-15	2.04E-13
PEX19	peroxisomal biogenesis factor 19	23.96	1.04E-15	2.05E-13
HSF2	heat shock transcription factor 2	-23.95	1.06E-15	2.05E-13
HSPE1P4	heat shock protein family E (Hsp10) member 1 pseudogene 4	23.95	1.08E-15	2.05E-13
SIGLEC15	sialic acid binding Ig like lectin 15	23.94	1.1E-15	2.05E-13
AC241377.4	novel pseudogene, similar to phosphodiesterase 4D interacting protein PDE4DIP	23.94	1.11E-15	2.05E-13
LINC00653	lysosome cell death regulator	-23.92	1.17E-15	2.11E-13
AC078883.1	PDK1 and ITGA6 antisense RNA 1	-23.9	1.24E-15	2.21E-13
M6PR	mannose-6-phosphate receptor, cation dependent	23.85	1.42E-15	2.49E-13
AC123595.2	novel transcript	23.83	1.5E-15	2.59E-13
SKP2	S-phase kinase associated protein 2	23.8	1.61E-15	2.73E-13
AC011815.1	novel transcript	23.8	1.64E-15	2.75E-13
ATXN7	ataxin 7	23.77	1.75E-15	2.88E-13
ALG13-AS1	ALG13 antisense RNA 1	23.77	1.78E-15	2.88E-13
F2R	coagulation factor II thrombin receptor	23.76	1.82E-15	2.91E-13
APBB3	amyloid beta precursor protein binding family B member 3	23.75	1.87E-15	2.94E-13
AURKC	aurora kinase C	23.74	1.91E-15	2.97E-13
NR2F6	nuclear receptor subfamily 2 group F member 6	-23.71	2.08E-15	3.18E-13
AC116913.1	novel transcript, antisense to MAP2K1	-23.7	2.11E-15	3.18E-13
RN7SL587P	RNA, 7SL, cytoplasmic 587, pseudogene	23.69	2.21E-15	3.24E-13

AL133230.1	novel transcript, antisense to PTPN1	23.68	2.25E-15	3.26E-13
WASF1	WASP family member 1	23.65	2.43E-15	3.47E-13
AL137779.2	novel transcript, antisense to PPP2R5C	23.65	2.45E-15	3.47E-13
AC103591.3	novel transcript, antisense to DNAJB4	-23.63	2.55E-15	3.56E-13
ENSG00000284862	coiled-coil domain containing 39	23.58	2.92E-15	4.02E-13
AC108078.1	UDP glucuronosyltransferase 2 family pseudogene	-23.56	3.05E-15	4.15E-13
POLR3F	RNA polymerase III subunit F	23.54	3.26E-15	4.38E-13
CHM	CHM Rab escort protein	-23.51	3.56E-15	4.72E-13
TPX2	TPX2 microtubule nucleation factor	23.44	4.24E-15	5.56E-13
ENSG00000247240	UBL7 divergent transcript	23.43	4.41E-15	5.72E-13
ATP6V1FNB	ATP6V1F neighbour	-23.39	4.87E-15	6.23E-13
SLC25A42	solute carrier family 25 member 42	-23.36	5.3E-15	6.72E-13
AL118516.1	TBC1D22A divergent transcript	23.32	5.85E-15	7.33E-13
AC113143.1	novel transcript	23.32	5.96E-15	7.37E-13
STK25	serine/threonine kinase 25	-23.29	6.33E-15	7.75E-13
CLEC9A	C-type lectin domain containing 9A	-23.26	6.9E-15	8.35E-13
ERMP1	endoplasmic reticulum metallopeptidase 1	23.22	7.71E-15	9.23E-13
OSGIN1	oxidative stress induced growth inhibitor 1	23.17	8.81E-15	1.04E-12
ZFYVE19	zinc finger FYVE-type containing 19	23.14	9.51E-15	1.11E-12
RAI2	retinoic acid induced 2	23.12	1.02E-14	1.18E-12
PRR7-AS1	PRR7 antisense RNA 1	-23.1	1.06E-14	1.21E-12
ABHD17C	abhydrolase domain containing 17C, depalmitoylase	23.08	1.13E-14	1.29E-12
ADAMTS7P4	ADAMTS7 pseudogene 4	-23.06	1.18E-14	1.32E-12
ENSG00000274376	ADAMTS7 pseudogene 1	-23.06	1.18E-14	1.32E-12
AC016949.1	novel transcript	23.04	1.25E-14	1.37E-12
AL355001.2	novel transcript	23.04	1.26E-14	1.38E-12